BASF Plant Science, L.P.

Petition for the Determination of Nonregulated Status for EPA+DHA Canola Event LBFLFK

The undersigned submits this petition under 7 CFR § 340.6 to request that the Administrator make a determination that the article should not be regulated under 7 CFR Part 340.

OECD Unique Identifier BPS-BFLFK-2

BASF Plant Science, L.P.
26 Davis Drive
Research Triangle Park, NC 27709

No CBI

Submitted by

Jordan Sottosanto

Prepared by

C. Andre, D.I. Arias, M. Bhatti, S. Breazeale, H. Fu, J. Klucinec, A. Lassen, E.A. Lipscomb, C. Martin, C.R. Moore, A.L. Olson, D.W. Roberts, T. Senger, S. Settlage, C. Wandelt, I. Wenderoth, P. Wu, M.K. Wyrick

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Release of Information

As part of the regulatory requirement in the United States, BASF Plant Science, L.P. is submitting the information in this petition for review by the USDA. The submission of this information by BASF Plant Science, L.P. does not constitute an authorization to release any information contained within it to any third party. In the event the USDA receives a Freedom of Information Act request, pursuant to 5 U.S.C., § 552, and 7 CFR Part 1, covering all or some of this information, BASF Plant Science, L.P. expects that USDA will provide BASF Plant Science, L.P. with a copy of the material proposed to be released and the opportunity to object to the release of any information based on appropriate legal grounds, e.g., responsiveness, confidentiality, and/or competitive concerns in advance of the release of the document(s). BASF Plant Science, L.P. authorizes the posting of the petition on the USDA Biotechnology Regulatory Services website when it is also published in the Federal Register for public comment. BASF Plant Science, L.P. understands that a copy of this information may be made available to the public by individual request, as part of a public comment period. Except in accordance with the foregoing, BASF Plant Science, L.P. does not authorize the release, publication or other distribution of this information (including website posting) without prior notice and consent by BASF Plant Science, L.P.

Certification

The undersigned certifies that, to the best knowledge of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioner that are unfavorable to the petition.

Jordan Sottosanto, Ph.D.
Regulatory Affairs Manager

Address:

BASF Plant Science, L.P.
26 Davis Drive
Research Triangle Park, NC 27709
Tel: (919) 547-2000
Email: jordan.sottosanto@basf.com

EXECUTIVE SUMMARY

BASF Plant Science, L.P. is submitting this request to USDA-APHIS for a determination of nonregulated status for the EPA+DHA canola (*Brassica napus* L.) event LBFLFK (OECD unique identifier BPS-BFLFK-2) and requests a determination from USDA-APHIS that LBFLFK canola and any progeny derived from LBFLFK canola by traditional breeding methods and naturally occurring crosses between LBFLFK canola and compatible species that are not regulated be considered nonregulated articles under 7 CFR part 340. As part of this petition, BASF is submitting phenotypic and genotypic experimental data, agronomic data, grain compositional data, safety assessment data, and other relevant information to fulfill the requirements of 7 CFR § 340.6 for an assessment of EPA+DHA canola event LBFLFK.

Product Description and Intended Uses

LBFLFK canola was produced using biotechnology to introduce newly expressed proteins. More specifically, recombinant DNA containing genes encoding fatty acid desaturase and elongase proteins and an herbicide resistant acetohydroxy acid synthase protein was introduced into the conventional canola variety Kumily by *Agrobacterium rhizogenes*-mediated transformation using a single transformation vector. The expression of these proteins in LBFLFK canola allows for the synthesis of long-chain omega-3 polyunsaturated fatty acids (LC-PUFAs), including EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), from oleic acid and for tolerance to treatment with an imidazolinone herbicide. LBFLFK canola provides a plant-based and scalable production system for omega-3 fatty acids and will be another source of EPA and DHA for consumers as either a food ingredient or as an aquaculture feed ingredient.

Currently, the omega-3 LC-PUFAs EPA and DHA are primarily consumed through seafood, including finfish (e.g., salmon, tuna, and trout) and shellfish (e.g., crab, mussels, and oysters). Numerous health organizations recommend adult intakes of 250 to 500 mg combined EPA and DHA per day. While this recommendation is met in some countries, many countries, including the United States, fall below the recommended average daily intake. The primary reason for this deficiency is that the supply of these fatty acids from marine animal and other sources is limited. There is a significant challenge in producing and distributing products containing EPA and DHA to consumers in adequate quantity. There is also high demand for fish oil as a primary ingredient for farmed fish, especially fatty fish like salmon or trout. This demand is not met to the point where the harvested fish now have reduced levels of omega-3 fatty acids compared to historical levels.

The herbicide tolerance trait of LBFLFK canola allows for selective post-emergence weed control during field production. Pending approval from the U.S. EPA for label use, Beyond® herbicide, containing active ingredient imazamox, an imidazolinone, will be used on LBFLFK canola as part of a weed management program. Rate, weed growth stage, adjuvants, spray volume and pressure, and nozzle use will follow all label directions. Agronomic practices will be similar to those used with Clearfield® canola, which also has imidazolinone herbicide tolerance, and provides a significant tool to growers for selective breeding and broad spectrum weed control. Clearfield® products have been widely adopted in North America, and herbicide applications for LBFLFK canola will follow established weed control practices.

Event LBFLFK will be cultivated within the United States and processed either in the United States or Canada as other available specialty canola varieties. To maintain the quality and ensure the segregation of LBFLFK canola seeds, grains, and processed products, an Identity Preservation System (IDP) will be implemented at every step of production and handling. Processing operations will be conducted either at dedicated facilities or at facilities with specific measures in place to ensure segregation from other products. Like other canola varieties, the products of LBFLFK canola will be processed into oil and defatted meal fractions. Oil and meal from canola can be manufactured into a variety of products for human and animal consumption or for industrial purposes. As a specialty canola with a fatty acid profile containing the LC-PUFAs EPA and DHA, the oil produced from LBFLFK canola will be sold specifically for the purpose of providing dietary omega-3 LC-PUFAs. The oil will be incorporated as an ingredient into consumer food items to provide individuals more options for dietary omega-3 LC-PUFAs. The refined oil may also be provided to dietary supplement manufacturers as an alternate source of omega-3 LC-PUFAs. The oil will also be used as an aquafeed input ingredient to international operations to provide omega-3 LC-PUFAs to farmed aquatic species. The defatted meal produced from LBFLFK canola, containing only a very small percent of fat, will not be sold as a specialty product and will be available for use in the same applications as conventional canola meal, including livestock feed.

Data and Information Presented Confirms the Lack of Plant Pest Potential and the Food and Feed Safety of LBFLFK Canola Compared to Conventional Canola

The data and information presented in this petition demonstrate LBFLFK canola is agronomically, phenotypically, and compositionally comparable to conventional canola, with the exception of the introduced proteins and the associated changes in the composition of fatty acids, including omega-3 LC-PUFAs. The data presented demonstrate LBFLFK canola is unlikely to pose an increased plant pest risk, including weediness characteristics or adverse environmental impacts, compared to conventional canola. The food, feed, and environmental safety of LBFLFK canola was confirmed based on multiple, well established lines of evidence:

- Canola is a familiar crop that has a history of safe consumption and serves as an appropriate basis for comparison for LBFLFK canola.

- A detailed molecular characterization of the introduced DNA in LBFLFK canola demonstrated two intact, stable copies of the intended T-DNA insert at two loci within the canola genome.
- A compositional assessment demonstrated that LBFLFK canola is compositionally comparable to conventional canola except for the intended increased levels of omega-3 LC-PUFAs and the associated changes to the levels of precursor and intermediary fatty acids.
- An evaluation of LBFLFK canola phenotypic and agronomic characteristics and environmental interactions demonstrated LBFLFK canola has no increased plant pest potential compared to conventional canola.
- An assessment of potential impact to organisms that associate with canola plants in the field indicated that LBFLFK canola does not have adverse effects on these organisms as compared to conventional canola.
- Evaluation of LBFLFK canola using intended and current cultivation and management practices for similar canola cultivars concluded that deregulation of LBFLFK will not significantly impact canola agronomic practices or land use.

Background on Canola and Use of Kumily as an Appropriate Comparator

Globally, canola production is concentrated in areas with dry weather and shorter growing seasons. There are spring and winter varieties of canola. Spring canola is a cool-season crop, is not very drought tolerant, and is the primary biotype grown in the northern regions of North America and Asia and the southern regions of Australia. Winter canola requires cold (vernalization) before flowering and is planted in the fall at more central latitudes, e.g., in the EU, Ukraine, Russia, and parts of China. Canola varieties of *B. napus* are the primary rapeseed varieties grown for oil production in North America. The major production areas are in the Canadian prairie provinces of Manitoba, Saskatchewan, and Alberta. In the U.S., canola is grown mostly in the northwestern region of the country where a drier, shorter growing season makes corn and soybean less attractive. According to the Food and Agriculture Organization of the United Nations, the total harvested area of rapeseed in North America in 2014 was about 9 million hectares with a production of about 17 million metric tons of oilseed. Most (> 90%) rapeseed production in North America is in Canada. Globally, North America, China, the European Union, and India are the major canola/rapeseed production regions.

In North America, production of canola is conducted under minimal or no-till systems to protect soil from erosion. Reduced tillage cultivation systems require pre- and post-emergent weed control. Most canola production in the U.S. and Canada utilizes at least one of three main groups of herbicide-tolerance traits – tolerance to glyphosate, tolerance to glufosinate (both derived from biotechnology), and tolerance to imidazolinone herbicides (derived from mutagenesis). As in other crops, herbicide tolerance traits in canola allow for simplified, effective chemical weed control programs.

Historically, rapeseed oil was used primarily for industrial purposes (lamp oil, soap-making), and it was produced from Brassicaceae species including *B. napus*, *B. rapa*, and *B. juncea*. Rapeseed breeding efforts to improve agronomic characteristics and oil content were intensified in Canada after its introduction from Europe in the 1940s. During the 1970s, low erucic acid rapeseed was developed from *B. napus* and *B. rapa* varieties to make rapeseed oil suitable for human consumption. A few years later, a “double-low” cultivar was developed by breeding with low erucic acid and low glucosinolate levels. These edible rapeseed varieties, collectively referred to as canola, have since been widely used for edible oil production and animal feed use.

The canola variety used for the introduction of the EPA+DHA canola trait was Kumily, a spring cultivar of *Brassica napus* L.

Intended Changes to the Fatty Acid Profile and Herbicide Tolerance

Enzyme-encoding genetic sequences and associated expression cassettes were introduced from a number of eukaryotic organisms (*Phytophthora sojae*, *Ostreococcus tauri*, *Thalassiosira pseudonana*, *Physcomitrella patens*, *Thraustochytrium* sp., *Phytophthora infestans*, *Pythium irregulare*, *Pavlova lutheri*, and *Arabidopsis thaliana*) to alter the production of specific fatty acids in canola, resulting in the production of omega-3 LC-PUFA fatty acids and providing tolerance to treatment with the herbicide active ingredient imazamox.

Canola normally produces primarily C18:1n-9 (oleic) and C18:2n-6 (linolenic) fatty acids in seeds through the combined efforts of enzymes involved in *de novo* fatty acid synthesis, elongation, and desaturation. The introduction of seven desaturases and three elongases to develop EPA+DHA canola event LBFLFK allows production of DHA and its biosynthetic intermediate EPA from these endogenous fatty acids through an aerobic pathway.

The herbicide tolerance trait is conferred through the introduction of a modified acetohydroxy acid synthase (AHAS) protein from *Arabidopsis thaliana.*

Molecular Characterization of EPA+DHA canola event LBFLFK

LBFLFK canola was generated via *Agrobacterium rhizogenes*-mediated transformation of *Brassica napus* cv. Kumily hypocotyl segments with a single plasmid vector to introduce fatty acid synthesis genes (desaturases and elongases) and an herbicide tolerance gene. The petition describes the transformation process, the origin, size, and function of each genetic element in the plasmid vector, and the location and orientation of the elements in the vector.

The molecular characterization of LBFLFK canola consisted of a combination of gene sequencing techniques (Next generation sequencing and Sanger sequencing), polymerase chain reaction, bioinformatic, and genetic segregation analysis. These molecular characterization efforts allowed the following conclusions to be made:

- Next generation sequencing of total genomic DNA and subsequent bioinformatic analysis demonstrated that LBFLFK has two inserts integrated at two separated loci (Insert1 and Insert2) and confirmed the absence of the transformation vector backbone sequences in event LBFLFK.
- Repeating the analysis for three generations demonstrated that the two inserts are stably integrated in the LBFLFK canola genome.
- Sanger sequencing analyses confirmed that each of the two inserts has the intended 13 gene expression cassettes and was identical to the vector T-DNA except for two single nucleotide changes in Insert1 and one nucleotide change in Insert2 that do not impact the functionality of the proteins produced. Both T-DNA inserts were intact, apart from a short rearrangement of 64 bp in the RB sequence of Insert1.
- A comparison to the sequence of the insertion site from the parental canola Kumily variety demonstrated that an 8-bp deletion was created at the genome integration site of Insert1 (Locus1) and a 31-bp deletion was created at the genome integration site of Insert2 (Locus2) in Kumily. No canola genomic sequence rearrangements were found at either integration site.
- A bioinformatics analysis identified 11 potential open reading frames that span the junctions between the T-DNA inserts and the flanking genomic DNA. None of the ORFs created by the insertion showed any significant homology to known allergens, protein toxins, and antinutrients.
- Segregation analysis of F2 and F3 LBFLFK offspring showed that Insert1 and Insert2 in LBFLFK are both independently inherited according to Mendelian principles.

Characterization and Quantification of Newly Expressed Proteins

Eleven proteins were introduced into canola event LBFLFK. This includes ten integral membrane proteins (desaturases and elongases), controlled by seed-specific promoters, that impact the content of omega-3 LC-PUFAs in the seeds. The 10 desaturases and elongases are a delta-12 desaturase from *Phytophthora sojae* (D12D(*Ps*)), a delta-6 desaturase from *Ostreococcus tauri* (D6D(*Ot*)), a delta-6 elongase from *Thalassiosira pseudonana* (D6E(*Tp*)), a delta-6 elongase from *Physcomitrella patens* (D6E(*Pp*)), a delta-5 desaturase from *Thraustochytrium* sp. (D5D(*Tc*)), an omega-3 desaturase from *Pythium irregulare* (O3D(*Pir*)), an omega-3 desaturase from *Phytophthora infestans* (O3D(*Pi*)), a delta-5 elongase from *Ostreococcus tauri* (D5E(*Ot*)), a delta-4 desaturase from *Thraustochytrium* sp. (D4D(*Tc*)), and a delta-4 desaturase from *Pavlova lutheri* (D4D(*Pl*)). The eleventh protein is the soluble, chloroplast-located acetohydroxy acid synthase from *Arabidopsis thaliana*, containing two amino acid substitutions (A122T and S653N). This enzyme, AHAS(*At*) [A122TS653N], under control of a constitutive promoter, confers tolerance to treatment with the herbicide active ingredient imazamox in event LBFLFK.

The safety assessment of crops improved through biotechnology includes a description of the nature and biochemical function of the newly expressed proteins. This typically includes characterization for identity and amino acid sequence, for apparent molecular weight and immunoreactivity, and for potential glycosylation and enzymatic activity. In general, this involves either isolation of the newly expressed proteins from the plant or production of the protein in a heterologous expression system, depending on the properties of the newly expressed proteins and levels of expression.

Integral membrane proteins, including fatty acid desaturases and elongases, that contain multiple transmembrane spanning domains are difficult to isolate or concentrate. Such intractable proteins are also generally not able to be produced at high levels in heterologous systems. In order to characterize the desaturase and elongase proteins introduced to LBFLFK canola, a membrane protein extract was prepared from developing plant embryos isolated from immature seeds. This membrane protein fraction, referred herein as plant-produced proteins (PPP), contains the highest concentration of the elongases and desaturases as active, complete proteins. Characterization studies of the introduced herbicide tolerant AHAS(*At*) [A122TS653N] protein using plant protein extracts were also performed.

The expression levels of each of the 11 newly introduced proteins were assessed using different plant tissue samples, including young whole plants (rosettes), flowering whole plants, root, leaf, pollen, immature seeds, and mature seeds. The expression data confirmed that the introduced elongases and desaturases, controlled by seed-specific promoters, were expressed only in seed tissue as expected. Additionally, two of the proteins, O3D(*Pi*) and D6E(*Pp*), were not found at detectable levels in any tissue sample of LBFLFK canola. The herbicide tolerant AHAS, controlled by a constitutive promoter, was found at highest concentrations in green plant tissues.

Safety Assessment of Newly Expressed Proteins

A weight-of-evidence approach, addressing the history of safe use and consumption, amino acid sequence similarity to known toxins, antinutrients, and allergens, digestibility, and degradation with exposure to elevated temperatures, was used to demonstrate the safety of the newly expressed proteins in LBFLFK canola.

The lack of adverse findings identified for these proteins and their donor organisms by a systematic literature search demonstrates a history of safe use based on uses in the food supply or from exposure routes other than intended food use. The protein sequence of each newly expressed protein was found to be structurally and functionally related to other proteins that are safely consumed by humans as food and by animals as feed. The wide distribution of proteins identified suggests that humans and animals have long been exposed as part of their diet and environment to proteins similar to the desaturases, elongases, or AHAS(*At*) [A122TS653N] present in LBFLFK without adverse effects. None of the newly expressed proteins in LBFLFK were shown by bioinformatic analysis to have significant homology to any known protein toxins, antinutrients, or allergens.

Digestive fate analysis was used to determine the sensitivity of the newly expressed proteins to simulated gastric (SGF) and intestinal (SIF) fluid digestion. Each of the newly expressed proteins present at sufficient levels to be assayed was found to be susceptible to digestion in SGF, SIF, or SGF followed by SIF.

Heat treatment sensitivity was evaluated by enzyme activity and structural integrity. The enzyme activity of D6D(*Ot*), D5D(*Tc*), O3D(*Pir*), and O3D(*Pi*) in response to heat treatment was not assessed because enzyme activity was not detectable in LBFLFK protein extract. The heat sensitivity of these proteins was assessed by structural integrity only or (in case of O3D(*Pi*)) was not assessed due to the low amount of protein in LBFLFK protein extract. Each of the newly expressed proteins assayed for structural integrity was heat-labile, and each of the newly expressed proteins assayed for enzymatic activity lost its activity, suggesting that enzyme activity is unlikely to remain after the conditions of commercial processing to produce oil and meal.

It is noted that the O3D(*Pi*) and D6E(*Pp*) proteins were not assessed for structural integrity to heat treatment or to digestibility in SGF or SIF as these proteins were not found at detectable levels in LBFLFK protein extract preparations or LBFLFK tissues. However, this low amount of O3D(*Pi*) and D6E(*Pp*) protein also demonstrates that they are unlikely to present any safety concern to humans or animals.

Overall, applying a weight-of-evidence approach, all newly expressed proteins in LBFLFK are considered to behave as any other dietary protein and thus do not raise any safety concerns with regard to human or animal health or the environment.

Agronomic, Phenotypic, and Environmental Interactions Assessment

LBFLFK canola, the parental variety Kumily, and six conventional reference varieties were cultivated during two seasons at a total of 14 locations representative of U.S. commercial canola production. The first season of cultivation occurred in the winter of 2014/15 at six locations in the southern U.S. The second season occurred in the spring of 2015 at eight locations in the northern U.S. As LBFLFK canola contains a trait that confers tolerance to the imidazolinone herbicide, plots treated with or without Beyond® herbicide were included in the randomized complete block design with four replicates per location. All other entries including LBFLFK canola (non-sprayed) received a standard herbicide treatment program.

The field trials, in combination with other collected data, support the conclusion that agronomic, phenotypic, and ecological characteristics of LBFLFK canola under typical growing conditions are similar to those of the parental control Kumily and are within the ranges found in conventional reference canola varieties, with the exception of seed germination characteristics.

LBFLFK canola performed similarly to Kumily when grown as a spring canola in the northern U.S., except for the characteristics of slightly decreased yield and increased seed moisture at harvest. The assessments during the 2014/15 winter season showed that the introduced fatty acid trait may have increased the sensitivity of LBFLFK canola to cold temperatures compared to conventional canola, resulting in site-specific differences during field emergence and early plant development. As further described in the petition, an assessment of the germination of harvested seed demonstrated that LBFLFK canola seed has a reduced and delayed seed germination rate, especially in cold conditions, as compared to Kumily and other conventional canola varieties. The reduced germination results from lower viability and not increased dormancy and, therefore, is not contributing to any increased weediness potential.

As LBFLFK canola is not engineered for pest resistance, there are not any 'target' or 'non-target' species. BASF Plant Science did assess whether exposure to LBFLFK canola might have an impact on pest species or species beneficial to agriculture. Evaluations of ecological interactions were conducted as part of the evaluation of agronomic and phenotypic characteristics of LBFLFK canola. Plots were monitored for disease and pest damage as well as damage from abiotic stresses. In addition, a dedicated assessment of the ecological interactions of LBFLFK canola was performed at three separate field locations during the spring season. These ecological evaluations did not show any consistent differences in organism abundance, including pests or diseases. Together the data support the conclusion that, compared to other canola varieties, LBFLFK canola is no more likely to result in the introduction or spread of a pest or disease, to be more susceptible to any pest or disease, or to otherwise impact pest or beneficial species.

In summary, LBFLFK canola does not present any different agronomic impacts than the cultivation of conventional canola varieties.

Compositional Analysis

Grain samples were harvested from the interior of each plot for compositional analysis. Grain samples were harvested and analyzed for composition, including proximates, fibers, amino acids, fatty acids, vitamins, minerals, antinutrients, and phytosterols. The components selected for analysis were based primarily on guidance provided in the consensus document for canola from the Organisation for Economic Co-operation and Development (OECD). A total of 112 components were measured in canola grain.

The composition of LBFLFK canola (sprayed and non-sprayed) was compared with the parental canola variety Kumily for each season. Data suitable for statistical analysis were subjected to mixed model analysis of variance. Across-site mean values were compared to the range of means generated from conventional canola reference varieties, the International Life Sciences Institute (ILSI) Crop Composition Database data, and peer-reviewed scientific literature to provide context for the comparative analyses and assess the broader biological relevance of the results.

As expected, the introduction of the EPA+DHA trait and the associated metabolic pathway in LBFLFK canola resulted in the presence of fatty acids not normally found in conventional canola. The EPA+DHA trait impacted the amount of fatty acids normally observed in conventional canola varieties in an expected way, with the content of oleic acid significantly reduced in LBFLFK canola across treatments and seasons relative to the parental control variety Kumily, as it serves as the primary starting substrate fatty acid for the newly introduced fatty acid synthesis pathway. The concentrations of measured fatty acids not associated with the introduced enzymatic pathway were not changed in LBFLFK canola, such as erucic acid, which remains low.

For the other grain components measured, the results of the comparative approach demonstrated that LBFLFK canola (either sprayed or non-sprayed) is equivalent to other commercially available canola varieties based on comparisons to the parental canola variety Kumily, the conventional reference varieties, the ILSI Crop Composition Database values, and peer-reviewed literature values.

Conclusion

Based on the data and information presented in this petition, BASF has concluded that EPA+DHA canola event LBFLFK is not likely to be a plant pest or to present unique risks to organisms beneficial to agriculture. Therefore, BASF requests a determination from USDA-APHIS that LBFLFK canola and any progeny derived from LBFLFK canola by traditional breeding methods and naturally occurring crosses between LBFLFK canola and compatible species that are not regulated be considered as articles that are not regulated under 7 CFR part 340.

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LIST OF ABBREVIATIONS AND DEFINITIONS

aa	Amino acid
AHAS	Acetohydroxy acid synthase
AHAS(*At*)	Large subunit of acetohydroxy acid synthase from *Arabidopsis thaliana*; refers to the AHAS enzyme large subunit comprising the A122T and S653N amino acid substitutions conferring tolerance to imidazolinone herbicide in the context of EPA+DHA canola
[A122TS653N]	Substitution of an alanine residue (A) with a threonine (T) at amino acid 122 and a serine residue (S) with an asparagine (N) at amino acid 653, relative to the *csr1-2* gene of *Arabidopsis thaliana*
a.i.	Active ingredient
ALS	Acetolactate synthase
ANOVA	Analysis of variance
AOSA	Association of Official Seed Analysts
APHIS	Animal and Plant Health Inspection Service
ARA	Arachidonic acid
B. napus	*Brassica napus* L.
BAC	Bacterial artificial chromosome
BBCH	Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie
bp(s)	Base pair(s)
CFIA	Canadian Food Inspection Agency
cv.	Cultivar
D12D(*Ps*)	Delta-12 desaturase from *Phytophthora sojae*
D4D(*Pl*)	Delta-4 desaturase from *Pavlova lutheri*
D4D(*Tc*)	Delta-4 desaturase from *Thraustochytrium* sp.
D5D(*Tc*)	Delta-5 desaturase from *Thraustochytrium* sp.
D5E(*Ot*)	Delta-5 elongase from *Ostreococcus tauri*
D6D(*Ot*)	Delta-6 desaturase from *Ostreococcus tauri*
D6E(*Pp*)	Delta-6 elongase from *Physcomitrella patens*
D6E(*Tp*)	Delta-6 elongase from *Thalassiosira pseudonana*
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DW	Dry weight

ELISA	Enzyme-linked immunosorbent assay
ELO	Elongation
EPA	Eicosapentaenoic acid
ETS	Excellence Through Stewardship®
EU	European Union
FAE	Fatty Acid Elongation
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FW	Fresh weight
gDNA	Genomic DNA
GLA	Gamma-linolenic acid
GRAS	Generally Recognized as Safe
HOSU	History of safe use
HT	Herbicide tolerant
IDP	Identity Preservation
ILSI	International Life Sciences Institute
kb	Kilobase
kDa	Kilodalton
LA	Linoleic acid
LB	T-DNA left border
LBFLFK	Event name for EPA+DHA canola, also known as Event LBFLFK, LBFLFK canola, LBFLFK event, EPA+DHA canola
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LC-PUFA(s)	Long-chain polyunsaturated fatty acid(s)
LOD	Limit of detection
LOQ	Limit of quantitation
LTM593	Plasmid vector used to transform *Brassica napus* hypocotyl cells to produce EPA+DHA canola event LBFLFK. Also known as LTM593-1qcz.
NGS	Next generation sequencing
O3D(*Pi*)	Omega-3 desaturase from *Phytophthora infestans*
O3D(*Pir*)	Omega-3 desaturase from *Pythium irregulare*
OA	Oleic acid
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
ORF(s)	Open reading frame(s)
PPP	Plant-produced proteins

QV	Quality value
RB	T-DNA right border
SD	Standard deviation
SE	Standard error
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
S-W	Shannon-Weaver
T_0	The parent transformed plant
T_1	The immediate progeny of the T_0 plant
T_2	The immediate progeny of the T_1 plant
T-DNA	Transfer DNA
TZ	Tetrazolium
USDA-ARS	United States Department of Agriculture – Agricultural Research Service
U.S.	United States of America
USDA	United States Department of Agriculture
UTR	Untranslated region

1. RATIONALE FOR THE DEVELOPMENT OF EPA+DHA CANOLA EVENT LBFLFK

1.1. Basis for the request for a determination of nonregulated status under 7 CFR § 340.6

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) is responsible under the Plant Protection Act (7 U.S.C. § 7701-7772) for preventing the introduction or dissemination of plant pests into the United States (U.S.). Under APHIS regulation 7 CFR § 340.6, an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted and unrestricted introduction of the article is permitted.

BASF Plant Science, L.P. is submitting this request to APHIS for a determination of nonregulated status for the EPA+DHA canola event LBFLFK (OECD unique identifier BPS-BFLFK-2) and requests a determination from APHIS that LBFLFK canola and any progeny derived from event LBFLFK canola by traditional breeding methods and naturally occurring crosses between event LBFLFK and compatible species that are not regulated be considered nonregulated articles under 7 CFR Part 340. As part of this petition, BASF is submitting phenotypic and genotypic experimental data, agronomic data, field test reports[1], and safety assessment data for canola event LBFLFK as described in 7 CFR § 340.6.

1.2. Rationale for the development of the new plant variety

LBFLFK canola was produced using modern biotechnology techniques to introduce newly expressed proteins. More specifically, recombinant DNA (transfer DNA, or T-DNA) containing genes encoding fatty acid desaturase and elongase proteins and an herbicide resistant acetohydroxy acid synthase protein was introduced into the conventional canola variety Kumily using *Agrobacterium rhizogenes*. The expression of these proteins in LBFLFK canola allows for the synthesis of long-chain polyunsaturated fatty acids (LC-PUFAs), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), from oleic acid and for tolerance to treatment with an imidazolinone herbicide. LBFLFK canola provides a plant-based and scalable production system for omega-3 fatty acids and will be another source of EPA and DHA for consumers, as either a food ingredient or as an aquaculture feed ingredient.

[1] A list of the field trials performed in the U.S. can be found in Appendix A.

Currently, the omega-3 LC-PUFAs EPA and DHA are primarily consumed through seafood, including finfish (e.g., salmon, tuna, and trout) and shellfish (e.g., crab, mussels, and oysters) (Blasbalg et al., 2011; Kitessa et al., 2014). Numerous health organizations recommend adult intakes of 250–500 mg combined EPA and DHA per day (Yi et al., 2014; Salem and Eggersdorfer, 2015). While this recommendation is met in some countries, many countries, including the U.S., fall below the recommended average for daily intakes (Gebauer et al., 2006; Kris-Etherton et al., 2009; Flock et al., 2013; Salem and Eggersdorfer, 2015). The primary reason for this deficiency is that the supply of the fatty acids from marine animals and other sources is limited. There is a significant challenge in producing and distributing products containing EPA and DHA to consumers in adequate quantity (Salem and Eggersdorfer, 2015). There is also high demand for fish oil as a primary ingredient for farmed fish, especially fatty fish like salmon or trout. This demand is not fully met, to the point where the harvested fish now have a reduced level of omega-3 fatty acids compared to historical levels (Kitessa et al., 2014; Tocher, 2015).

The herbicide tolerance trait of LBFLFK canola will allow for selective post-emergence weed control during field production. The use of herbicide with the active ingredient imazamox (an imidazolinone) will follow the same agronomic practices as used for Clearfield® canola. Clearfield® canola products have been widely adopted in North America (Canola Council of Canada, 2017), and herbicide applications for LBFLFK canola production will follow established weed control practices.

Event LBFLFK will be cultivated within the U.S. and processed either in the U.S. or Canada as a specialty canola variety. To maintain the quality and ensure the segregation of LBFLFK canola seeds, grains, and processed products, an Identity Preservation System (IDP) will be implemented at every step of production and handling. Processing operations will be conducted either at dedicated facilities or at facilities with specific measures in place to ensure segregation from other products. Like other canola varieties, the products of LBFLFK canola will be processed into oil and defatted meal fractions. Oil and meal from canola can be manufactured into a variety of products for human and agricultural consumption or for industrial purposes (OECD, 2011b). As a specialty canola with a fatty acid profile containing the LC-PUFAs EPA and DHA, the oil produced from LBFLFK canola will be consumed specifically for the purpose of providing dietary omega-3 LC-PUFAs. The oil will be incorporated as an ingredient into consumer food items to provide individuals more options for dietary omega-3 LC-PUFAs (see 21 CFR part 184.1472(a)(3) for categories of food where EPA- and DHA-containing oil from LBFLFK canola may be incorporated as an ingredient). The refined oil may also be provided to dietary supplement manufacturers as an alternate source of omega-3 LC-PUFA fatty acids. Additionally, the oil will be used as an input to aquafeed operations to provide omega-3 LC-PUFAs to farmed aquatic species. Defatted canola meal produced from LBFLFK canola will be

available for use in the same applications as conventional canola meal. The primary application for canola meal is as a feed ingredient for livestock. The defatted meal produced from LBFLFK canola will not be sold as a source of omega-3 LC-PUFAs as the oil content of the meal will be too low to be a significant contribution to the nutrition of the livestock.

The herbicide tolerance trait will provide a tool to growers for selective breeding and weed control during field production. The herbicide tolerance trait will allow for application of Beyond® herbicide (active ingredient imazamox) during breeding and commercial production of LBFLFK canola.

1.3. Submissions to other regulatory agencies

Under the Coordinated Framework for Regulation of Biotechnology (USDA-APHIS, 1986), the Office of Science and Technology Policy describes the U.S. Government agencies responsible for oversight of the products of agricultural modern biotechnology including the USDA Animal and Plant Health Inspection Service (USDA-APHIS), the U.S. Environmental Protection Agency (U.S. EPA), and the Department of Health and Human Services' Food and Drug Administration (FDA).

As the FDA is responsible for ensuring the safety and proper labeling of all plant-derived food and feed, including those developed through modern biotechnology, BASF Plant Science, L.P., in accordance with this policy, will initiate a consultation with the FDA and will submit molecular and protein data, compositional and nutrition data, as well as other food and feed safety assessment data related to EPA+DHA canola event LBFLFK as part of this consultation.

The U.S. EPA, through a registration process, regulates the sale, distribution, and use of pesticides to protect human and animal health and the environment. As event LBFLFK does not contain a plant-incorporated protectant, it will not be submitted to the U.S. EPA for review under the Coordinated Framework. The U.S. EPA also sets tolerance limits for residues of pesticides on and in food and feed or establishes an exemption from the requirement for a tolerance under the Federal Food, Drug, and Cosmetic Act. As event LBFLFK contains a trait of herbicide tolerance, BASF will petition the U.S. EPA to update the label for Beyond® herbicide to allow for the field application on EPA+DHA canola event LBFLFK.

Consistent with a commitment to Excellence Through Stewardship®, BASF will meet applicable regulatory requirements for LBFLFK canola in the country of intended production and for key import countries with functioning regulatory systems based on a market and trade assessment and the intended use of the product. This will assure regulatory compliance, maintain product integrity, and assist in minimizing the potential for trade disruptions.

2. THE PRODUCTION AND BIOLOGY OF CANOLA

The biology and history of canola or oilseed rape (specifically *Brassica napus* L.) is described in several comprehensive references including the biology documents published by the Organisation for Economic Co-operation and Development (OECD) (OECD, 2012a), Canadian Food Inspection Agency (CFIA) (CFIA, 2012), and Australian Government Department of Health (OGTR, 2016). These documents present information on *Brassica napus* taxonomy, morphology, agronomic practices, reproductive biology, ecological and environmental considerations, and uses as a crop plant.

2.1. Canola as a crop

Globally, canola production is concentrated in areas with dry weather and shorter growing seasons (USDA-ERS, 2016). There are spring and winter varieties of canola. Spring canola is a cool-season crop, not very drought tolerant, and the primary biotype grown in the northern regions of North America and Asia and the southern regions of Australia. Winter canola requires cold (vernalization) before flowering. It is planted in the fall at more central latitudes, e.g., in the EU, Ukraine, Russia, and parts of China (USDA-ERS, 2016). Canola varieties of *B. napus* are the primary rapeseed varieties grown for oil production in North America. The major production areas are in the Canadian prairie provinces of Manitoba, Saskatchewan, and Alberta. In the U.S., canola is grown mostly in the northwestern region of the country where a drier, shorter growing season makes corn and soybean less attractive (USDA-ERS, 2016). According to the Food and Agriculture Organization of the United Nations (FAO, 2016), the total harvested area of rapeseed in North America in 2014 was about 9 million hectares with a production of about 17 million metric tons of oilseed (Table 1). Most (> 90%) rapeseed production in North America is in Canada. Globally, North America, China, the European Union, and India are the major rapeseed production regions (USDA-FAS, 2016).

Table 1. Leading Rapeseed Producing Countries

Countries or Regions	Area Harvested (thousand hectares)	Production Quantity (thousand metric tons)
Canada	8075	15555
China	7588	14772
EU-28[1]	6716	24291
India[2]	6646	7877
Australia	2721	3832
Russian Federation	1062	1338
Ukraine	865	2198
United States	630	1140
Total (leading countries)	**34303**	**71003**

[1] Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, UK.
[2] Rapeseed production in India is predominantly *B. juncea* (Indian mustard).
Source: (FAO, 2016)

In North America, production of canola (*Brassica napus* L.) is conducted under minimal or no-till systems to protect the soil from erosion. Reduced tillage cultivation systems require pre- and post-emergent weed control. Most canola production in the U.S. and Canada utilizes at least one of three main groups of herbicide-tolerance traits: tolerance to glyphosate, tolerance to glufosinate (both derived from biotechnology), and tolerance to imidazolinone herbicides (derived from mutagenesis) (Canola Council of Canada, 2016b). As in other crops, herbicide tolerance traits in canola allow for simplified, effective chemical weed control programs.

2.1.1. The history of canola

Historically, rapeseed oil was used primarily for industrial purposes (e.g., lamp oil, soap-making), and it was produced from Brassicaceae species including *B. napus*, *B. rapa*, and *B. juncea*. Rapeseed breeding efforts to improve agronomic characteristics and oil content were intensified in Canada after its introduction from Europe in the 1940s. During the 1970s, low erucic acid rapeseed was developed from *B. napus* and *B. rapa* varieties to make rapeseed oil suitable for human consumption. A few years later, a "double-low" cultivar was developed by breeding for low erucic acid and glucosinolate levels (OGTR, 2016). These edible rapeseed varieties, collectively referred to as canola, have since been widely used for edible oil production and animal feed use (OECD, 2011b). Defined quality specifications for canola oil stipulate that the "oil shall contain less than 2% erucic acid in its fatty acid profile and the solid component shall contain less than 30 micromoles of any one or any mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate per gram of air-dry, oil-free solid" (Canola Council, 2016).

2.1.2. The taxonomy and genetics of canola

Oilseed rape belongs to the botanical family Brassicaceae, also commonly known as the mustard or cabbage family. *Brassica napus* L. (n = 19, AA and CC genomes) is a natural tetraploid containing the diploid chromosome set of both parents, also known as amphidiploid (OGTR, 2016). Common names used for oil-producing *B. napus* varieties include rape, rapeseed, oilseed rape, and turnip. The taxonomic hierarchy for this member of the mustard family (taxonomic serial number 23060) (ITIS, 2016) is as follows:

Kingdom	Plantae – plants
Subkingdom	Viridiplantae – green plants
Division	Tracheophyta – vascular plants
Subdivision	Spermatophytina – seed plants
Class	Magnoliopsida – flowering plants
Order	Brassicales
Family	Brassicaceae – mustard family
Genus	*Brassica* L. – mustard
Species	*Brassica napus* L. – turnip, rape

2.1.3. Morphology of cultivated canola

Spring and winter varieties of canola typically grow to heights ranging from 70–170 cm and 120–210 cm, respectively. A normal plant produces between 10 and 15 leaves (OGTR, 2016). The oldest leaves at the base are the largest, forming a rosette that is up to 50 cm wide. Its leaves are smooth, bluish-green, and the base of the upper leaves clasp the stem. Plants have one main supporting stem with about 15–30 nodes at a spacing of 5–10 cm and a taproot system to a maximum depth of about 120 cm (OGTR, 2016).

2.1.4. Reproductive biology and hybridization with cultivated canola and related species

Brassica napus is an amphidiploid that may have arisen in the Mediterranean or European west coastal regions as the result of a cross between two diploid *Brassica* species, *Brassica rapa* (A genome) and *Brassica oleracea* (C genome) (OECD, 2012a). *Brassica napus* is sexually compatible with other *Brassica* and related plant species, e.g., *Sinapis arvensis*, *Eruca sativa*, and *Raphanus raphanistrum*, among others (OECD, 2012a).

Brassica napus reproduces through seeds. There are no reports of vegetative reproduction under field conditions. The flowers of *B. napus* are bisexual, self-compatible, and mainly self-pollinated. The flowers develop in indeterminate simple racemes beginning at the lowest part of the main raceme and auxiliary branches and continuing towards the top of the raceme, which may attain a length of 1–2 m. *Brassica* pollen, while heavy and slightly sticky, can still become air-borne due to its small size (30–40 µm). Like any other pollen, *Brassica* pollen viability is affected by environmental conditions, especially high temperature and humidity (OGTR, 2016).

Brassica napus is mostly self-pollinated, with an average of 70% of the seeds resulting from self-pollination and 30% from cross-pollination occurring over very short distances (less than 10 m). *Brassica* pollen dispersal is mainly by wind. Its dispersal is described as presenting a leptokurtic distribution, a term that refers to the dispersal as showing a more acute peak and fatter tails than found in a normal statistical distribution. Due to this type of distribution, any foreign pollen in each site will quickly be dispersed into the massive local pollen emission. Nevertheless, low to very low pollen movements can occur at long distances depending on topographical and environmental conditions, making complete genetic isolation difficult to attain (OGTR, 2016). There are multiple models that could guess the level of gene flow that might be expected among *B. napus* fields and feral *Brassica* populations. However, since gene flow is affected by many biotic and abiotic factors, these models can only provide an approximation (OECD, 2012a). In addition to wind, insects (specifically honey bees), physical contact between flowers of neighboring plants, and animals, including humans, can act as a means of pollen dispersal (OECD, 2012a).

Seeds of *Brassica napus* develop in two-celled 6–9 cm elongated siliques or pods. Seeds are yellow to brown and black, spherical, and about 1–2 mm wide (OGTR, 2016). Seeds are released as siliques dry out. This phenomenon, known as pod shattering, is a factor that causes seed loss and impacts crop yield. Shattering is one of many traits where breeding efforts have been focused (OGTR, 2016).

2.2. Weediness potential of cultivated canola

Canola is a domesticated *Brassica* species. Canola is not identified as a noxious weed in the Federal Noxious Weed List nor does it appear in any state weed lists (USDA-NRCS, 2017). However, canola does possess a few attributes commonly associated with weeds, such as a large seed crop and harvest yield loss (Thomas et al., 1991; Brown et al., 1995), prolonged seed dormancy of 2–5 years, and an ability to persist as feral populations in disturbed habitats (Gulden et al., 2004).

2.3. Characteristics of the recipient canola variety

The host plant for EPA+DHA canola event LBFLFK is Kumily, a canola cultivar of *Brassica napus* L. The parental canola variety Kumily is a spring variety of *B. napus* developed as a doubled haploid by Svalöf Weibull AB (Svalöv, Sweden) as the result of crossing in 1996. The selection criteria for the crossing was double low quality of erucic acid and glucosinolates, early flowering and maturity, stalk stiffness, blackleg resistance, high oil, and high protein levels and yield (CFIA, 2016). It is listed as a variety with granted Plant Breeders' Rights as administered by the Canadian Food Inspection Agency, and its holder is Lantmännen SW Seed AB, certificate number 4053, valid until March 31, 2029 (CFIA, 2016). Additionally, Kumily has been evaluated by the United Kingdom Agriculture and Horticulture Development Board and has historically been on the recommended list of varieties by the Home Grown Cereals Authorities (UK Agriculture and Horticulture Development Board, 2013).

2.4. Breeding history of EPA+DHA canola event LBFLFK

The breeding history of LBFLFK, outlining the generations that were used in regulatory studies, is described below in Figure 1. A summary is presented in Table 2.

Figure 1. Breeding History of LBFLFK

T_0 corresponds to the transformed plant, and F_1 corresponds to the first filial generation of offspring of a cross between event LBFLFK and the parental canola variety Kumily. The # index in T# and F# indicate further generations resulting from self-pollination. T4A and T4B were independently propagated from the same pool of T3 seed.

⊗ designates self-pollination; × designates backcrossing.

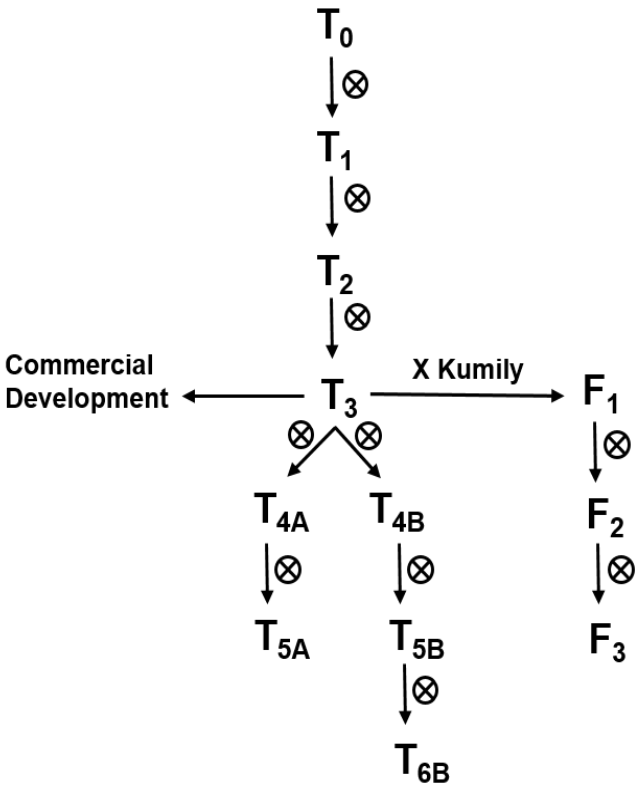


Table 2. LBFLFK Canola Starting Seed Used in Regulatory Studies

Generation	Regulatory Study
T3	• Next Generation (NGS) and Sanger sequencing, BAC clone production for molecular characterization • Agronomic and phenotypic characterization (Winter 2014/15 field trials) • Nutrient composition (Winter 2014/15 field trials) • Immature seed production for protein characterization
T4A	• Newly expressed protein levels in field produced plant tissues • Agronomic and phenotypic characterization (Spring 2015 field trials) • Nutrient composition (Spring 2015 field trial)
T4B	• NGS for molecular characterization (T-DNA insert generational stability) • Assessment of environmental interaction in the United States
T5A	• Pollen protein expression analysis
T5B	• NGS for molecular characterization (T-DNA insert generational stability) • Pollen germination, viability, morphology
F2	• Mendelian inheritance
F3	• Mendelian inheritance

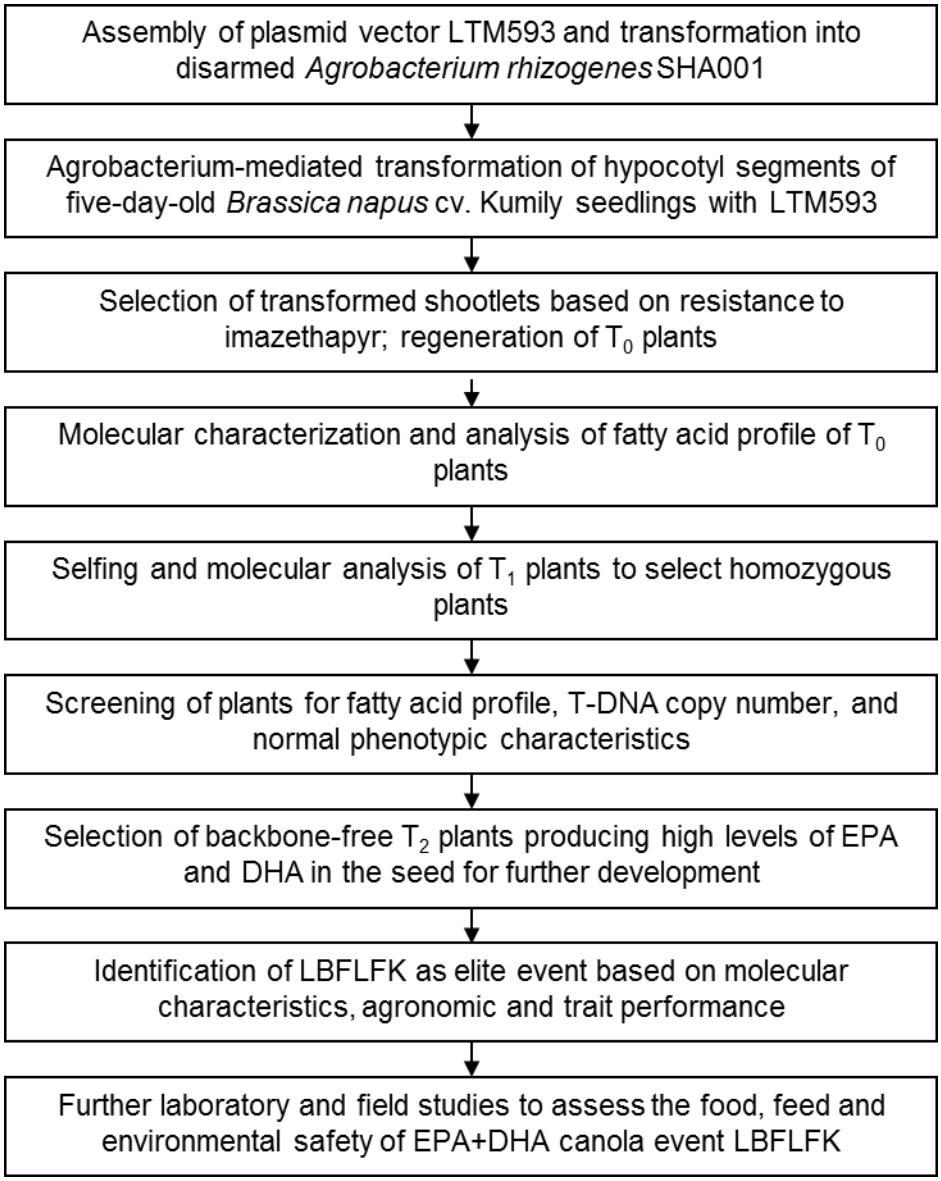
3. DESCRIPTION OF THE INTENDED GENETIC MODIFICATION

EPA+DHA canola event LBFLFK was generated via *Agrobacterium rhizogenes*-mediated transformation of *Brassica napus* cv. Kumily with the plasmid vector LTM593 to introduce fatty acid synthesis genes and an herbicide tolerance gene. This section describes the *Agrobacterium*-mediated canola transformation process, the origin, size, and function of each genetic element in plasmid vector LTM593, and the location and orientation of the elements in the plasmid.

3.1. The transformation system

Transformation was performed following a modified De Block protocol (De Block et al., 1989). Four to seven-millimeter hypocotyl segments of five-day-old Kumily seedlings were inoculated with disarmed *Agrobacterium rhizogenes* strain SHA001 containing LTM593. After three days of co-cultivation, explants were transferred to plant growth medium containing the antibiotic carbenicillin to prevent *Agrobacterium* growth. Following seven days of recovery, the explants were transferred to selection medium containing imazethapyr (an imidazolinone herbicide) and cultured for two weeks for selective regeneration of shoots. Herbicide-resistant shootlets were placed on medium facilitating shoot elongation and root development, and rooted shoots were transferred to soil for growth and further analysis. Transgenic plants (T_0 plants) regenerated via *Agrobacterium*-mediated transformation and tissue culture and subsequent T_1 and T_2 generations produced by selfing were characterized by molecular analyses, fatty acid profiles, agronomic evaluations, and herbicide efficacy analyses. Those plants that produced higher levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the seed, that were imidazolinone herbicide tolerant, free of any vector backbone from LTM593, and that displayed normal phenotypic characteristics were advanced for further development. Based on its superior characteristics, event LBFLFK was selected as an elite candidate and evaluated further. A schematic overview of the transformation and development process of EPA+DHA canola event LBFLFK is depicted in Figure 2.

Figure 2. Schematic Depicting the Process of Canola Transformation and Development of EPA+DHA Canola Event LBFLFK



3.2. The transformation vector LTM593

Plasmid vector LTM593 was used to transform canola to produce EPA+DHA canola event LBFLFK. A map of plasmid LTM593 is shown in Figure 3, and the location, size, origin, and function of the genetic elements included in the plasmid are described in Table 3. LTM593 is 60,074 base pairs (bp) in length and contains one T-DNA of 44,010 bp. The T-DNA is delineated by left border and right border sequences that are derived from the *Agrobacterium tumefaciens* octopine-type plant tumor-inducing plasmid pTi15955 (Barker et al., 1983). The T-DNA contains 13 expression cassettes. There are 12 fatty acid synthesis cassettes encoding 10 different fatty acid desaturases and elongases, of which D5D(*Tc*) and O3D(*Pir*) coding sequences are present twice, in two different expression cassettes. In addition, vector LTM593 encodes a mutant AHAS(*At*) protein with two amino acid substitutions (A122T and S653N) to confer tolerance to imidazolinone herbicides (Tan et al., 2005). The backbone region outside of the T-DNA contains genetic elements for the maintenance and selection of the plasmid in bacteria.

The origin, function, and position of each genetic element present in LTM593 are listed in Table 3. The coding sequences are described individually below followed by a description of the corresponding regulatory elements for each coding sequence.

Figure 3. Circular Map of Plasmid LTM593

Plasmid LTM593 (also indicated as LTM593-1qcz) was used in Agrobacterium-mediated transformation to generate EPA+DHA canola event LBFLFK. Coding sequences are indicated by boxes with an arrowhead, with the orientation of the arrow showing the transcription direction. Arcs with arrowheads in the inner circle indicate the 13 expression cassettes, which are named after their coding sequences. In those instances where the same coding sequence is used twice (*D5D*(*Tc*) and c-*O3D*(*Pir*)), a suffix number is added to the cassette name.

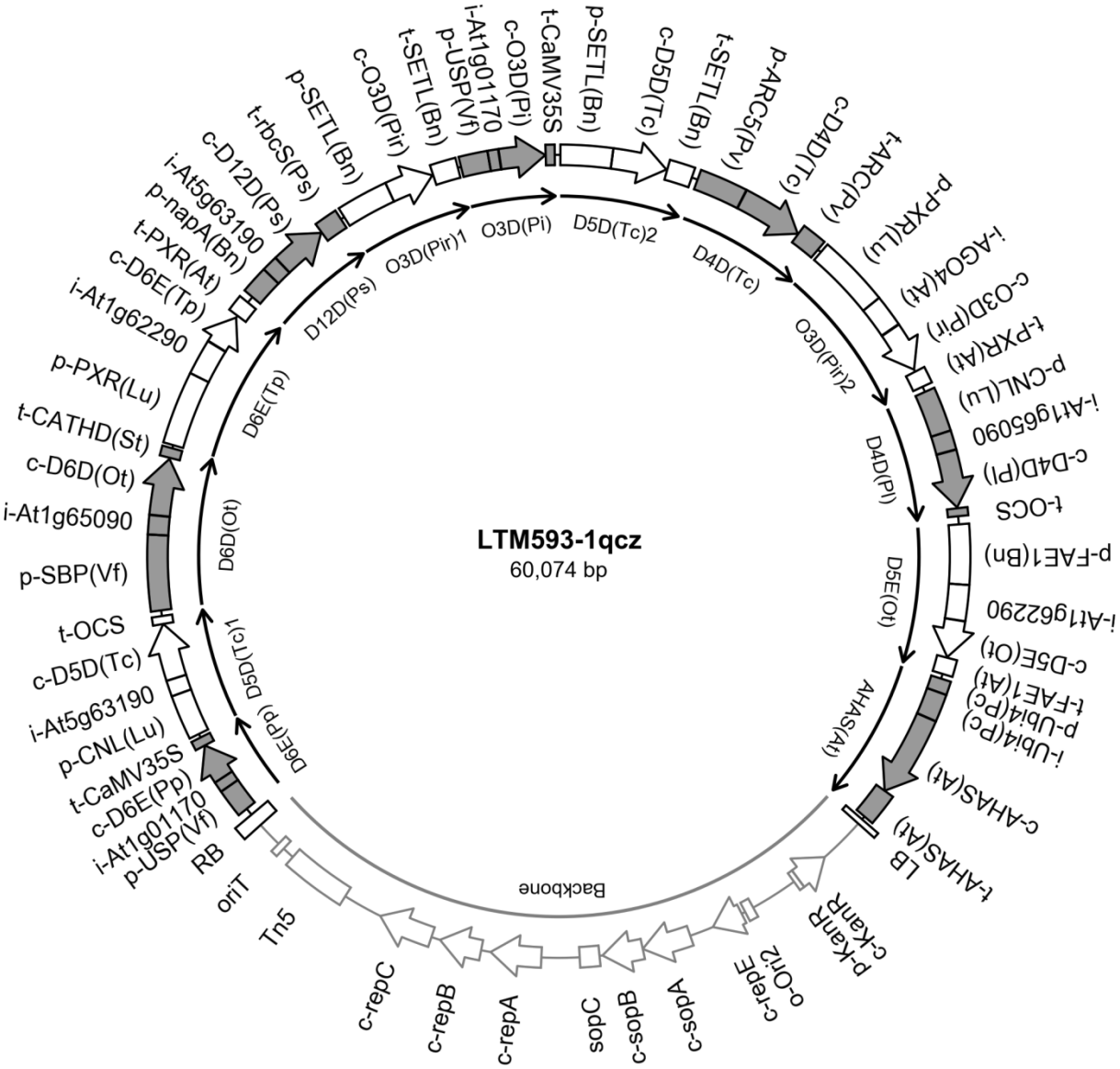


Table 3. Summary of Genetic Elements in LTM593

Prefixes used in the table: p-, promoter; i-, intron-containing 5' untranslated region (UTR); c-, coding sequence; t-, transcription terminator; o-, origin of replication.

Genetic element	Location in construct (size in base pairs)	Origin and function (reference)
T-DNA		
RB	1–328 (328)	*Agrobacterium tumefaciens*, octopine-type Ti plasmid pTi15955, right T-DNA border region, identical to section of GenBank nucleotide accession number AF242881 (Barker et al., 1983)
intervening sequence	329–508 (180)	Region required for cloning of genetic elements
p-*USP*(*Vf*)	509–1192 (684)	*Vicia faba*, promoter region of unknown seed protein gene *USP* (Bäumlein et al., 1991), identical to section of GenBank nucleotide accession number HJ187156, and highly homologous to section of GenBank nucleotide accession number X56240
i-*At1g01170*	1,193–1,444 (252)	*Arabidopsis thaliana*, intron-containing 5' UTR of locus At1g01170 (Nakabayashi et al., 2005)
intervening sequence	1,445–1,446 (2)	Region required for cloning of genetic elements
c-*D6E*(*Pp*)	1,447–2,319 (873)	*Physcomitrella patens*, delta-6 elongase (originally named as polyunsaturated fatty acid specific elongation enzyme 1, *PSE1*), codon optimized based on GenBank nucleotide accession number AF428243 (Zank et al., 2000; Zank et al., 2002)
t-*CaMV35S*	2,320–2,535 (216)	Cauliflower mosaic virus, CaMV35S terminator region, identical to section of GenBank nucleotide accession number AF234316 (Hajdukiewicz et al., 1994)
intervening sequence	2,536–2,627 (92)	Region required for cloning of genetic elements
p-*CNL*(*Lu*)	2,628–3,691 (1064)	*Linum usitatissimum*, seed-specific promoter of *conlinin* gene (Truksa et al., 2003), identical to section of GenBank nucleotide accession number HJ187156
i-*At5g63190*	3,692–4,068 (377)	*Arabidopsis thaliana*, intron-containing 5' UTR of locus At5g63190 (Sharma et al., 2007; Wang et al., 2008)
intervening sequence	4,069–4,071 (3)	Region required for cloning of genetic elements
c-*D5D*(*Tc*)1	4,072–5,391 (1320)	*Thraustochytrium* sp., delta-5 desaturase, codon optimized based on GenBank nucleotide accession number AF489588 (Qiu et al., 2001)
t-*OCS*	5,392–5,583 (192)	*Agrobacterium tumefaciens*, octopine-type Ti plasmid pTi15955, terminator of octopine synthase gene (MacDonald et al., 1991), identical to section of the GenBank nucleotide accession number NC_002377
intervening sequence	5,584–5,718 (135)	Region required for cloning of genetic elements
p-*SBP*(*Vf*)	5,719–7,517 (1799)	*Vicia faba*, promoter of a sucrose-binding protein-related gene (Grimes et al., 1992; Heim et al., 2001), active at late seed development stage, identical to GenBank nucleotide accession number LQ576466
i-*At1g65090*	7,518–7,972 (455)	*Arabidopsis thaliana*, intron-containing 5' UTR of locus At1g65090 (Braybrook et al., 2006)

Genetic element	Location in construct (size in base pairs)	Origin and function (reference)
intervening sequence	7,973–7,981 (9)	Region required for cloning of genetic elements
c-*D6D*(*Ot*)	7,982–9,352 (1371)	*Ostreococcus tauri*, delta-6 desaturase, codon optimized based on GenBank nucleotide accession number AY746357 (Domergue et al., 2005)
intervening sequence	9,353–9,379 (27)	Region required for cloning of genetic elements
t-*CATHD*(*St*)	9,380–9,614 (235)	*Solanum tuberosum*, terminator of cathepsin D inhibitor gene (Hannapel, 1993), identical to section of GenBank nucleotide accession number HJ187168
intervening sequence	9,615–9,692 (78)	Region required for cloning of genetic elements
p-*PXR*(*Lu*)	9,693–11,419 (1727)	*Linum usitatissimum*, seed-specific promoter of peroxiredoxin like protein gene *PXR* (Duwenig and Loyall, 2006), identical to GenBank nucleotide accession number HL700593
i-*At1g62290*	11,420–12,265 (846)	*Arabidopsis thaliana*, intron-containing 5' UTR of locus At1g62290 (Chen et al., 2002)
intervening sequence	12,266–12,278 (13)	Region required for cloning of genetic elements
c-*D6E*(*Tp*)	12,279–13,097 (819)	*Thalassiosira pseudonana*, delta-6 elongase (Ambrust et al., 2004), codon optimized based on GenBank nucleotide accession number XM_002288445
intervening sequence	13,098–13,152 (55)	Region required for cloning of genetic elements
t-*PXR*(*At*)	13,153–13,552 (400)	*Arabidopsis thaliana*, terminator of peroxiredoxin (PXR)-like protein gene *PER1* (GenBank nucleotide accession number HL700651, At1g48130, (Haslekås et al., 1998))
intervening sequence	13,553–13,721 (169)	Region required for cloning of genetic elements
p-*napA*(*Bn*)	13,722–14,385 (664)	*Brassica napus*, seed-specific promoter of seed storage protein napin A/B gene (Ellerström et al., 1996; Rask et al., 1998), identical to GenBank nucleotide accession number LQ576463
i-*At5g63190*	14,386–14,762 (377)	*Arabidopsis thaliana*, intron-containing 5' UTR of locus At5g63190 (Sharma et al., 2007; Wang et al., 2008)
intervening sequence	14,763–14,768 (6)	Region required for cloning of genetic elements
c-*D12D*(*Ps*)	14,769–15,965 (1197)	*Phytophthora sojae*, delta-12 desaturase, codon optimized based on GenBank accession number GY508423 (Cirpus and Bauer, 2006)
intervening sequence	15,966–15,983 (18)	Region required for cloning of genetic elements
t-*rbcS*(*Ps*)	15,984–16,541 (558)	*Pisum sativum*, terminator of RuBisCO small subunit gene (*rbcS*) E9 (Coruzzi et al., 1984; Smigocki, 1991), identical to section of GenBank nucleotide accession number AY572837
intervening sequence	16,542–16,633 (92)	Region required for cloning of genetic elements
p-*SETL*(*Bn*)	16,634–17,867 (1234)	*Brassica napus*, seed-specific promoter of *SETL* gene (Bauer and Senger, 2010), identical to a section of GenBank nucleotide accession number HC307781
intervening sequence	17,868–17,869 (2)	Region required for cloning of genetic elements

Genetic element	Location in construct (size in base pairs)	Origin and function (reference)
c-*O3D*(*Pir*)1	17,870–18,961 (1092)	*Pythium irregulare*, omega-3 desaturase, codon optimized based on GenBank nucleotide accession number FB753541 (Cheng et al., 2010)
intervening sequence	18,962–18,982 (21)	Region required for cloning of genetic elements
t-*SETL*(*Bn*)	18,983–19,596 (614)	*Brassica napus*, terminator of *SETL* gene (Bauer and Senger, 2010), identical to GenBank nucleotide accession number HC307782
intervening sequence	19,597–19,674 (78)	Region required for cloning of genetic elements
p-*USP*(*Vf*)	19,675–20,358 (684)	*Vicia faba*, promoter region of unknown seed protein gene *USP* (Bäumlein et al., 1991), identical to section of GenBank nucleotide accession number HJ187156, and highly homologous to section of GenBank nucleotide accession number X56240
i-*At1g01170*	20,359–20,610 (252)	*Arabidopsis thaliana*, intron-containing 5' UTR of locus At1g01170 (Nakabayashi et al., 2005)
intervening sequence	20,611–20,620 (10)	Region required for cloning of genetic elements
c-*O3D*(*Pi*)	20,621–21,706 (1086)	*Phytophthora infestans*, omega-3 desaturase, codon optimized based on GenBank nucleotide accession number XM_002902553 (Wu et al., 2005)
intervening sequence	21,707–21,714 (8)	Region required for cloning of genetic elements
t-*CaMV35S*	21,715–21,930 (216)	Cauliflower mosaic virus, CaMV35S terminator region, identical to section of GenBank nucleotide accession number AF234316 (Hajdukiewicz et al., 1994)
intervening sequence	21,931–22,065 (135)	Region required for cloning of genetic elements
p-*SETL*(*Bn*)	22,066–23,299 (1234)	*Brassica napus*, seed-specific promoter of *SETL* gene (Bauer and Senger, 2010), identical to a section of GenBank nucleotide accession number HC307781
intervening sequence	23,300–23,301 (2)	Region required for cloning of genetic elements
c-*D5D*(*Tc*)2	23,302–24,621 (1320)	*Thraustochytrium* sp., delta-5 desaturase, codon optimized based on GenBank nucleotide accession number AF489588 (Qiu et al., 2001)
intervening sequence	24,622–24,642 (21)	Region required for cloning of genetic elements
t-*SETL*(*Bn*)	24,643–25,256 (614)	*Brassica napus*, terminator of *SETL* gene (Bauer and Senger, 2010), identical to GenBank nucleotide accession number HC307782
intervening sequence	25,257–25,402 (146)	Region required for cloning of genetic elements
p-*ARC5*(*Pv*)	25,403–26,553 (1151)	*Phaseolus vulgaris*, seed–specific *Arcelin-5* gene promoter, identical to GenBank nucleotide accession number JC056714, and homologous to GenBank nucleotide accession number Z50202 (Goossens et al., 1994; Goossens et al., 1999)
intervening sequence	26,554–26,563 (10)	Region required for cloning of genetic elements

Genetic element	Location in construct (size in base pairs)	Origin and function (reference)
c-*D4D*(*Tc*)	26,564–28,123 (1560)	*Thraustochytrium* sp., delta-4 desaturase, codon optimized based on GenBank nucleotide accession number GN042654 (Qiu et al., 2001)
intervening sequence	28,124–28,136 (13)	Region required for cloning of genetic elements
t-*ARC*(*Pv*)	28,137–28,736 (600)	*Phaseolus vulgaris*, terminator of *Arc5* gene, identical to section of GenBank nucleotide accession number Z50202 (Goossens et al., 1994; Goossens et al., 1999)
intervening sequence	28,737–28,828 (92)	Region required for cloning of genetic elements
p-*PXR*(*Lu*)	28,829–30,555 (1727)	*Linum usitatissimum*, seed-specific promoter of peroxiredoxin like protein gene *PXR* (Duwenig and Loyall, 2006), identical to GenBank nucleotide accession number HL700593
i-*AGO4*(*At*)	30,556–31,313 (758)	*Arabidopsis thaliana*, intron-containing 5' UTR of gene *AGO4*(*At*) (Zilberman et al., 2003)
intervening sequence	31,314–31,328 (15)	Region required for cloning of genetic elements
c-*O3D*(*Pir*)2	31,329–32,420 (1092)	*Pythium irregulare*, omega-3 desaturase, codon optimized based on GenBank nucleotide accession number FB753541 (Cheng et al., 2010)
intervening sequence	32,421–32,476 (56)	Region required for cloning of genetic elements
t-*PXR*(*At*)	32,477–32,876 (400)	*Arabidopsis thaliana*, terminator of peroxiredoxin (PXR)-like protein gene *PER1* (GenBank nucleotide accession number HL700651, At1g48130 (Haslekås et al., 1998))
intervening sequence	32,877–33,011 (135)	Region required for cloning of genetic elements
p-*CNL*(*Lu*)	33,012–34,075 (1064)	*Linum usitatissimum*, seed-specific promoter of *conlinin* gene (Truksa et al., 2003), identical to section of GenBank nucleotide accession number HJ187156
i-*At1g65090*	34,076–34,530 (455)	*Arabidopsis thaliana*, intron-containing 5' UTR of locus At1g65090 (Braybrook et al., 2006)
intervening sequence	34,531–34,539 (9)	Region required for cloning of genetic elements
c-*D4D*(*Pl*)	34,540–35,877 (1338)	*Pavlova lutheri*, delta-4 desaturase, codon optimized based on GenBank nucleotide accession number AY332747 (Tonon et al., 2003)
intervening sequence	35,878–35,898 (21)	Region required for cloning of genetic elements
t-*OCS*	35,899–36,090 (192)	*Agrobacterium tumefaciens*, octopine-type Ti plasmid pTi15955, terminator of octopine synthase gene (MacDonald et al., 1991), identical to section of the GenBank nucleotide accession number NC_002377
intervening sequence	36,091–36,283 (193)	Region required for cloning of genetic elements
p-*FAE1*(*Bn*)	36,284–37,713 (1430)	*Brassica napus*, promoter of fatty acid elongase (*FAE1.1*) gene, identical to section of GenBank nucleotide accession number HC474755, and highly homologous to section of GenBank nucleotide accession number AF275254 (Han et al., 2001)
i-*At1g62290*	37,714–38,560 (847)	*Arabidopsis thaliana*, intron-containing 5' UTR of locus At1g62290 (aspartyl protease family protein) (Chen et al., 2002)

Genetic element	Location in construct (size in base pairs)	Origin and function (reference)
intervening sequence	38,561–38,567 (7)	Region required for cloning of genetic elements
c-*D5E*(*Ot*)	38,568–39,470 (903)	*Ostreococcus tauri*, delta-5 elongase (Zank et al., 2005), codon optimized based on GenBank nucleotide accession number CS020159
intervening sequence	39,471–39,486 (16)	Region required for cloning of genetic elements
t-*FAE1*(*At*)	39,487–39,886 (400)	*Arabidopsis thaliana*, terminator of fatty acid elongase gene (*FAE1*) (Rossak et al., 2001), identical to section of GenBank nucleotide accession number HV571989
intervening sequence	39,887–40,004 (118)	Region required for cloning of genetic elements
p-*Ubi4*(*Pc*)	40,005–40,398 (394)	*Petroselinum crispum*, ubiquitin (Pcubi4-2) promoter, identical to section of GenBank nucleotide accession number X64345 (Kawalleck et al., 1993)
i-*Ubi4*(*Pc*)	40,399–40,986 (588)	*Petroselinum crispum*, ubiquitin gene intron in the 5' UTR, identical to section of GenBank nucleotide accession number JC289689, and highly homologous to section of GenBank nucleotide accession number X64345 (Kawalleck et al., 1993)
intervening sequence	40,987–40,993 (7)	Region required for cloning of genetic elements
c-*AHAS*(*At*)	40,994–43,006 (2013)	*Arabidopsis thaliana*, acetohydroxy acid synthase large-subunit (Mazur et al., 1987) with S653N substitution and A122T substitution, highly homologous to GenBank nucleotide accession number NM_114714
t-*AHAS*(*At*)	43,007–43,786 (780)	*Arabidopsis thaliana*, terminator of *AHAS*(*At*) gene (Mazur et al., 1987), highly homologous to a segment in GenBank nucleotide accession number CP002686
intervening sequence	43,787–43,874 (88)	Region required for cloning of genetic elements
LB	43,875–44,010 (136)	*Agrobacterium tumefaciens*, octopine-type Ti plasmid pTi15955, left T-DNA border region, identical to section of GenBank nucleotide accession number AF242881 (Barker et al., 1983)
Vector backbone		
intervening sequence	44,011–45,141 (1131)	Bases 44170 to 44835 is a partial chloramphenicol acetyltransferase gene, including its promoter and partial coding sequence, identical to section of GenBank nucleotide accession number HQ245711
c-*KanR*	Complement 45,142–45,957 (816)	*E. coli*, aminoglycoside 3'-phosphotransferase of kanamycin resistance transposon Tn*903* (Oka et al., 1981; Naumovski and Friedberg, 1983), identical to a section of GenBank nucleotide accession number V00359
p-*KanR*	Complement 45,958–46,078 (121)	*E. coli*, promoter for aminoglycoside 3'-phosphotransferase gene of kanamycin resistance transposon Tn*903* (Naumovski and Friedberg, 1983), identical to a section of GenBank nucleotide accession number NZ_CP009789
intervening sequence	46,079–47,230 (1152)	Region required for cloning of genetic elements

Genetic element	Location in construct (size in base pairs)	Origin and function (reference)
o-*Ori2*	47,231–47,447 (217)	*E. coli*, replication origin (ori-2) of the F plasmid (Murotsu et al., 1984), identical to section of GenBank nucleotide accession number AP001918
intervening sequence	47,448–47,540 (93)	Native intergenic sequence from the *E. coli* F plasmid
c-*repE*	47,541–48,296 (756)	*E. coli*, *repE* gene of the F plasmid
intervening sequence	48,297–48,874 (578)	Native intergenic sequence between c-repE and c-sopA
c-*sopA*	48,875–50,050 (1176)	*E. coli*, *sopA* gene of the F plasmid (Mori et al., 1986)
c-*sopB*	50,050–51,021 (972)	*E. coli*, *sopB* gene of the F plasmid
intervening sequence	51,022–51,093 (72)	Native intergenic sequence between *sopB* and *sopC*
sopC	51,094–51,567 (474)	*E. coli*, partial *sopC* region of the F plasmid required for plasmid partition (Helsberg and Eichenlaub, 1986; Mori et al., 1986)
intervening sequence	51,568–52,480 (913)	Sequence flanking the repABC operon from pTiC58, contains the promoter of operon *repABC* (Li and Farrand, 2000)
c-*repA*	52,481–53,698 (1218)	*Agrobacterium tumefaciens*, *repA* gene from pTiC58 replicon (Li and Farrand, 2000)
intervening sequence	53,699–53,927 (229)	Native intergenic sequence in the *repABC* operon
c-*repB*	53,928–54,938 (1011)	*Agrobacterium tumefaciens*, *repB* gene from pTiC58 replicon (Li and Farrand, 2000)
intervening sequence	54,939–55,152 (214)	Native intergenic sequence in the *repABC* operon
c-*repC*	55,153–56,472 (1320)	*Agrobacterium tumefaciens*, *repC* gene from pTiC58 replicon (Li and Farrand, 2000)
intervening sequence	56,473–57,429 (957)	Region required for cloning of genetic elements
Tn*5*	57,430–58,991 (1562)	*E. coli*, transposon Tn*5* sequence (Beck et al., 1982), not required for the functionality of LTM593
intervening sequence	58,992–59,286 (295)	Region required for cloning of genetic elements
oriT	59,287–59,455 (169)	*Agrobacterium tumefaciens*, origin of conjugal transfer from pRK310 (Marx and Lidstrom, 2001)
intervening sequence	59,456–60,074 (619)	Region required for cloning of genetic elements

3.3. Donor genes

The coding sequences in the T-DNA of plasmid vector LTM593 are derived from different eukaryotic organisms (Table 4). To achieve optimal translation rate, the sequences encoding the fatty acid synthesis enzymes were optimized for codon usage in *B. napus*. In addition, the nucleotide sequences were modified to remove the following elements: (i) additional open reading frames (ORFs) longer than 90 bp in sense and anti-sense direction, (ii) ORFs within 30 bp after the start codon in sense direction, (iii) internal TATA-boxes, chi sequences, and ribosomal entry sites, (iv) AT-rich or GC-rich sequence stretches, (v) RNA instability motifs, (vi) RNA secondary structures and repeat sequences, and (vii) possible cryptic intron splice donor and acceptor sites in higher eukaryotes. Regarding the AHAS(*At*) coding sequence, a few nucleotide changes were introduced to eliminate unwanted restriction sites. These changes did not result in a change to the amino acid sequence of the protein. In addition, the coding sequence contains two mutations, which result in the desired amino acid substitutions A122T and S653N[2] that confer herbicide tolerance. In the following, the fatty acid desaturases and elongases are listed in order of the introduced biosynthesis pathway (Figure 4).

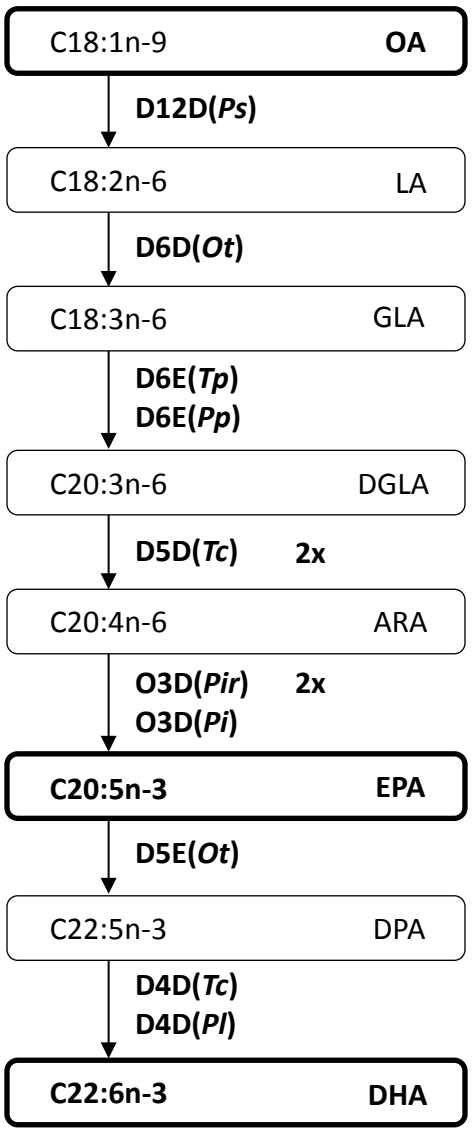
Table 4. Designation and Donor Organisms of the Newly Expressed Proteins

Coding sequence designation	Enzyme full name	Enzyme abbreviation	Donor organism
c-*D12D*(*Ps*)	Delta-12 desaturase (*Ps*)	D12D(*Ps*)	*Phytophthora sojae*
c-*D6D*(*Ot*)	Delta-6 desaturase (*Ot*)	D6D(*Ot*)	*Ostreococcus tauri*
c-*D6E*(*Tp*)	Delta-6 elongase (*Tp*)	D6E(*Tp*)	*Thalassiosira pseudonana*
c-*D6E*(*Pp*)	Delta-6 elongase (*Pp*)	D6E(*Pp*)	*Physcomitrella patens*
c-*D5D*(*Tc*)	Delta-5 desaturase (*Tc*)	D5D(*Tc*)	*Thraustochytrium* sp.
c-*O3D*(*Pir*)	Omega-3 desaturase (*Pir*)	O3D(*Pir*)	*Pythium irregulare*
c-*O3D*(*Pi*)	Omega-3 desaturase (*Pi*)	O3D(*Pi*)	*Phytophthora infestans*
c-*D5E*(*Ot*)	Delta-5 elongase (*Ot*)	D5E(*Ot*)	*Ostreococcus tauri*
c-*D4D*(*Pl*)	Delta-4 desaturase (*Pl*)	D4D(*Pl*)	*Pavlova lutheri*
c-*D4D*(*Tc*)	Delta-4 desaturase (*Tc*)	D4D(*Tc*)	*Thraustochytrium* sp.
c-*AHAS*(*At*)	Acetohydroxy acid synthase	AHAS(*At*)	*Arabidopsis thaliana*

[2] Substitution of an alanine residue (A) with a threonine (T) at amino acid 122 and a serine residue (S) with an asparagine (N) at amino acid 653, relative to the native *Arabidopsis thaliana* protein.

Figure 4. Diagram of the Fatty Acid Synthesis Pathway Introduced into EPA+DHA Canola Event LBFLFK

The diagram depicts the EPA and DHA biosynthesis pathway from oleic acid (OA) in EPA+DHA canola event LBFLFK. Lipid numbers are indicated in the form Ca:b, where a is the number of carbon atoms and b is the number of double bonds in the fatty acid. The n−x nomenclature indicates that a double bond is located on the xth carbon–carbon bond, counting from the terminal omega (methyl) carbon. Enzyme abbreviations are D12D: delta-12 desaturase; D6D: delta-6 desaturase; D6E: delta-6 elongase; D5D: delta-5 desaturase; O3D: omega-3 desaturase; D5E: delta-5 elongase; D4D: delta-4 desaturase. The abbreviation in the parenthesis following each enzyme indicates the donor organism of the corresponding enzyme, *Ps*: *Phytophthora sojae*; *Ot*: *Ostreococcus tauri*; *Tp*: *Thalassiosira pseudonana*; *Pp*: *Physcomitrella patens*; *Tc*: *Thraustochytrium* sp.; *Pir*: *Pythium irregulare*; *Pi*: *Phytophthora infestans*; *Pl*: *Pavlova lutheri*. OA: Oleic acid; LA: Linoleic acid; GLA: Gamma-linolenic acid; DGLA: Dihomo-gamma-linolenic acid; ARA: Arachidonic acid; EPA: Eicosapentaenoic acid; DPA: Docosapentaenoic acid; DHA: Docosahexaenoic acid; 2x: the same coding sequence is used in two different expression cassettes.



To demonstrate that the nucleotide sequences in LTM593 would have the same translated amino acid sequences of the encoded proteins as those found in the donor organisms, pairwise amino acid sequence alignment using the ClustalW interface was applied (Thompson et al., 1994). A comparison between the deduced amino acid sequences of the fatty acid desaturases and elongases present in LTM593 and the respective amino acid sequences from the donor organisms established that no changes were introduced in the plasmid for all but one coding sequence. The c-*D6E*(*Tp*) sequence encodes for a serine at position 196 while the published sequence (Ambrust et al., 2004) from the donor organism encodes a proline at position 196. This P196S substitution does not occur in any known conserved domains responsible for the functionality of the D6E(*Tp*) protein. Pairwise amino acid sequence alignment between the deduced amino acid sequence of AHAS(*At*) present in LTM593 and the respective amino acid sequence from Arabidopsis showed that only the intended amino acid changes (A122T and S653N) were introduced.

3.3.1. D12D(*Ps*) coding sequence

The D12D(*Ps*) coding sequence (c-*D12D*(*Ps*)) encodes the delta-12 desaturase protein, a polypeptide of 398 amino acids approximately 46 kDa in size. The c-*D12D*(*Ps*) sequence (Cirpus and Bauer, 2006) is derived from the common oomycete (water mold) species *Phytophthora sojae*. The D12D(*Ps*) protein creates a double bond at the 12th position from the carboxyl end of oleic acid (OA) and catalyzes the conversion of OA to linoleic acid (LA).

3.3.2. D6D(*Ot*) coding sequence

The D6D(*Ot*) coding sequence (c-*D6D*(*Ot*)) encodes the delta-6 desaturase protein, a polypeptide of 456 amino acids approximately 52 kDa in size. The c-*D6D*(*Ot*) sequence (Domergue et al., 2005) is derived from the unicellular marine green alga species *Ostreococcus tauri*. The D6D(*Ot*) protein creates a double bond at the sixth position from the carboxyl end of LA and catalyzes the conversion of LA to γ-linolenic acid (GLA).

3.3.3. D6E(*Tp*) coding sequence

The D6E(*Tp*) coding sequence (c-*D6E*(*Tp*)) encodes the delta-6 elongase protein, a polypeptide of 272 amino acids approximately 32 kDa in size. The c-*D6E*(*Tp*) sequence (Ambrust et al., 2004) is derived from unicellular marine diatom alga species *Thalassiosira pseudonana*. The D6E(*Tp*) protein adds two carbon-hydrogen groups to the carboxyl end of GLA and catalyzes the conversion of GLA to dihomo-gamma-linolenic acid (DGLA). The c-*D6E*(*Tp*) sequence introduced using LTM593 includes a sequence substitution such that the translated protein has one amino acid difference compared to the published protein sequence from the donor organism (Ambrust et al., 2004). The c-*D6E*(*Tp*) sequence encodes for a serine at position 196 while the published sequence from the donor organism encodes a proline at position 196. This P196S substitution does not occur in any known conserved domains responsible for the functionality of the D6E(*Tp*) protein (Leonard et al., 2004; Meyer et al., 2004).

3.3.4. D6E(*Pp*) coding sequence

The D6E(*Pp*) coding sequence (c-*D6E*(*Pp*)) encodes the delta-6 elongase protein, a polypeptide of 290 amino acids approximately 34 kDa in size. The c-*D6E*(*Pp*) sequence (Zank et al., 2000; Zank et al., 2002) is derived from the moss species *Physcomitrella patens*. The D6E(*Pp*) protein adds two carbon-hydrogen groups to the carboxyl end of GLA and catalyzes the conversion of GLA to DGLA.

3.3.5. D5D(*Tc*) coding sequence

The D5D(*Tc*) coding sequence (c-*D5D*(*Tc*)) encodes the delta-5 desaturase protein, a polypeptide of 439 amino acids approximately 50 kDa in size. The c-*D5D*(*Tc*) sequence (Qiu et al., 2001) is derived from the marine protist *Thraustochytrium* sp. The D5D(*Tc*) protein creates a double bond at the fifth position from the carboxyl end of DGLA and catalyzes the conversion of DGLA to arachidonic acid (ARA). The c-*D5D*(*Tc*) sequence is present in two different expression cassettes in the T-DNA of LTM593.

3.3.6. O3D(*Pir*) coding sequence

The O3D(*Pir*) coding sequence (c-*O3D*(*Pir*)) encodes the omega-3 desaturase protein, a polypeptide of 363 amino acids approximately 40 kDa in size. The c-*O3D*(*Pir*) sequence (Cheng et al., 2010) is from the common oomycete (water mold) species *Pythium irregulare*. The O3D(*Pir*) protein creates a double bond at the third position from the omega (methyl) end of ARA and catalyzes the conversion of ARA to EPA. The c-*O3D*(*Pir*) coding sequence is present in two different expression cassettes in the T-DNA of LTM593.

3.3.7. O3D(*Pi*) coding sequence

The O3D(*Pi*) coding sequence (c-*O3D*(*Pi*)) encodes the omega-3 desaturase protein, a polypeptide of 361 amino acids approximately 41 kDa in size. The c-*O3D*(*Pi*) sequence (Wu et al., 2005) is from the common oomycete (water mold) species *Phytophthora infestans*. The O3D(*Pi*) protein creates a double bond at the third position from the omega (methyl) end of ARA and catalyzes the conversion of ARA to EPA.

3.3.8. D5E(*Ot*) coding sequence

The D5E(*Ot*) coding sequence (c-*D5E*(*Ot*)) encodes the delta-5 elongase protein, a polypeptide of 300 amino acids approximately 34 kDa in size. The c-*D5E*(*Ot*) sequence (Zank et al., 2005) is from the unicellular marine green alga species *Ostreococcus tauri*. The D5E(*Ot*) protein adds two carbon-hydrogen groups to the carboxyl end of EPA and catalyzes the conversion of EPA to docosapentaenoic acid (DPA).

3.3.9. D4D(*Tc*) coding sequence

The D4D(*Tc*) coding sequence (c-*D4D*(*Tc*)) encodes the delta-4 desaturase protein, a polypeptide of 519 amino acids approximately 59 kDa in size. The c-*D4D*(*Tc*) sequence (Qiu et al., 2001) is from the marine protist *Thraustochytrium* sp. The D4D(*Tc*) protein creates a double bond at the fourth position from the carboxyl end of DPA and catalyzes the conversion of DPA to DHA.

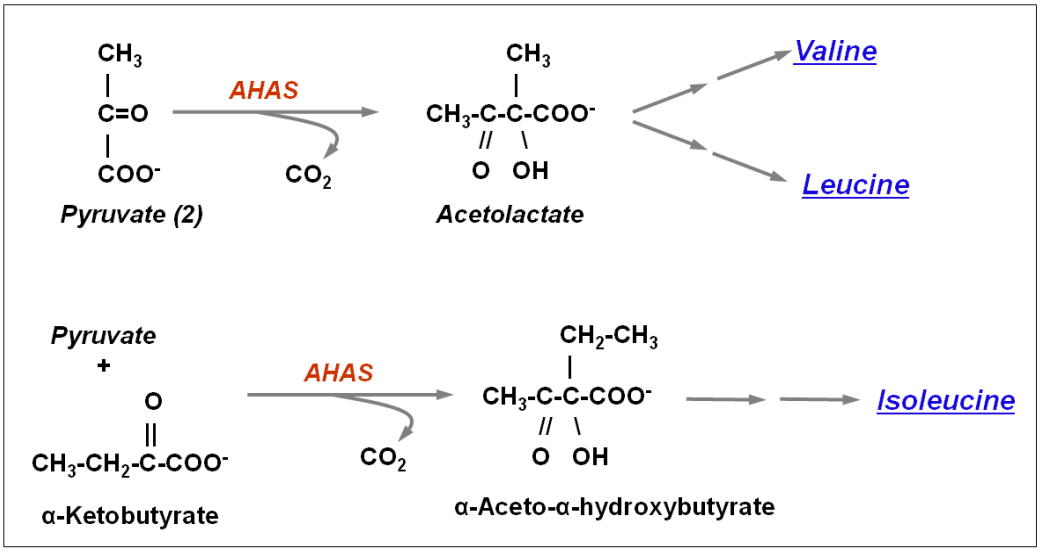
3.3.10. D4D(*Pl*) coding sequence

The D4D(*Pl*) coding sequence (c-*D4D*(*Pl*)) encodes the delta-4 desaturase protein, a polypeptide of 445 amino acids approximately 49 kDa in size. The c-*D4D*(*Pl*) sequence (Tonon et al., 2003) is from the unicellular marine photosynthetic alga species *Pavlova lutheri*. The D4D(*Pl*) protein creates a double bond at the fourth position from the carboxyl end of DPA and catalyzes the conversion of DPA to DHA.

3.3.11. AHAS(*At*) coding sequence

The AHAS(*At*) coding sequence (c-*AHAS*(*At*)) encodes the large subunit of acetohydroxy acid synthase, a polypeptide of 670 amino acids approximately 73 kDa in size. The c-*AHAS*(*At*) sequence (Mazur et al., 1987) is derived from the plant species *Arabidopsis thaliana.* The AHAS(*At*) protein catalyzes the first step in the biosynthesis of branched-chain amino acids (Figure 5). The c-*AHAS*(*At*) sequence introduced using LTM593 includes sequence substitutions such that the translated protein has an alanine substituted with a threonine at amino acid position 122 (A122T) and a serine substituted with an asparagine at amino acid position 653 (S653N). These amino acid substitutions impair imidazolinone binding to the AHAS(*At*) large subunit protein, rendering plants containing the protein tolerant to treatment with imidazolinone herbicides (Haughn and Somerville, 1990).

Figure 5. Enzymatic Reactions Catalyzed by Acetohydroxy Acid Synthase (AHAS)



3.4. Regulatory sequences

Expression of the c-*D12D*(*Ps*) coding sequence is driven by p-*napA*(*Bn*), a seed-specific promoter from *B. napus* (Ellerström et al., 1996; Rask et al., 1998). The intron-containing 5' untranslated region (UTR) used (i-At5g63190) is from Arabidopsis locus At5g63190 (Sharma et al., 2007; Wang et al., 2008). Polyadenylation and termination of transcription is directed by t-*rbcS*(*Ps*), the E9 3' UTR of the *rbcS* gene from garden pea (*Pisum sativum*) encoding the small subunit of ribulose bisphosphate carboxylase protein (Coruzzi et al., 1984; Smigocki, 1991).

Expression of the c-*D6D*(*Ot*) coding sequence is driven by p-*SBP*(*Vf*), a seed-specific sucrose-binding protein-related gene promoter from faba bean (*Vicia faba*) (Grimes et al., 1992; Heim et al., 2001). The intron-containing 5' UTR (i-At1g65090) is from Arabidopsis locus At1g65090 (Braybrook et al., 2006). Polyadenylation and termination of transcription is directed by t-*CATHD*(*St*), the 3' UTR of potato (*Solanum tuberosum*) cathepsin D inhibitor gene (Hannapel, 1993).

Expression of the c-*D6E*(*Tp*) coding sequence is driven by p-*PXR*(*Lu*), the seed-specific peroxiredoxin like protein gene *PXR* promoter of flax (*Linum usitatissimum*) (Duwenig and Loyall, 2006). The intron-containing 5' UTR (i-At1g62290) is from Arabidopsis locus At1g62290 (Chen et al., 2002). Polyadenylation and termination of transcription is directed by t-*PXR*(*At*), the 3' UTR of *Arabidopsis thaliana* peroxiredoxin (PXR)-like protein gene *PER1* (Haslekås et al., 1998).

Expression of the c-*D6E*(*Pp*) coding sequence is under the control of p-*USP*(*Vf*), the promoter of a seed protein gene of unknown function from faba bean (*Vicia faba*) (Bäumlein et al., 1991). The intron-containing 5' UTR (i-At1g01170) is from Arabidopsis locus At1g01170 (Nakabayashi et al., 2005). Polyadenylation and termination of transcription is directed by *t-CaMV35S*, the 35S 3' UTR derived from the Cauliflower Mosaic Virus (Hajdukiewicz et al., 1994).

There are two expression cassettes for c-*D5D*(*Tc*). For the first, designated D5D(*Tc*)1, expression is driven by p-*CNL*(*Lu*), the seed-specific *conlinin* gene promoter from flax (*Linum usitatissimum*) (Truksa et al., 2003). The intron-containing 5' UTR (i-At5g63190) is from Arabidopsis locus At5g63190 (Sharma et al., 2007; Wang et al., 2008). Polyadenylation and termination of transcription is directed by t-*OCS*, the 3' UTR of the octopine synthase gene (MacDonald et al., 1991).

For the second expression cassette of c-*D5D*(*Tc*), designated D5D(*Tc*)2, expression is driven by p-*SETL*(*Bn*), the seed-specific *B. napus SETL* gene promoter (Bauer and Senger, 2010). Transcription termination is directed by t-*SETL*(*Bn*), the terminator of the *B. napus SETL* gene (Bauer and Senger, 2010).

Expression of the c-*O3D*(*Pi*) coding sequence is driven by p-*USP*(*Vf*), the promoter of a seed protein gene of unknown function from faba bean (*Vicia faba*) (Bäumlein et al., 1991). The intron-containing 5' UTR (i-At1g01170) is from Arabidopsis locus At1g01170 (Nakabayashi et al., 2005). Polyadenylation and termination of transcription is directed by t-*CaMV35S*, the 3' UTR derived from the Cauliflower Mosaic Virus (Hajdukiewicz et al., 1994).

There are two expression cassettes for c-*O3D*(*Pir*). For the first, designated O3D(*Pir*)1, expression is driven by p-*SETL*(*Bn*), the seed-specific *B. napus SETL* gene promoter (Bauer and Senger, 2010). Transcription termination is directed by t-*SETL*(*Bn*), the terminator of the *B. napus SETL* gene (Bauer and Senger, 2010).

For the second expression cassette of c-*O3D*(*Pir*), designated O3D(*Pir*)2, expression is driven by p-*PXR*(*Lu*), the seed-specific peroxiredoxin like protein gene *PXR* promoter of flax (*Linum usitatissimum*) (Duwenig and Loyall, 2006). The intron-containing 5' UTR (i-*AGO4*(*At*)) is from Arabidopsis gene *AGO4*(*At*) (Zilberman et al., 2003). Polyadenylation and termination of transcription is directed by t-*PXR*(*At*), the 3' UTR of *Arabidopsis thaliana* peroxiredoxin (PXR)-like protein gene *PER1* (Haslekås et al., 1998).

Expression of the c-*D5E*(*Ot*) coding sequence is driven by p-*FAE1*(*Bn*), a seed-specific gene promoter for a fatty acid elongase from *B. napus* (Han et al., 2001). The intron-containing 5' UTR (i-At1g62290) is from Arabidopsis locus At1g62290 (Chen et al., 2002). Polyadenylation and termination of transcription is directed by t-*FAE1*(*At*), the 3' UTR of a fatty acid elongase gene from *Arabidopsis thaliana* (Rossak et al., 2001).

Expression of the c-*D4D*(*Tc*) coding sequence is driven by p-*ARC5*(*Pv*), the seed-specific *Arcelin-5* (*Arc5*) gene promoter from kidney bean (*Phaseolus vulgaris*) (Goossens et al., 1994; Goossens et al., 1999). Polyadenylation and termination of transcription is directed by the *Phaseolus vulgaris Arc5* gene 3' UTR (Goossens et al., 1994; Goossens et al., 1999).

Expression of the c-*D4D*(*Pl*) coding sequence is driven p-*CNL*(*Lu*), the seed-specific *conlinin* gene promoter from flax (*Linum usitatissimum*) (Truksa et al., 2003). The intron-containing 5' UTR (i-At1g65090) is from Arabidopsis locus At1g65090 (Braybrook et al., 2006). Polyadenylation and termination of transcription is directed by t-*OCS*, the 3' UTR of the octopine synthase gene (MacDonald et al., 1991).

Expression of the c-*AHAS*(*At*) coding sequence is driven by p-*Ubi4*(*Pc*), a ubiquitin promoter from parsley (*Petroselinum crispum*) (Kawalleck et al., 1993). The intron-containing 5' UTR is i-*Ubi4(Pc*) from the *P. crispum* ubiquitin promoter (Kawalleck et al., 1993). Polyadenylation and termination of transcription is directed by t-*AHAS*(*At*), the 3' UTR of the *Arabidopsis thaliana* AHAS large subunit gene (Mazur et al., 1987).

3.5. T-DNA borders and genetic elements outside the T-DNA borders

Approximately 16 kb of vector backbone region is located outside of the T-DNA borders of the LTM593 vector. This backbone region of LTM593 contains a bacterial origin of replication (o-*Ori2*), the replication initiation gene (c-*repE*)*,* and plasmid partition genes (*c-sopA, c-sopB*, *and sopC*) from the *E. coli* F plasmid. With the proteins encoded by c-*repE*, c-*sopA*, and c-*sopB* and the cis-acting sequence *sopC*, in combination with the o-*Ori2* origin of replication, plasmids of up to 300 kb can be stably maintained in *E. coli* (Murotsu et al., 1984; Masson and Ray, 1986; Mori et al., 1986; Shizuya et al., 1992).

The operon *repABC* (c-*repA*, c-r*epB*, c-*repC*) is derived from the *Agrobacterium tumefaciens* plasmid pTiC58 (Li and Farrand, 2000). The encoded proteins are responsible for plasmid segregation and replication in Agrobacteria (Oka et al., 1981; Tabata et al., 1989; Gerdes et al., 2000; Pinto et al., 2012). The origin of conjugal transfer (*ori*T) is the place at which plasmid transfer initiates (Marx and Lidstrom, 2001). The operon *repABC* (c-*repA*, c-r*epB*, c-*repC*) is derived from the *Agrobacterium tumefaciens* plasmid pTiC58 (Li and Farrand, 2000).

In addition, the backbone region of LTM593 also contains a bacterial selectable marker (*KanR*) that comprises a bacterial promoter and coding sequence of transposon Tn*903*, which confers kanamycin resistance in *E. coli* (Oka et al., 1981; Naumovski and Friedberg, 1983) and *Agrobacterium* (Gardner et al., 1986) to aid in selection during the cloning process.

4. MOLECULAR CHARACTERIZATION

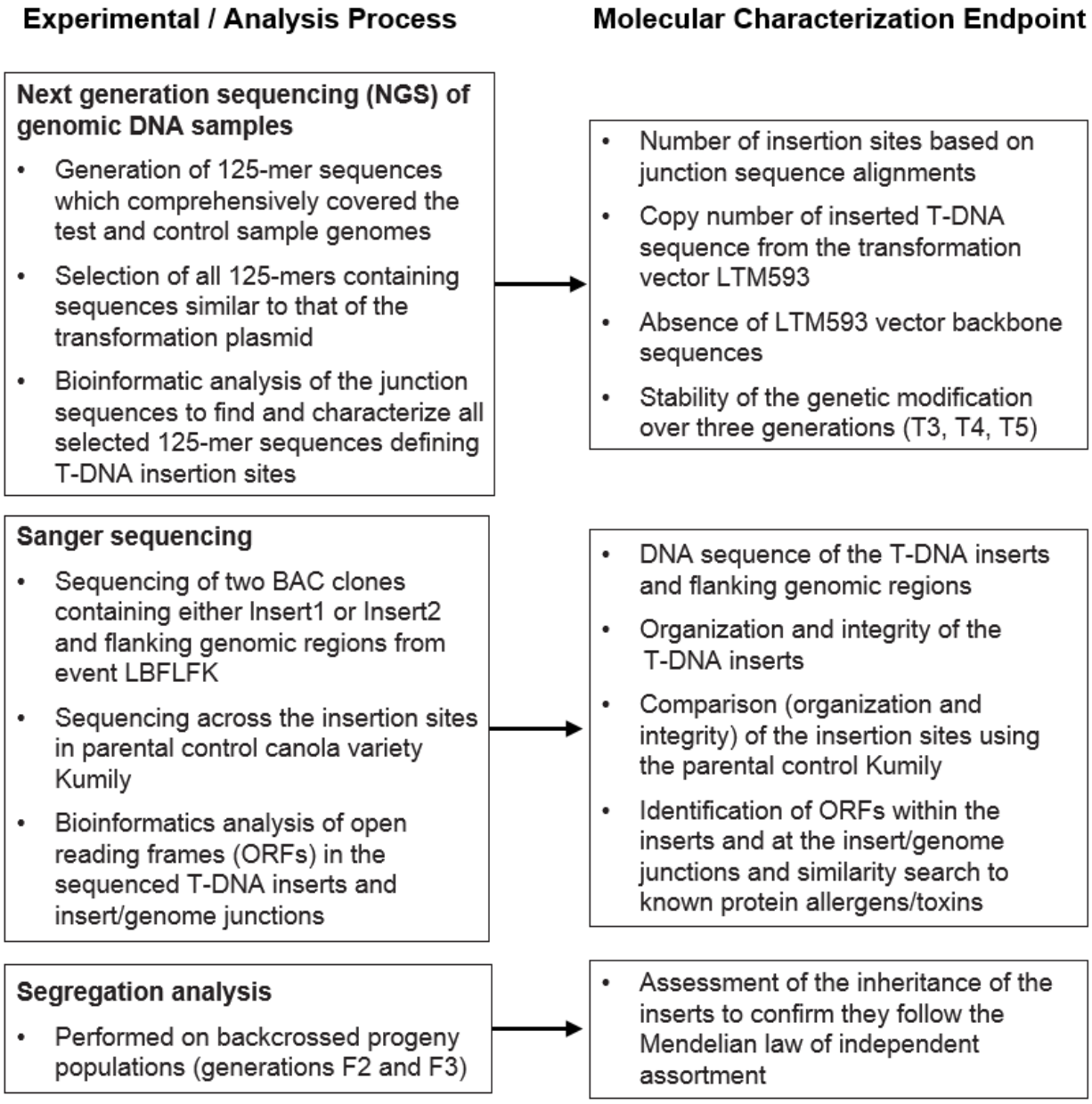
A comprehensive molecular characterization of the genetic modification in EPA+DHA canola event LBFLFK was performed. The molecular characterization consisted of a combination of different sequencing techniques, polymerase chain reaction (PCR), bioinformatic analysis, and genetic segregation studies and determined:

- the number of insertion sites and the number of inserts at each insertion site.
- the organization and integrity of the inserts at each insertion site and whether genomic rearrangements have occurred upon integration.
- the characterization of the DNA sequence of all inserted DNA, including flanking regions in the host genome.
- the absence of transformation vector backbone sequences in the LBFLFK genome.
- the identification of open reading frames (ORFs) created by the insertions with contiguous plant genomic DNA.
- the stability of the genetic modification through three generations and the pattern of inheritance.

A schematic representation of the methodology and the basis of the molecular characterization of LBFLFK canola is illustrated in Figure 6.

Figure 6. Molecular Characterization Strategy

The left text boxes identify the major experimental and analytical processes that were used to achieve the LBFLFK molecular characterization endpoints, which are listed in the right text boxes.



Molecular Characterization Background

For next generation sequencing (NGS), genomic DNA (gDNA) was isolated from three generations of LBFLFK (generations T3, T4, and T5). Using an Illumina® HiSeq™ 2500 system, hundreds of millions of randomly-distributed sequence reads of approximately 125 DNA bp were produced. The sequence reads were mapped to the transformation vector LTM593 using bioinformatics tools. The reads that had sequence similarity to LTM593 were selected and characterized. The number of inserts and insertion sites were then determined by analyzing the selected sequence reads for novel sequence junctions containing sequence from both LTM593 and the canola genome. In addition, the presence or absence of unintended vector backbone sequences was demonstrated by a lack of sequence reads that matched the LTM593 backbone. The stability of inserted DNA over multiple generations (generations T3, T4, and T5) was demonstrated by comparing the novel junctions and distribution of sequencing reads over the T-DNA insert sequences in those three generations.

To determine the DNA sequences of the inserted material and the flanking regions of the host canola genome, locus-specific PCR and Sanger sequencing were used. To isolate each locus, LBFLFK gDNA was used to prepare a bacterial artificial chromosome (BAC) library. Two BAC clones were identified that separately contained Insert1 and Insert2 along with flanking genomic DNA sequences on each side of the inserts. DNA isolated from these two BACs was used for PCR and sequencing analysis of the two inserts of event LBFLFK along with approximately 1 kb of all flanking sequences. The resulting sequences demonstrated the organization and integrity of the T-DNA inserts. To assess whether rearrangements or deletions had occurred in the canola genome at the insertion sites, sequences corresponding to each LBFLFK insertion site were isolated and analyzed using the gDNA of the parental control variety Kumily and applying PCR followed by Sanger sequencing.

The sequence results from both loci in event LBFLFK were further analyzed using bioinformatics to identify ORFs within the inserts and at the junctions between the T-DNA inserts and the canola genome. The deduced amino acid sequences of these ORFs were compared against known protein allergens and toxins.

Finally, segregation analysis of F2 and F3 offspring after a cross of LBFLFK with Kumily was conducted to demonstrate the stable and independent inheritance (according to Mendelian principles) of the two inserts.

The materials and methods used for achieving the endpoints of the molecular characterization of LBFLFK are summarized in Table 5. For further details of the materials and methods used, refer to Appendix B.

Table 5. Materials and Methods Used to Achieve Specific Endpoints in the Molecular Characterization of Canola Event LBFLFK

Characterization Endpoint	Material	Method
• Number of T-DNA inserts • Number of insertion sites	gDNA LBFLFK, T3 generation	NGS/bioinformatics to map sequence reads and identify split-read clusters partially aligning to T-DNA in LTM593 and partially aligning to host gDNA
• Number of insertion sites	gDNA Kumily	NGS/bioinformatics to identify false positive junctions (genomic canola sequences that are also present in the T-DNA construct)
• Confirm absence of vector backbone	gDNA LBFLFK, T3 generation	NGS/bioinformatics to check alignment of sequence reads with vector backbone sequence)
• Confirm absence of vector backbone	gDNA Kumily spiked LTM593 plasmid DNA	NGS/bioinformatics to demonstrate sufficient sensitivity of NGS/bioinformatics pipeline
• Sequence of T-DNA inserts and flanking regions (organization and integrity)	BAC containing LBFLFK Insert1 and LBFLFK Insert2, generated from T3 leaf material	Sanger sequencing for locus-specific PCR and sequence analysis
• Check for rearrangements at insertion sites	gDNA Kumily	PCR/Sanger DNA sequencing of Locus1 and Locus2
• Similarity of unintended ORFs to known toxins and allergens	BACs containing LBFLFK Insert1 and LBFLFK Insert2	Bioinformatics analysis of DNA sequence obtained by Sanger sequencing
• Stability of genetic modification over multiple generations	gDNA LBFLFK, generations T3, T4, T5	NGS/bioinformatics to confirm no generational changes in split reads
• Mendelian inheritance	Seeds of F2 and F3 generations	Segregation analysis by locus-specific PCR

4.1. The organization of the inserted genetic material at each insertion site, including copy number and absence of vector backbone

4.1.1. Background information on use of next generation sequencing (NGS)

Genomic DNA from LBFLFK and Kumily was sequenced using an NGS approach. The usefulness of NGS data as an alternative to Southern blots in the characterization of DNA insertions has been shown previously (Kovalic et al., 2012; Zastrow-Hayes et al., 2015).

Safety assessments of biotechnology-derived crops include a detailed molecular characterization of the inserted DNA (Codex Alimentarius Commission, 2009). Historically, molecular characterization has relied on Southern blot analysis along with targeted sequencing of PCR products spanning any inserted DNA to establish the number of loci and T-DNA inserts as well as to determine the absence of vector backbone. Improvements in sequencing technologies, such as through the use of NGS (Shendure and Ji, 2008; Zhang et al., 2011), have enabled alternative methods for molecular characterization that do not require Southern blot analysis. NGS in combination with bioinformatics analysis is used to address molecular characterization endpoints in an equivalent way to those achieved with Southern blot-based methods.

There are multiple advantages to using NGS and bioinformatics, most notably the robustness, simplicity, and consistency of the method compared with Southern blot studies, which require a customized experimental design for every transformation event. New sequencing-based methods overcome many technical challenges inherent in Southern blot analyses (e.g., false positive hybridization bands resulting from incomplete digestion or star activity, the need for radioactively-labeled probes) and provide higher reproducibility because they are less dependent on complex lab-based procedures.

4.1.2. Quality of the NGS analysis method

The computational pipeline used to analyze NGS data combined bioinformatics tools and parsing algorithms to assess the number of T-DNA inserts (section 4.1.3), absence of transformation vector backbone (section 4.1.4), and stability of the inserted T-DNA over three generations (section 4.5).

NGS of a gDNA library results in millions of short DNA sequences (reads) that are derived from all possible positions of the gDNA. The term “read depth” indicates the number of reads that map to a given genomic position. It has been demonstrated that a ≥ 75X read depth of a genome is adequate to provide comprehensive coverage (Kovalic et al., 2012).

Six single-copy endogenous reference genes were used for read uniformity analysis and to demonstrate that the gDNA was sequenced without bias. The minimum average read depth was 160X across both the T-DNA and each of the six single-copy endogenous reference genes for all samples. One hundred percent read breadth with at least 50X read depth for T-DNA in LBFLFK samples and for the six single-copy endogenous reference genes in all analyzed gDNA samples was also obtained. Additionally, 100% coverage of vector LTM593 in a Kumily sample spiked with 0.1X equivalent copies of the vector plasmid DNA further demonstrated the sensitivity of the NGS analysis.

4.1.3. Determination of the number of insertion sites and insert copy number

The number of insertion sites of LTM593 DNA in LBFLFK was assessed in T3 seed by NGS technology and subsequent bioinformatics analysis.

Any genomic insertion of DNA sequence from the transformation vector will produce two junctions between the plant genome sequence and the T-DNA insert, one at the 5′ end and one at the 3′ end of the inserted T-DNA. These junction sequences (also called “split-reads”) are partially aligned to the host genome and partially aligned to the T-DNA sequence of the transformation vector (Kovalic et al., 2012). Therefore, the number of insertion sites can be deduced from the number of unique junction sequence classes found.

It is noted that, because the LTM593 transformation vector T-DNA contains sequences derived from the canola genome (i.e. the seed-specific promoters), false positive (not unique) junction sequences were identified using Kumily gDNA. These false-positive junctions, found in all LBFLFK and Kumily sequence samples, were eliminated as a step of the NGS bioinformatics pipeline.

Four unique classes of split-read clusters that partially aligned to the T-DNA of vector LTM593 and partially aligned to the host genome sequences were identified in LBFLFK. Two of the four classes of unique junctions aligned partially to the left border (LB) of the vector LTM593 T-DNA, and the other two classes aligned partially to the right border (RB) of the vector LTM593 T-DNA. This indicates the presence of two T-DNA insertion sites in event LBFLFK. The insertion sites were mapped to different chromosomes, demonstrating that two inserts are integrated at two separate loci in LBFLFK.

In addition to the four unique junctions between T-DNA and canola genome sequences, another sequence junction was identified that was produced by a rearrangement of RB sequences of the LTM593 T-DNA in Insert1. This indicated that a minor rearrangement of the RB sequence had occurred during T-DNA insertion. No additional junctions were identified, indicating that each T-DNA insertion site in LBFLFK consists of a single copy of the T-DNA from LTM593 without rearrangements of the introduced gene expression cassette sequences.

These junction sequences, including the RB rearrangement in Insert1, were also confirmed by Sanger DNA sequence analysis (see section 4.2).

4.1.4. Absence of vector backbone

The NGS and bioinformatics also confirmed that event LBFLFK contained no vector backbone sequences as no matching sequences were detected in the genomic DNA.

4.2. DNA sequence of LBFLFK Insert1 and Insert2 and respective flanking regions

Locus1 and Locus2 were individually isolated, cloned, and analyzed using Sanger sequencing. A BAC library was generated from event LBFLFK gDNA, and the BAC clones were screened for the presence of LBFLFK T-DNA insert sequences. A BAC clone containing the complete Insert1 with flanking gDNA and another BAC clone containing the complete Insert2 with flanking gDNA were isolated. The DNA from these BACs was independently subjected to locus-specific PCR followed by Sanger DNA sequence analysis. The obtained DNA sequences demonstrated the organization and integrity of the two T-DNA inserts, including any rearrangements or nucleotide changes as compared to the vector LTM593 T-DNA.

The obtained LBFLFK insert and flanking sequences were assembled based on the sequences of overlapping PCR products. Every base pair in the consensus sequences was independently determined at least four times, and a Quality Value (QV, similar to *phred*) (Ewing and Green, 1998) of 70 or more was confirmed for each base pair, correlating to an expected error probability of at most 1 bp in 10,000,000 bp.

Figure 7 depicts a diagram of the T-DNA inserts and flanking regions at Locus1 (Panel A) and at Locus2 (Panel B) of event LBFLFK. The corresponding expression cassettes are defined in Panel C. T-DNA Insert1 and Insert2 have a length of 43,818 bp and 43,779 bp, respectively. Compared to the 44,010-bp long T-DNA sequence in vector LTM593, Insert1 had a 184-bp truncation of the 5′ end of the RB and a 72-bp truncation of the 3′ end of the LB. In addition, the first 64 bp in the RB of Insert1 was determined to be a rearrangement of short T-DNA RB-derived repeats as seen also from the NGS analysis. Insert2 had a 184-bp truncation of the 5′ end of the RB with a 53-bp truncation of the 3′ end of the LB.

No other rearrangements besides the RB rearrangement in Insert1 were present in either insert. Both T-DNA Insert1 and Insert2 contained all 13 intended gene expression cassettes. The cassette sequences were determined to be identical to the T-DNA sequence of LTM593 except for two single nucleotide changes in Insert1 and one nucleotide change in Insert2. In Insert1, one cytosine to adenine nucleotide change was in the coding sequence of the delta-12 desaturase gene, c-*D12D*(*Ps*), which resulted in a phenylalanine to leucine amino acid substitution (F83L) in the D12D(*Ps*) protein. Another cytosine to adenine nucleotide change was found in Insert1 in the promoter sequence p-*PXR*(*Lu*), which is part of an expression cassette containing the c-*O3D*(*Pir*) coding sequence. This nucleotide change does not result in an amino acid substitution. Lastly, there was a guanine to thymine nucleotide change in Insert2 that is in the coding sequence of the delta-4 desaturase gene, c-*D4D*(*Pl*). This change resulted in an alanine to serine amino acid substitution (A102S) in the D4D(*Pl*) protein. The two amino acid changes have no impact on the function or activity of the respective proteins (section 5.2).

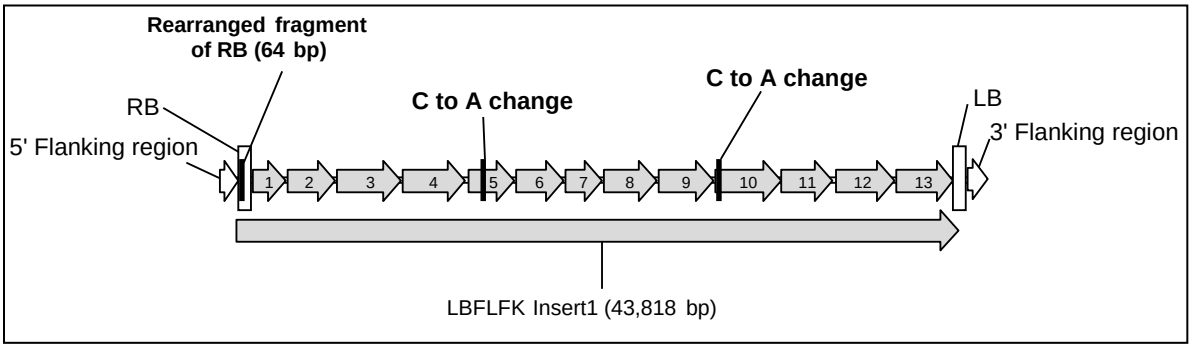
Figure 7. Diagrams of Insert1 and Insert2 and Flanking Regions in LBFLFK

Organization of LBFLFK expression cassettes and genomic flanking regions in Locus1 (panel A) and Locus2 (panel B). The flanking regions are depicted as open arrows. The grey numbered arrows represent the T-DNA expression cassettes (numbered 1–13). The open boxes represent the right border (RB) at the 5´ end and the left border (LB) at the 3´ end of the T-DNA, respectively, as labeled. Thick black lines indicate regions where the T-DNA sequence differs from the sequence of the transformation vector LTM593 with the nucleotide changes indicated. “C to A” indicates a cytosine to adenine change, and “G to T” indicates a guanine to thymine change.

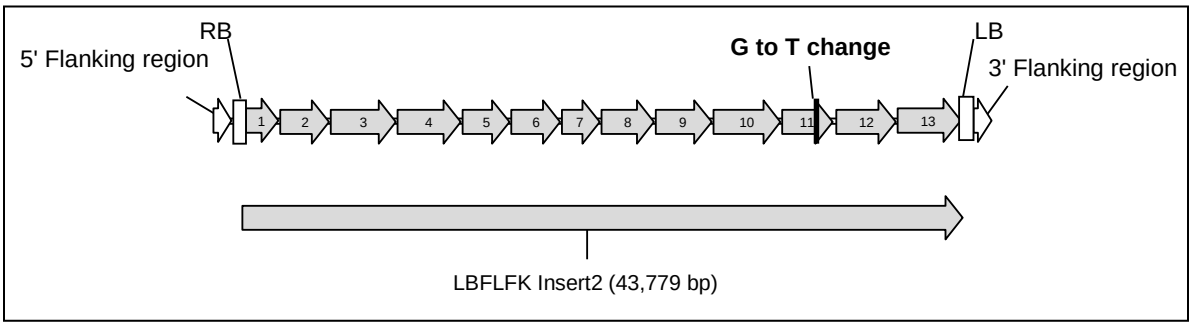
The insertion of Insert1 introduced an 8-bp deletion in the host genome (not shown); the insertion of Insert2 introduced a 31-bp deletion (not shown).

Panel C lists the genetic elements that make up the individual expression cassettes.

A



B



C

Cassette #	Promoter	Intron	CDS	Terminator
1	p-*USP(Vf)*	i-*At1g01170*	c-*D6E(Pp)*	t-*CaMV35S*
2	p-*CNL(Lu)*	i-*At5g63190*	c-*D5D(Tc)1*	t-*OCS*
3	p-*SBP(Vf)*	i-*At1g65090*	c-*D6D(Ot)*	t-*CATHD(St)*
4	p-*PXR(Lu)*	i-*At1g62290*	c-*D6E(Tp)*	t-*PXR(At)*
5	p-*napA(Bn)*	i-*At5g63190*	c-*D12D(Ps)*	t-*rbcS(Ps)*
6	p-*SETL(Bn)*		c-*O3D(Pir)1*	t-*SETL(Bn)*
7	p-*USP(Vf)*	i-*At1g01170*	c-*O3D(Pi)*	t-*CaMV35S*
8	p-*SETL(Bn)*		c-*D5D(Tc)2*	t-*SETL(Bn)*
9	p-*ARC5(Pv)*		c-*D4D(Tc)*	t-*ARC(Pv)*
10	p-*PXR(Lu)*	i-*AGO4(At)*	c-*O3D(Pir)2*	t-*PXR(At)*
11	p-*CNL(Lu)*	i-*At1g65090*	c-*D4D(PI)*	t-*OCS*
12	p-*FAE1(Bn)*	i-*At1g62290*	c-*D5E(Ot)*	t-*FAE1(At)*
13	p-*Ubi4(Pc)*	i-*Ubi4(Pc)*	c-*AHAS(At)*	t-*AHAS(At)*

4.3. Sequence and integrity of the insertion sites

The sequence of the insertion sites in Kumily were analyzed in order to reveal any effects due to the insertion of the T-DNAs in event LBFLFK on the canola genome. PCR was performed on genomic Kumily DNA across the insertion sites (Locus1 and Locus2) using a forward primer corresponding to the genomic sequence flanking the RB and a reverse primer corresponding to the genomic sequence flanking the LB of the respective T-DNA inserts. The PCR products were sequenced, and the resulting Kumily sequences were subjected to homology searches against the *B. napus* genome sequence from cultivar Darmor-*bzh* (Chalhoub et al., 2014; Centre National de Séquençage, 2017).

LBFLFK Insert1 was determined to be integrated into chromosome “Cnn random,” and LBFLFK Insert2 was determined to be integrated into the “C03” chromosome. A comparison of the sequences obtained from Kumily with those from the 3’ and 5’ flanking regions of the two T-DNA inserts in LBFLFK revealed an 8-bp deletion of the canola genome at the integration site of Insert1 and a 31-bp deletion at the integration site of Insert2. Short sequence deletions are common occurrences during Agrobacterium-mediated T-DNA integration (Gheysen et al., 1991). The remaining flanking sequences in LBFLFK were identical to Kumily, and no genomic sequence rearrangements were found at either genomic integration site.

4.4. Identification of any unintended open reading frames created by the T-DNA insertions

A bioinformatic analysis based on the DNA sequence obtained for both T-DNA inserts via Sanger sequencing was conducted to predict the presence of any potential ORFs created at the genomic junctions that could lead to the expression of any unintended proteins. Amino acid sequence alignments were made to determine whether any of the putative polypeptides from any identified ORFs show homology to any known protein allergen, toxin, or antinutrient.

In the context of this analysis, an ORF was defined as any contiguous nucleic acid sequence that contains a string of 30 translated codons between two in-frame termination codons (i.e. TAA, TAG, or TGA) from any of the six potential reading frames (three forward and three reverse reading frames). The deduced amino acid sequences were used as input sequences for the alignments.

A total of 11 ORFs were identified at the four junctions between the canola genome and the T-DNA inserts: one at the Insert1 5´ end, three at the Insert1 3´ end, three at the Insert2 5´ end, and four at the Insert2 3´ end.

To determine the similarity of the identified ORFs to known allergens, the Food Allergy Research and Resource Program (FARRP) Allergen Protein Database (Food Allergy Research and Resource Program, 2017) was used. It was determined that none of the ORFs created by the insertions shared > 35% identity over 80 amino acids with a known allergen, none had a sequence of eight or more consecutive identical amino acids with a known allergen, and none had any significant overall homology to a known allergen.

Additionally, the National Center for Biotechnology Information (NCBI) GenBank® non-redundant peptide sequence database (NCBI Resource Coordinators, 2016) was searched by applying the Basic Local Alignment Search algorithm for protein-to-protein comparisons (BLASTP, NCBI Version 2.6.0+ Jan. 10, 2017) (Altschul et al., 1997) to determine the similarity of the identified ORFs to known toxins and antinutrients. None of the ORFs created by the insertion showed significant homology to known protein toxins as defined in the U.S. Code of Federal Regulations (40 CFR 725.421, 2016) or showed significant homology to known antinutrients of canola (OECD, 2011b), maize (OECD, 2002a), rice (OECD, 2016), soybean (OECD, 2012b), sugar beet (OECD, 2002b), or sugarcane (OECD, 2011a).

4.5. Stability of the T-DNA inserts across multiple generations

The stability of inserted T-DNA in LBFLFK across three generations was evaluated using NGS combined with bioinformatic analysis. Two additional generations of event LBFLFK (T4 and T5) were assessed to determine the genetic stability of the two inserts over multiple generations. Read depth distribution patterns across the entire T-DNA were similar in all three analyzed generations of LBFLFK, and the same four unique genome/insert junctions were found in all generations. This indicates that the T-DNA inserts were stably inherited.

4.6. Mendelian Inheritance of the T-DNA inserts

During development of LBFLFK, genotypic segregation data were recorded to assess the inheritance pattern using Chi-square statistical analysis over two generations. The analysis is based on comparing the observed segregation ratio to the segregation ratio that is expected according to Mendelian laws for two independent loci, as found in LBFLFK canola.

The inheritance of the two LBFLFK T-DNA insertion loci was assessed in F2 and F3 generations using segregating F2 and F3 seed material derived from hemizygous parental plants. Figure 8 shows the full breeding history of LBFLFK and details the generation of materials for the segregation analysis. The zygosity of plants in the T3, F1, and F2 generations was assessed via real-time TaqMan® end-point PCR assays. T3 plants were crossed to the parental variety Kumily to produce hemizygous F1 seeds. After zygosity was confirmed, the F1 plants were self-pollinated to produce segregating F2 seeds. These F2 seeds were pooled, and the resulting seed lot was randomly sampled for segregation analysis. A subset of the F2 seeds was planted, and hemizygous F2 plants were selected for self-pollination to produce segregating F3 seeds. These F3 seeds were also pooled, and the resulting seed lot was again sampled at random for segregation analysis. The expected ratios for two independently segregating loci are described in Table 6.

Figure 8. Schematic Presentation of the Breeding Tree for Event LBFLFK for the Purpose of Segregation Analysis

T_3 represents the T_3 generation. F# are the filial generations.
⊗ designates self-pollination; × designates backcrossing.

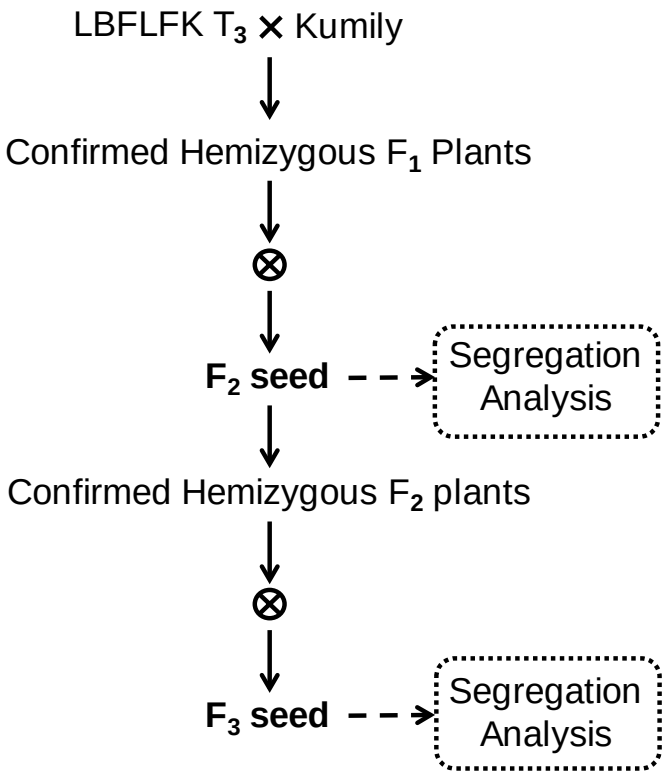


Table 6. Expected Genotype Distribution in the F2 and F3 Generations if Inheritance follows Mendelian Principles

Genotype	AABB	AABb	AAbb	AaBB	AaBb	Aabb	aaBB	aaBb	aabb
Expected segregation ratio	1/16	2/16	1/16	2/16	4/16	2/16	1/16	2/16	1/16

A Pearson's Chi-square (χ^2) analysis was used to statistically compare the observed segregation ratios of the LBFLFK inserts to the expected Mendelian ratios. The Chi-square is calculated as:

$$\chi^2 = \sum \frac{(\text{Observed Value - Expected Value})^2}{\text{Expected Value}}$$

A significance level of 0.05 for accepting the hypothesis that inheritance followed Mendelian inheritance was used for all statistical tests. A p-value ≥ 0.05 indicates, at a 95% confidence level, that there is no statistically significant evidence for non-Mendelian segregation.

F2 seeds were randomly picked and subjected to genotyping by locus-specific PCR and subsequent data analysis. Chi-square analysis was carried out with the data obtained from 768 F2 seeds, and a p-value of 0.543 was obtained (Table 7). Based on this p-value, the hypothesis that segregation is in accordance to Mendel's laws was accepted.

F3 seeds were randomly selected and subjected to genotyping PCR and subsequent data analysis. Chi-square analysis was carried out with the data obtained from 763 F3 seeds, and a p-value of 0.974 was obtained (Table 8). Based on this p-value, the hypothesis that segregation is in accordance to Mendel's laws was accepted.

In conclusion, Locus1 and Locus2 segregate as predicted by Mendelian laws, indicating that Insert1 and Insert2 are inherited independently at an equal frequency.

Table 7. Results of the Segregation Analysis of Single F2 Seeds

Genotypes were successfully determined for 768 out of 792 randomly picked F2 seeds. The observed frequencies were compared with the expected frequencies using the Chi-Square procedure. According to the obtained p-value, the analyzed seed population was segregating as expected.

Genotypes	Number of Seeds (Observed)	Segregation Ratio *	Number of Seeds (Expected)	(Observed – Expected)^2 / Expected
AaBb	200	4/16 or 0.25	192	0.333
AABb	76	2/16 or 0.125	96	4.167
AaBB	98	2/16 or 0.125	96	0.042
Aabb	97	2/16 or 0.125	96	0.010
aaBb	105	2/16 or 0.125	96	0.844
AABB	45	1/16 or 0.0625	48	0.188
AAbb	54	1/16 or 0.0625	48	0.750
aaBB	50	1/16 or 0.0625	48	0.083
aabb	43	1/16 or 0.0625	48	0.521
Total	768		**Chi-Square**	6.938
			p-value	0.543

* Expected frequency according to Mendelian Laws

Table 8. Results of the Segregation Analysis of Single F3 Seeds

Genotypes were successfully determined for 763 out of 792 randomly picked F3 seeds. The observed frequencies were compared with the expected frequencies using the Chi-Square procedure. According to the obtained p-value, the analyzed seed population was segregating as expected.

Genotypes	Number of Seeds (Observed)	Segregation Ratio *	Number of Seeds (Expected)	(Observed – Expected)^2 / Expected
AaBb	189	4/16 or 0.25	190.75	0.016
AABb	98	2/16 or 0.125	95.375	0.072
AaBB	84	2/16 or 0.125	95.375	1.357
Aabb	100	2/16 or 0.125	95.375	0.224
aaBb	100	2/16 or 0.125	95.375	0.224
AABB	48	1/16 or 0.0625	47.688	0.002
AAbb	49	1/16 or 0.0625	47.688	0.036
aaBB	45	1/16 or 0.0625	47.688	0.151
aabb	50	1/16 or 0.0625	47.688	0.112
Total	763		**Chi-Square**	2.195
			p-value	0.974

* Expected frequency according to Mendelian Laws

4.7. Conclusion on the molecular characterization

The molecular characterization efforts described above allow the following conclusions to be made:

- NGS of total genomic DNA and subsequent bioinformatics analysis demonstrated that LBFLFK has two inserts integrated at two separated loci and confirmed the absence of LTM593 vector backbone sequences in the genome of LBFLFK.
- Repeating the analysis for three generations demonstrated that the two inserts are stably integrated in the LBFLFK genome.
- Sanger sequencing analyses confirmed that each of the two inserts has the intended 13 gene expression cassettes. All cassettes were found to be identical to the LTM593 T-DNA except for two single nucleotide changes in Insert1 and one nucleotide change in Insert2. Both T-DNA inserts were intact, apart from a short rearrangement of 64 bp in the RB sequence of Insert1.
- A comparison to the sequence of the insertion site from the parental variety Kumily demonstrated that an 8-bp deletion was created at the genome integration site of Insert1 (Locus1) and a 31-bp deletion was created at the genome integration site of Insert2 (Locus2) in Kumily. No genomic sequence rearrangements were found at either integration site.
- Eleven ORFs were identified that span the junctions between the T-DNA inserts and the flanking genomic DNA. None of the ORFs created by the insertion showed significant homology to known allergens, protein toxins, and antinutrients.
- Segregation analysis of F2 and F3 LBFLFK offspring showed that Insert1 and Insert2 in LBFLFK are both independently inherited according to Mendelian principles.

5. CHARACTERIZATION AND SAFETY ASSESSMENT OF THE NEWLY EXPRESSED PROTEINS

The safety assessment of crops improved through biotechnology includes a description of the nature and biochemical function of the newly expressed proteins (Codex Alimentarius Commission, 2009). This typically includes characterization for identity and amino acid sequence, apparent molecular weight and immunoreactivity, and an assessment of potential glycosylation and enzymatic activity. In general, depending on the properties of the newly expressed proteins and their levels of expression in the genetically modified plant, this may require either the isolation of the newly expressed proteins from the plant or production in a heterologous expression system.

Eleven proteins are newly expressed in EPA+DHA canola event LBFLFK. These include ten integral membrane proteins, desaturase and elongase enzymes, which together impact the content of omega-3 long-chain polyunsaturated fatty acids in the seeds. The eleventh protein is the soluble, chloroplast-located AHAS(*At*) [A122TS653N] enzyme that confers tolerance to treatment with an imidazolinone herbicide.

Integral membrane proteins, containing multiple transmembrane-spanning domains, are generally intractable, meaning they are difficult to isolate, concentrate, and quantify from tissues or difficult to produce at high levels in heterologous systems (Madduri et al., 2012; Bushey et al., 2014). The protein characterizations of the integral membrane desaturase and elongase proteins were performed with a membrane fraction purified from crude extracts of developing embryos that was isolated from immature seeds of event LBFLFK. This detergent-free membrane fraction (referred to herein as “plant-produced proteins” or PPP), contains active and full-length elongase and desaturases from the plant. It is noted that there was also sufficient AHAS(*At*) [A122TS653N] present in PPP to perform several of the characterization studies for the soluble protein. Further enrichment by immunopurification from PPP was needed for glycosylation analysis and confirmation of protein identity. Additionally, AHAS(*At*) [A122TS653N] molecular weight, immunoreactivity, and enzyme activity were demonstrated using both leaf protein extracts and PPP. Feedback regulation of AHAS(*At*) [A122TS653N] activity by branched chain amino acids and a decreased sensitivity to imazamox were confirmed using leaf protein extracts.

5.1. Biochemistry and function of the newly expressed proteins

Fatty acid elongases

Fatty acid elongases extend existing C18 or longer fatty acids by C2 units. The process requires the input of electrons in a four-step reaction cycle and produces water and carbon dioxide as biproducts (Leonard et al., 2004; Jakobsson et al., 2006; Haslam and Kunst, 2013). Elongation enzymes are membrane-bound, localized in the endoplasmic reticulum, and the fatty acid substrate is bound to Coenzyme-A (Leonard et al., 2004). All the elongase proteins newly expressed in EPA+DHA canola event LBFLFK perform the ketoacyl synthase step of the four step elongase reaction cycle. Fatty acid substrate specificity of the elongation complex is controlled by the ketoacyl synthase component and, therefore, elongation can be manipulated by substituting only the ketoacyl synthase component of the elongation complex.

Additional information on the biochemistry and function of the introduced fatty acids elongases can be found in Appendix C.

Fatty acid desaturases

Fatty acid desaturases catalyze the formation of a double bond in a fatty acid substrate at a defined position of the acyl chain. The process requires the abstraction of an electron from the fatty acid substrate and results in the formation of the desaturated fatty acid and water (Shanklin et al., 1994; Buist, 2004; Shanklin et al., 2009). All the desaturases introduced into EPA+DHA canola event LBFLFK are eukaryotic integral membrane desaturases that are membrane-bound and localized in the endoplasmic reticulum. Substrate and product specificity are intrinsic features of each desaturase protein and, therefore, the degree of desaturation and position of double bonds in fatty acids can be manipulated by simply introducing a desaturase with the desired specificity.

Additional information on the biochemistry and function of the introduced fatty acids desaturases can be found in Appendix C.

Acetohydroxy acid synthase

The mechanism of action of imidazolinone herbicides on weeds and non-tolerant plants is by inhibition of the enzyme acetohydroxy acid synthase (AHAS) (Sathasivan et al., 1991). AHAS catalyzes the first common step in branched-chain amino acid biosynthesis that is specific to plants and microorganisms. The AHAS enzyme is composed of a large catalytic subunit and a small regulatory subunit. The enzyme catalyzes the condensation of two molecules of pyruvate to form acetolactate, the precursor of valine and leucine, or the condensation of a molecule of pyruvate with a molecule of 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate, an intermediate in isoleucine biosynthesis (Figure 5) (Singh

and Shaner, 1995; Duggleby and Pang, 2000). AHAS is the key control enzyme within the biosynthetic pathway whose feedback-inhibition, which is completely dependent on the small regulatory subunit, is caused by the presence of the end-product amino acids valine, leucine, and isoleucine. Imidazolinone herbicides such as imazamox, once inside the plant cells, readily inhibit the activity of AHAS (Duggleby and Pang, 2000), resulting in plant death.

Previous studies have shown that specific amino acid substitutions in the large subunit of the AHAS protein, resulting from single-point nucleotide changes in the *AHAS* coding sequence, confer tolerance to imidazolinone herbicides by altering the binding site for these herbicides but with no effect on the normal biosynthetic function of the enzyme or feedback regulation by branched-chain amino acids (Newhouse et al., 1991; Newhouse et al., 1992; Tan et al., 2005).

5.2. Characteristics of newly expressed proteins

To characterize each of the newly expressed proteins in LBFLFK canola, a series of biochemical experiments were conducted to determine for each protein: (1) the deduced amino acid sequence; (2) the protein quantity in analyzed tissues; (3) the apparent molecular weight; (4) the immunoreactivity; (5) the identity; (6) the glycosylation; and (7) the enzymatic activity.

The results of the biochemical and functional characterization are summarized in the following sections and presented in Table 9. Materials and methods and more detailed results are described in Appendix C.

5.2.1. Delta-12 desaturase (*Ps*) characterization

Event LBFLFK contains two T-DNA inserts with the respective D12D(*Ps*) coding sequences differing by one nucleotide. This nucleotide difference results in two newly expressed proteins, D12D(*Ps*) and D12D(*Ps*) [F83L]. The *in silico* predicted protein structure for D12D(*Ps*) has been described and enzyme function presented based on published *in vivo* experiments in yeast (Yilmaz et al., 2017). Bioinformatic analysis indicated the D12D(*Ps*) protein is a methyl-end integral membrane desaturase, and the *in vivo* yeast experiments confirmed this enzymatic function for both D12D(*Ps*) and D12D(*Ps*) [F83L]. The enzymes convert C18:1n-9 into C18:2n-6.

The D12D(*Ps*) proteins were characterized using LBFLFK PPP. Western blot analysis confirmed the D12D(*Ps*) proteins were immunoreactive to an anti-D12D(*Ps*) antibody, and the apparent molecular weight was in good agreement to the calculated molecular mass. The antibody used to detect the D12D(Ps) proteins is expected to detect both D12D(*Ps*) and D12D(*Ps*) [F83L] with similar sensitivity, and the results apply to both D12D(*Ps*) proteins. Tryptic peptide mapping using liquid chromatography tandem mass spectrometry (LC-MS/MS) positively identified 10 distinct peptides (> 9 amino acids (aa)) corresponding to the deduced amino acid sequence of both D12D(*Ps*) proteins and one peptide (> 9 aa) specific to D12D(*Ps*) without the F83L substitution. The D12D(*Ps*) proteins were found to be not glycosylated. Delta-12 desaturation activity, i.e. the

introduction of a double bond in C18:1n-9 generating C18:2n-6, was observed in an *in vitro* enzyme assay.

5.2.2. Delta-6 desaturase (*Ot*) characterization

Event LBFLFK contains two T-DNA inserts encoding two identical copies of the D6D(*Ot*) protein. The *in silico* predicted protein structure was described and enzyme function was based on published *in vivo* experiments in yeast (Domergue et al., 2005; Yilmaz et al., 2017). Bioinformatic analysis indicated the protein is a front-end integral membrane desaturase, and the *in vivo* yeast experiments confirmed this enzymatic function. The enzyme converts C18:2n-6 fatty acids into C18:3n-6 fatty acids.

The D6D(*Ot*) protein was characterized using LBFLFK PPP. Western blot analysis confirmed the protein was immunoreactive to an anti-D6D(*Ot*) antibody. The apparent molecular weight was in good agreement with the calculated molecular mass of the D6D(*Ot*) protein. Tryptic peptide mapping using LC-MS/MS positively identified 11 distinct peptides (> 9 aa) corresponding to the deduced amino acid sequence of the D6D(*Ot*) protein. The D6D(*Ot*) protein was found to be not glycosylated. Delta-6 desaturation activity, i.e. the introduction of a double bond in C18:2n-6 generating C18:3n-6, was not detected in an *in vitro* enzyme assay, likely due to relatively high activity of enzymes competing for the C18:2n-6-CoA substrate in LBFLFK PPP. However, the presence of C18:3n-6 in event LBFLFK seeds indicates that the D6D(*Ot*) has the intended *in vivo* activity in event LBFLFK.

5.2.3. Delta-6 elongase (*Tp*) characterization

Event LBFLFK contains two T-DNA inserts encoding two identical copies of the D6E(*Tp*) protein. The *in silico* predicted protein structure was described and the enzyme function was presented based on published *in vivo* experiments in yeast (Meyer et al., 2004; Yilmaz et al., 2017). Bioinformatic analysis indicates the protein is an ELO-type integral membrane elongase, and the *in vivo* yeast experiments confirmed this enzymatic function. The enzyme catalyzes the decarboxylation Claisen-like condensation of two carbons from malonyl-CoA to C18:3n-6-CoA generating C20:3n-6-β-keto-CoA, which is then converted to C20:3n-6-CoA by endogenous enzymes.

The D6E(*Tp*) protein was characterized using LBFLFK PPP. Western blot analysis confirmed the protein was immunoreactive to an anti-D6E(*Tp*) antibody. The apparent molecular weight was in good agreement with the calculated molecular mass of the D6E(*Tp*) protein. Tryptic peptide mapping using LC-MS/MS positively identified two distinct peptides (> 9 aa) corresponding to the deduced amino acid sequence of the D6E(*Tp*) protein. The D6E(*Tp*) protein was found to be not glycosylated. Delta-6 elongation activity, i.e. condensation of two carbons from malonyl-CoA to C18:3n-6-CoA generating C20:3n-6-β-keto-CoA, which was ultimately converted to C20:5n-6-CoA by endogenous canola enzymes, was observed in an *in vitro* enzyme assay.

5.2.4. Delta-6 elongase (*Pp*) characterization

Event LBFLFK contains two T-DNA inserts encoding two identical copies of the D6E(*Pp*) protein. The *in silico* predicted protein structure was based on published *in vivo* experiments in yeast (Zank et al., 2000; Zank et al., 2002; Domergue et al., 2003; Yilmaz et al., 2017). Bioinformatic analysis indicated the protein is an ELO-type integral membrane elongase, and the *in vivo* yeast experiments confirmed this enzymatic function. The enzyme catalyzes the decarboxylation Claisen-like condensation of two carbons from malonyl-CoA to C18:3n-6-CoA generating C20:3n-6-β-keto-CoA, which is then converted to C20:3n-6-CoA by endogenous enzymes.

The D6E(*Pp*) protein was characterized using LBFLFK PPP. The D6E(*Pp*)-specific antibody could not detect a protein in agreement with the calculated molecular mass of the D6E(*Pp*); therefore, the immunoreactivity and molecular weight of this protein could not be determined. Tryptic peptide mapping using LC-MS/MS did not identify the D6E(*Pp*) protein. The D6E(*Pp*)-specific antibody could not detect a protein in agreement with the calculated molecular mass of the D6E(*Pp*); therefore, the glycosylation status of this protein could not be determined. Delta-6 elongation activity, i.e. of condensation of two carbons from malonyl-CoA to C18:3n-6-CoA generating C20:3n-6-β-keto-CoA, which was ultimately converted to C20:5n-6-CoA by endogenous canola enzymes, was observed in an *in vitro* enzyme assay.

5.2.5. Delta-5 desaturase (*Tc*) characterization

Event LBFLFK contains two T-DNA inserts encoding four identical copies of the D5D(*Tc*) protein. The *in silico* predicted protein structure was described and enzyme function was based on published *in vivo* experiments in yeast (Qiu et al., 2001; Yilmaz et al., 2017). Bioinformatic analysis indicates the protein is a front-end integral membrane desaturase, and the *in vivo* yeast experiments confirmed this enzymatic function. The enzyme converts C20:3n-6 fatty acids into C20:4n-6 fatty acids.

The D5D(*Tc*) protein was characterized using LBFLFK PPP. Western blot analysis confirmed the protein was immunoreactive to an anti-D5D(*Tc*) antibody. The apparent molecular weight was in good agreement with the calculated molecular mass of the D5D(*Tc*) protein. Tryptic peptide mapping using LC-MS/MS positively identified seven distinct peptides (> 9 aa) corresponding to the deduced amino acid sequence of the D5D(*Tc*) protein. The D5D(*Tc*) protein was found to be not glycosylated. Delta-5 desaturation activity, i.e. the introduction of a double bond in C20:3n-6 generating C20:4n-6, was not detected in an *in vitro* enzyme assay, likely due to relatively high activity of enzymes competing for the C20:3n-6-CoA substrate in LBFLFK PPP. However, the presence of C20:4n-6 fatty acids in event LBFLFK seeds indicates the D5D(*Tc*) has the intended *in vivo* activity in event LBFLFK.

5.2.6. Omega-3 desaturase (*Pir*) characterization

Event LBFLFK contains two T-DNA inserts encoding four identical copies of the O3D(*Pir*) protein. The *in silico* predicted protein structure was described and the enzyme function was based on published *in vivo* experiments in yeast (Yilmaz et al., 2017). Bioinformatic analysis indicates the protein is a methyl-end integral membrane desaturase, and the *in vivo* yeast experiments confirmed this enzymatic function. The enzyme converts C20:4n-6 into C20:5n-3.

The O3D(*Pir*) protein was characterized using LBFLFK PPP. Western blot analysis confirmed the protein was immunoreactive to an anti-O3D(*Pir*) antibody. The apparent molecular weight was in good agreement with the calculated molecular mass of the O3D(*Pir*) protein. Tryptic peptide mapping using LC-MS/MS positively identified eight distinct peptides (> 9 aa) corresponding to the deduced amino acid sequence of the O3D(*Pir*) protein. The O3D(*Pir*) protein was found to be not glycosylated. Omega-3 desaturation activity, i.e. the introduction of a double bond in C20:4n-6 fatty acids generating C20:5n-3 fatty acids, was not detected in an *in vitro* enzyme assay. However, the presence of the C20:5n-3 fatty acids in event LBFLFK seeds indicates that there is *in vivo* O3D activity in event LBFLFK.

5.2.7. Omega-3 desaturase (*Pi*) characterization

Event LBFLFK contains two T-DNA inserts encoding two identical copies of the O3D(*Pi*) protein. The *in silico* predicted protein structure was described and enzyme function was based on published *in vivo* experiments in yeast (Yilmaz et al., 2017). Bioinformatic analysis indicates the protein is a methyl-end integral membrane desaturase, and the *in vivo* yeast experiments confirmed this enzymatic function. The enzyme converts C20:4n-6 into C20:5n-3.

The O3D(*Pi*) protein was characterized using LBFLFK PPP. The O3D(*Pi*)-specific antibody could not detect a protein in agreement with the calculated molecular mass of the O3D(*Pi*); therefore, the immunoreactivity and molecular weight of this protein could not be determined. Tryptic peptide mapping using LC-MS/MS did not identify the O3D(*Pi*) protein. The O3D(*Pi*)-specific antibody could not detect a protein in agreement with the calculated molecular mass of the O3D(*Pi*); therefore, the glycosylation status of this protein could not be determined. Omega-3 desaturation activity, i.e. the introduction of a double bond in C20:4n-6 generating C20:5n-3, was not detected in an *in vitro* enzyme assay. However, the presence of the C20:5n-3 fatty acids in event LBFLFK seeds indicates that there is *in vivo* O3D activity in event LBFLFK.

5.2.8. Delta-5 elongase (*Ot*) characterization

Event LBFLFK contains two T-DNA inserts encoding two identical copies of the D5E(*Ot*) protein. The *in silico* predicted protein structure was described and enzyme function was based on published *in vivo* experiments in yeast (Meyer et al., 2004; Yilmaz et al., 2017). Bioinformatic analysis indicates the protein is an ELO-type integral membrane elongase, and the *in vivo* yeast experiments confirmed this enzymatic function. The enzyme catalyzes the decarboxylation Claisen-like condensation of two carbons from malonyl-CoA to C20:5n-3-CoA generating C22:5n-3-β-keto-CoA, which is then converted to C22:5n-3-CoA by endogenous enzymes.

The D5E(*Ot*) protein was characterized using LBFLFK PPP. Western blot analysis confirmed the protein was immunoreactive to an anti-D5E(*Ot*) antibody. The apparent molecular weight was in good agreement with the calculated molecular mass of the D5E(*Ot*) protein. Tryptic peptide mapping using LC-MS/MS positively identified four distinct peptides (> 9 aa) corresponding to the deduced amino acid sequence of the D5E(*Ot*) protein. The D5E(*Ot*) protein was found to be not glycosylated. Delta-5 elongation activity, i.e. of condensation of two carbons from malonyl-CoA to C20:5n-3-CoA generating C22:5n-3-β-keto-CoA, which was ultimately converted to C22:5n-3-CoA by endogenous canola enzymes, was observed in an *in vitro* enzyme assay.

5.2.9. Delta-4 desaturase (*Tc*) characterization

Event LBFLFK contains two T-DNA inserts encoding two identical copies of the D4D(*Tc*) protein. The *in silico* predicted protein structure was described and the enzyme function was based on published *in vivo* experiments in yeast (Yilmaz et al., 2017). Bioinformatic analysis indicates the protein is a front-end integral membrane desaturase, and the *in vivo* yeast experiments confirmed this enzymatic function. The enzyme converts C22:5n-3 into C22:6n-3.

The D4D(*Tc*) protein was characterized using LBFLFK PPP. Western blot analysis confirmed the protein was immunoreactive to an anti-D4D(*Tc*) antibody. The apparent molecular weight was in good agreement with the calculated molecular mass of the D4D(*Tc*) protein. Tryptic peptide mapping using LC-MS/MS positively identified nine distinct peptides (> 9 aa) corresponding to the deduced amino acid sequence of the D4D(*Tc*) protein. The D4D(*Tc*) protein was found to be not glycosylated. Delta-4 desaturation activity, i.e. the introduction of a double bond in C22:5n-3 fatty acids generating C22:6n-3 fatty acids, was observed in an *in vitro* enzyme assay.

5.2.10. Delta-4-desaturase (*Pl*) characterization

EPA+DHA canola event LBFLFK contains two T-DNA inserts with the respective D4D(*Pl*) coding sequences differing by a single nucleotide. This nucleotide difference results in the two newly expressed proteins D4D(*Pl*) and D4D(*Pl*) [A102S]. The *in silico* predicted protein structure for D4D(*Pl*) has been described and enzyme function shown based on published *in vivo* experiments in yeast (Yilmaz et al., 2017). Bioinformatic analysis indicated the D4D(*Pl*) protein is a front-end integral membrane desaturase, and the *in vivo* yeast experiments confirmed this enzymatic function for both D4D(*Pl*) and D4D(*Pl*) [A102S]. The enzymes convert C22:5n-3 into C22:6n-3.

The D4D(*Pl*) proteins were characterized using LBFLFK PPP. Western blot analysis confirmed the proteins were immunoreactive to an anti-D4D(*Pl*) antibody. The apparent molecular weight was in good agreement with the calculated molecular mass of the D4D(*Pl*) proteins. The antibody used to detect the D4D(*Pl*) proteins is expected to detect both D4D(*Pl*) and D4D(*Pl*) [A102S] with similar sensitivity; therefore, results are applied to both D4D(*Pl*) proteins. Tryptic peptide mapping using LC-MS/MS positively identified three distinct peptides (> 9 aa) corresponding to the deduced amino acid sequence of both D4D(*Pl*) proteins. The D4D(*Pl*) proteins were found to be not glycosylated. Delta-4 desaturation activity, i.e. the introduction of a double bond in C22:5n-3 generating C22:6n-3, was observed in an *in vitro* enzyme assay.

5.2.11. Acetohydroxy acid synthase (*At*) characterization

Event LBFLFK contains two T-DNA inserts encoding two identical copies of the AHAS(*At*) [A122TS653N] protein. The AHAS(*At*) [A122TS653N] protein represents the large subunit of acetohydroxy acid synthase. The chloroplast transit peptide is removed upon import into the chloroplast, which results in mature AHAS(*At*) [A122TS653N] enzyme. Western blot analysis confirmed the protein was immunoreactive to an anti-AHAS(*At*) [A122TS653N] antibody. The apparent molecular weight was in good agreement with the calculated molecular mass of the mature AHAS(*At*) [A122TS653N] protein lacking 64 amino acids of the N-terminus of the immature protein. Tryptic peptide mapping using LC-MS/MS positively identified six distinct peptides (> 9 aa) corresponding to the deduced amino acid sequence of the AHAS(*At*) [A122TS653N] protein. The AHAS(*At*) [A122TS653N] protein was found to be not glycosylated. The enzymatic activity of the AHAS protein was demonstrated in both LBFLFK PPP and leaf extract via an *in vitro* assay for the AHAS protein, confirming the biosynthetic function of the AHAS(*At*) [A122TS653N] enzyme. Additionally, AHAS activity assessed in LBFLFK leaf tissue extracts displayed a greater tolerance to the herbicide imazamox compared to Kumily leaf extracts, confirming the reduced binding of imazamox to AHAS in LBFLFK due to the introduced AHAS(*At*) [A122TS653N] protein. Both LBFLFK and Kumily leaf extracts showed similar feedback inhibition when incubated with the amino acids leucine and valine, which confirms that the amino acid substitutions in the AHAS(*At*) [A122TS653N] protein did not impact the feedback regulation by branched-chain amino acids.

Table 9. Characteristics of the Newly Expressed Proteins in PPP isolated from EPA+DHA Canola Event LBFLFK

Protein	Apparent Molecular Weight (kDa)	Immuno-reactivity	Identity	Newly Expressed Protein Concentration Mean ± SD		Observed Activity	Glycosylation Status
				Wet Weight (µg/ml)	Dry Weight (µg/g)		
D12D(*Ps*)	41.8	Confirmed	Confirmed	7.88 ± 0.38	175.05 ± 27.69	Yes	Negative
D6D(*Ot*)	55.6	Confirmed	Confirmed	38.12 ± 2.09	793.36 ± 153.44	ND	Negative
D6E(*Tp*)	25	Confirmed	Confirmed	657.57 ± 107.49	16386.31 ± 2192.83	Yes	Negative
D6E(*Pp*)	ND	ND	ND	ND	NA		NA
D5D(*Tc*)	46.8	Confirmed	Confirmed	6.82 ± 0.81	395.11 ± 103.03	ND	Negative
O3D(*Pir*)	38.1	Confirmed	Confirmed	156.67 ± 13.67	4029.87 ± 117.09	ND	Negative
O3D(*Pi*)	ND	ND	ND	ND	NA		NA
D5E(*Ot*)	30.8	Confirmed	Confirmed	8.88 ± 0.11	220.42 ± 5.08	Yes	Negative
D4D(*Tc*)	63.1	Confirmed	Confirmed	34.74 ± 1.79	593.63 ± 13.88	Yes	Negative
D4D(*Pl*)	50.7	Confirmed	Confirmed	11.24 ± 0.40	194.54 ± 64.81		Negative
AHAS(*At*) [A122TS653N]	79.2	Confirmed	Confirmed	0.54 ± 0.04	24.28 ± 1.29	Yes	Negative

ND = Not detected or below detection limit, NA = Not applicable (assay not performed)
Characterization experiments of AHAS(*At*) [A122TS653N] in leaf extracts confirmed the immunoreactivity of a protein with an apparent molecular weight of 79.2 kDa, with observable enzyme activity and protein concentrations of 1.07 ± 0.67 µg/ml (wet weight) and 7.41 ± 1.67 µg/ml (dry weight).

5.3. Levels of expression of newly expressed proteins

The safety assessment of crops improved through biotechnology typically includes the quantification of the newly expressed proteins to determine their level and site of expression. This also demonstrates that they are expressed as expected in the appropriate tissues in a manner and at levels that are consistent with the associated regulatory sequences driving the expression of the corresponding gene (Codex Alimentarius Commission, 2009).

As described earlier, eleven proteins were introduced into EPA+DHA canola event LBFLFK. The ten desaturases and elongases introduced are controlled by seed-specific promoters, and the soluble, chloroplast-located acetohydroxy acid synthase AHAS(*At*) [A122TS653N] is under the control of a constitutive promoter.

The amounts of each of the newly expressed proteins in EPA+DHA canola tissues were measured by validated quantitative immunoassays. Analyzed canola tissues included whole plants at rosette and flowering stages, leaf and root from early maturity plants, immature seed, mature seed, and pollen. Except for pollen, tissue was collected from plants grown in the U.S. at four field sites during the 2015 growing season. Pollen was collected from plants grown in greenhouses. Each trial site consisted of three plots: LBFLFK sprayed with Beyond® herbicide (active ingredient imazamox), LBFLFK not sprayed with Beyond® herbicide, and the parental control variety Kumily. LBFLFK treated with Beyond® herbicide is referred to herein as LBFLFK (sprayed) and untreated as LBFLFK (non-sprayed). Each plot was otherwise managed using standard herbicide practices applied equally to all plots. The materials and methods used to determine the concentration of the newly expressed proteins, as well as the summary results per protein across locations and for all plant tissues analyzed, are presented in Appendix D.

In summary, eleven immunoassays were developed and validated for the purpose of quantifying each of the newly expressed proteins in canola plant tissues. Each protein was measured with either an enzyme-linked immunosorbent assay (ELISA) or a capillary-based quantitative western blot method.

ELISAs were used to determine the amounts of D12D(*Ps*), D6E(*Pp*), D5D(*Tc*), and D5E(*Ot*) present in the samples. Quantitative western blot methods were used to determine the amounts of D6D(*Ot*), D6E(*Tp*), O3D(*Pir*), O3D(*Pi*), D4D(*Pl*), D4D(*Tc*), and AHAS(*At*) [A122TS653N] present in the samples.

The choice of method was dependent on the sensitivity, specificity, and reproducibility that was obtainable. Due to the intractable nature of the newly expressed membrane proteins, specific detergents and denaturing reagents were necessary for the extraction of the proteins from tissue samples (Bushey et al., 2014). The concentrations of these components can interfere with antibody-antigen interactions and were therefore incompatible with ELISA-based methods, which are sensitive to these effects. For such intractable newly expressed proteins, quantitative western blot methods were used.

For the AHAS(*At*) [A122TS653N] protein, a quantitative western blot method was applied to allow the use of a single antibody with a preferred specificity compared to the endogenous AHAS protein.

The parental variety Kumily served as a negative control.

Mean protein levels for each tissue type were determined on a fresh weight (FW) basis and converted to a dry weight (DW) basis after adjusting for the moisture content. The expression levels of each of the newly expressed proteins on a FW and DW basis for both LBFLFK (sprayed) and LBFLFK (non-sprayed) samples in each of the seven canola tissue types are summarized and presented in Appendix D.

Table 10 presents a summary of the expression analysis for each protein across locations in mature and immature seed. Quantification of the newly expressed elongases and desaturases did not occur in any other tissue sample type, which aligns with the seed-specific promoters that control expression. As intended, each of the newly expressed integral membrane proteins under the control of a seed-specific promoter, with the exception of D6E(*Pp*) and O3D(*Pi*), was quantified[3] in immature and mature seed across field sites. The level of expression for the integral membrane proteins was below the limit of quantification (< LOQ) in all other tissues analyzed. The newly expressed AHAS(*At*) [A122TS653N] protein, driven by a constitutive promoter, was quantifiable in every tissue except mature seed[4], and the expression data for AHAS(*At*) [A122TS653N] are shown separately for every tissue analyzed in Table 11.

[3] D5D(*Tc*) and D5E(*Ot*) were only quantified in mature seed samples.
[4] The AHAS(*At*) [A122TS653N] protein was quantified in a single LBFLFK mature seed sample but was otherwise < LOQ in all mature seed samples.

Table 10. Summary of Protein Expression Levels in Immature and Mature Seed of EPA+DHA Canola Event LBFLFK

Protein	Tissue[3]	LBFLFK (non–sprayed)[1]		LBFLFK (sprayed)[2]		Method Quantification and Detection Limits	
		Mean (SD)[4] min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	Mean (SD) min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	LOQ LOD (µg/g FW)	LOQ LOD (µg/g DW)
D12D(*Ps*)	**Immature Seed**	1.25 (0.32) < LOQ–1.93	3.98 (1.30) < LOQ–6.56	1.15 (0.44) < LOD–1.97	3.72 (1.69) < LOD–6.99	1.02 0.24	2.97 0.70
	Mature Seed	0.79 (0.19) 0.51–1.10	0.93 (0.23) 0.57–1.24	0.71 (0.19) < LOQ–1.12	0.83 (0.23) < LOQ–1.29	0.51 0.12	0.55 0.13
D6D(*Ot*)	**Immature Seed**	8.97 (4.86) < LOQ–20.00	29.90 (17.71) < LOQ–68.00	9.16 (4.80) < LOD–18.18	30.89 (18.45) < LOD–64.54	5.40 1.80	15.77 5.26
	Mature Seed	34.65 (15.46) 18.16–65.96	40.22 (16.87) 21.43–74.53	35.76 (14.27) 19.17–66.05	41.80 (16.11) 23.00–75.96	10.80 3.60	11.77 3.92
D6E(*Tp*)	**Immature Seed**	180.40 (42.33) < LOQ–301.84	600.86 (181.74) < LOQ–1038.33	185.25 (48.05) < LOD–271.99	626.21 (194.98) < LOD–900.29	153.60 79.56	448.51 232.33
	Mature Seed	793.02 (232.25) 399.59–1181.34	936.43 (309.07) 471.52–1488.49	779.21 (229.40) 372.33–1229.15	915.86 (276.98) 446.80–1376.65	344.06 222.78	375.03 242.83
D6E(*Pp*)	**Immature Seed**	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	5.70 0.95	16.65 2.78
	Mature Seed	NA < LOD	NA < LOD	NA < LOD	NA < LOD	5.70 0.95	6.22 1.04
D5D(*Tc*)	**Immature Seed**	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	9.60 3.84	28.03 11.21
	Mature Seed	1.19 (0.35) < LOD–1.66	1.33 (0.40) < LOD–1.88	1.38 (0.46) < LOD–2.56	1.53 (0.55) < LOD–2.94	1.20 0.48	1.31 0.52
O3D(*Pir*)	**Immature Seed**	47.66 (18.21) < LOQ–77.41	162.36 (70.06) < LOQ–266.29	48.06 (24.43) <LOD–109.14	169.26 (86.39) <LOD–361.25	29.97 11.43	87.51 33.37
	Mature Seed	428.26 (140.79) 168.54–623.80	504.38 (169.24) 188.76–704.89	474.27 (157.65) 188.70–693.86	561.61 (196.20) 211.34–832.63	107.89 34.29	117.60 37.37
O3D(*Pi*)	**Immature Seed**	NA < LOD	NA < LOD	NA < LOD	NA < LOD	6.52 2.48	19.03 7.26
	Mature Seed	NA < LOD	NA < LOD	NA < LOD	NA < LOD	65.16 24.85	71.02 27.08

Protein	Tissue[3]	LBFLFK (non–sprayed)[1]		LBFLFK (sprayed)[2]		Method Quantification and Detection Limits	
		Mean (SD)[4] min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	Mean (SD) min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	LOQ LOD (µg/g FW)	LOQ LOD (µg/g DW)
D5E(*Ot*)	**Immature Seed**	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	4.46 0.97	13.03 2.84
	Mature Seed	13.32 (3.75) 7.63–18.27	15.48 (4.00) 9.00–20.65	13.14 (3.35) 6.99–18.27	15.36 (3.63) 8.39–21.01	4.46 0.97	4.87 1.06
D4D(*Pl*)	**Immature Seed**	4.19 (1.72) < LOD–7.04	16.16 (6.18) < LOD–27.80	3.68 (1.78) < LOD–7.06	13.28 (6.67) < LOD–25.06	1.93 0.92	5.63 2.68
	Mature Seed	3.74 (1.91) < LOD–7.24	4.16 (2.18) < LOD–8.18	3.60 (1.96) < LOD–7.28	4.03 (2.29) < LOD–8.37	3.86 1.83	4.21 2.00
D4D(*Tc*)	**Immature Seed**	7.88 (7.63) < LOD–30.05	29.02 (26.96) < LOD–102.17	6.29 (5.03) < LOD–18.96	22.53 (18.72) < LOD–62.76	3.02 1.66	8.83 4.84
	Mature Seed	10.55 (4.75) < LOD–20.36	11.81 (5.43) < LOD–23.01	9.66 (3.12) < LOD–15.89	10.88 (3.71) < LOD–18.27	8.47 3.49	9.23 3.80
AHAS(*At*) [A122TS653N]	**Immature Seed**	2.97 (0.68) < LOQ–4.52	11.72 (2.92) < LOQ–15.38	3.53 (0.96) 2.13–5.27	14.12 (3.68) 8.37–19.98	2.10 0.44	6.12 1.28
	Mature Seed	NA < LOQ	NA < LOQ	NA < LOQ–2.92	NA < LOQ–3.50	2.80 0.59	3.05 0.64

< LOQ = below the limit of quantitation; < LOD = below the limit of detection; NA = not applicable; mean and standard deviation were not calculated for those datasets that did not contain two or more values ≥ LOQ.

[1] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[3] n = 20.

[4] Mean, standard deviation (SD), and range (min – max) of protein concentrations are reported on a µg/g fresh weight (FW) and dry weight (DW) basis.

Table 11. AHAS(*At*) [A122TS653N] Protein Levels in EPA+DHA Canola Event LBFLFK Tissues

Tissue[3]	LBFLFK (non–sprayed)[1]		LBFLFK (sprayed)[2]		Method Quantification and Detection Limits	
	Mean (SD)[4] min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	Mean (SD) min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	LOQ LOD (µg/g FW)	LOQ LOD (µg/g DW)
Whole Plant (Rosette Stage)	1.48 (0.57) < LOQ–2.44	14.00 (4.89) < LOQ–22.01	1.81 (0.85) 0.89–3.40	16.27 (6.62) < LOQ[6]–28.63	0.87 0.18	8.96 1.88
Whole Plant (Flowering Stage)	1.41 (0.29) < LOD–1.63	13.03 (2.66) < LOD–< LOQ[5]	1.50 (0.12) < LOQ–1.91	NA < LOQ[6]	1.46 0.18	13.62 1.72
Leaf	0.74 (0.09) < LOQ–0.95	NA < LOQ[6]	1.07 (0.67) < LOQ–2.49	7.41 (1.67) < LOQ–11.48	0.70 0.15	6.55 1.38
Root	0.10 (0.04) < LOD–0.21	0.42 (0.09) < LOD–0.68	0.13 (0.05) < LOD–0.24	0.49 (0.19) < LOD–1.13	0.08 0.05	0.39 0.25
Immature Seed	2.97 (0.68) < LOQ–4.52	11.72 (2.92) < LOQ–15.38	3.53 (0.96) 2.13–5.27	14.12 (3.68) 8.37–19.98	2.10 0.44	6.12 1.28
Mature Seed	NA < LOQ	NA < LOQ	NA < LOQ–2.92	NA < LOQ–3.50	2.80 0.59	3.05 0.64
Pollen	30.63 (5.72) 22.95–39.25	33.39 (6.28) 25.25–43.18	ND ND	ND ND	9.31 2.94	10.24 3.23

< LOQ = below the limit of quantitation; < LOD = below the limit of detection; ND = not determined; NA = not applicable; mean and standard deviation were not calculated for those datasets that did not contain two or more values ≥ LOQ.

[1] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[3] n = 20 for whole plant at rosette stage, whole plant at flowering stage, immature seed, leaf, root, and mature seed non-sprayed and sprayed (with Beyond® herbicide) datasets; n = 2 for pollen dataset, non-sprayed only.

[4] Mean, standard deviation (SD), and range (min – max) of protein concentrations are reported on a µg/g fresh weight (FW) and dry weight (DW) basis.

[5] The DW min or max value is reported as < LOQ. Some DW values were < LOQ due to use of sample dry weight conversion factors in converting FW results to DW results. < LOQ values were substituted with the LOQ for the purposes of determining the mean DW level.

[6] The DW range value is reported as < LOQ. All DW values were < LOQ due to use of sample dry weight conversion factors in converting FW results to DW results.

5.4. Safety assessment of newly expressed proteins

The safety assessment of agricultural products produced through biotechnology includes an evaluation of the safety of the newly expressed proteins (Codex Alimentarius Commission, 2009). This assessment is accomplished by applying a weight-of-evidence approach to the data generated from *in silico* and experimental studies. A weight-of-evidence approach is used because no single assay or biochemical characteristic can identify a protein as a hazard and, scientifically, it is not possible to prove a lack of hazard with complete certainty (Delaney et al., 2008).

The weight-of-evidence approach for the safety assessment of newly expressed proteins prioritizes the testing into two tiers (Delaney et al., 2008). Tier I testing (potential hazard identification) includes establishing a history of safe use (HOSU) and consumption, amino acid sequence similarity between the newly expressed protein and known protein toxins and antinutrients, biological function of the protein in the plant, stability to heat or conditions of commercial processing, degradation in appropriate representative gastric and intestinal model systems, and expression level and dietary intake. Tier II testing (hazard classification), which may be performed on a case-by-case basis if the first tier does not provide sufficient evidence to make a determination of safety, might include acute or repeated dose toxicity testing of the newly expressed proteins or hypothesis-based studies.

The safe use of the newly expressed proteins and their donor organisms was evaluated by a systematic literature search that identified relevant documents related to establishing dietary or environmental exposure or toxicity of the newly expressed proteins or donor organisms. In addition, the amino acid sequence of each newly expressed protein was compared to the amino acid sequences of proteins present in consumed food or feed to show sequence identity to proteins that are already safely consumed.

Additionally, bioinformatic analysis of the amino acid sequence of each newly expressed protein was performed to determine the similarity of the amino acid sequence of the newly expressed proteins to known protein toxins or antinutrients. Specifically, for LBFLFK, nucleotide sequences from both LBFLFK Insert1 and LBFLFK Insert2 were translated into amino acid sequences, and bioinformatic analysis was performed on both LBFLFK Insert1 and LBFLFK Insert2 sequences.

Lastly, heat stability[5] and digestibility studies of the newly expressed desaturase and elongase proteins in LBFLFK were performed with a membrane fraction purified from crude extracts of developing embryos (plant-produced proteins, or PPP) isolated from immature seeds. The digestive fate assessment of AHAS(*At*) [A122TS653N] was performed with AHAS(*At*) [A122TS653N] protein present in both LBFLFK PPP and

[5] Assessment of structural integrity and/or enzyme activity at 30, 50, 70, and 90°C for 5 and 20 minutes. For those proteins tested, the lowest temperature where structural change or loss of enzyme activity was observed is noted in the individual protein assessments (Section 5.4.1).

LBFLFK leaf isolates, and the heat stability of AHAS(*At*) [A122TS653N] was performed just with LBFLFK PPP.

The results of the safety assessment for the individual newly expressed proteins are summarized below, followed by the conclusions reached.

5.4.1. Individual protein safety assessments

5.4.1.a. Delta-12-desaturase (*Ps*) safety assessment

The HOSU of D12D(*Ps*) and its donor organism *Phytophthora sojae* was evaluated. *P. sojae* is not known to produce or contain toxins or antinutrients. Likewise, *P. sojae* has not been reported to cause disease in humans or animals. Delta-12 desaturases are found in commodity crops, including soybean (Heppard et al., 1996), cotton (Liu et al., 1999; Pirtle et al., 2001; Zhang et al., 2009), peanut (Jung et al., 2000; Lopez et al., 2000), and flax (Krasowska et al., 2007) but are not found in mammals, which are dependent on dietary intake of delta-12 desaturated fatty acids. No reports of adverse effects were identified due to exposure to delta-12 desaturases. The lack of adverse findings for D12D(*Ps*) and its donor organism substantiates their HOSU.

The amino acid sequence of D12D(*Ps*) was compared with other desaturases present in consumed food or feed. For this assessment, only the intended amino acid sequence of D12D(*Ps*) from LBFLFK Insert2, without the single nucleotide change (section 5.2.1), was used. The proteins identified with the highest sequence identity to D12D(*Ps*) originated from plants and fungi. The protein found to have the most identity to D12D(*Ps*) is from *Hordeum vulgare* (barley, 46.7% identity) followed closely by *Oryza brachyantha* (ancestor of rice, 46.4% identity) and *Zea mays* (corn, 46.3% identity). The fungal protein is from *Mortierella alpina* (45% identity), which is used to produce arachidonic acid-rich oil for human consumption that is Generally Recognized as Safe (GRAS) (FDA, 2001). This protein from *Mortierella alpina* has been shown to have delta-12 desaturase activity (Huang et al., 1999). Thus, D12D(*Ps*) is structurally and functionally related to other desaturases that are safely consumed by humans as food and by animals as feed.

Bioinformatic analysis was used to determine whether the amino acid sequence of D12D(*Ps*) had significant sequence similarity to known protein toxins or antinutrients. The amino acid sequences from both LBFLFK Insert1 and LBFLFK Insert2 were used for bioinformatic analysis. For D12D(*Ps*), LBFLFK Insert1 includes a nucleotide change in the coding region, which results in the F83L amino acid substitution (section 5.2.1). D12D(*Ps*) did not show significant homology to proteins that are toxic to humans (40 CFR 725.421, 2016). D12D(*Ps*) did not show significant homology to known antinutrients (OECD, 2002a, b, 2011a, b, 2012b, 2016).

To determine the impact of commercial processing, D12D(*Ps*) enzyme activity and structural integrity following heat treatment were evaluated. The desaturase assay does not discriminate between the intended D12D(*Ps*) and the D12D(*Ps*) [F83L]. No D12D(*Ps*) enzyme activity could be observed after heat treatment, and the proteins underwent aggregation at ≥ 50°C within 5 minutes (min). These results demonstrate that D12D(*Ps*)

proteins are heat-labile and the enzymatic activity is unlikely to remain after commercial processing.

The digestibility of D12D(*Ps*) in SGF (simulated gastric fluid, which contains pepsin) and in SIF (simulated intestinal fluid, which contains pancreatin) was evaluated at 37°C during a 60-min time course. Protein degradation was then analyzed by western blot analysis using a D12D(*Ps*)-specific antibody. It was estimated that ≥ 90.4% of the full-length D12D(*Ps*) was digested within 0.5 min with SGF. It was also estimated that ≥ 89.2% of the full-length D12D(*Ps*) was digested within 0.5 min with SIF. These results demonstrate that D12D(*Ps*) is susceptible to digestion and rapidly degraded in SGF and SIF.

5.4.1.b. **Delta-6 desaturase (*Ot*) safety assessment**

The HOSU of D6D(*Ot*) and its donor organism *Ostreococcus tauri* was evaluated. *O. tauri* is not known to produce or contain toxins or antinutrients. Likewise, *O. tauri* has not been reported to cause disease in humans or animals. Delta-6 desaturases are found in all vertebrates, lower plants, insects, and some other invertebrates (Cook and McMaster, 2002). No reports of adverse effects were identified due to exposure to delta-6 desaturases. The lack of adverse findings for D6D(*Ot*) and its donor organism substantiates their HOSU.

The amino acid sequence of D6D(*Ot*) was compared with other desaturases present in consumed food or feed. The proteins identified with the highest sequence identity to D6D(*Ot*) originated from mollusks *Lingula anatina* (lamp shell, 27.4% identity) and *Octopus vulgaris* (common octopus, 27.3% identity). Other similar sequences, which are all ~25% identical to D6D(*Ot*), originate from diverse sources, including fish (*Tachysurus fulvidraco*, yellowhead catfish), plants (*Oryza sativa*, rice), and birds (*Meleagris gallopavo*, turkey). While specific delta-6 desaturase activity has not been identified in these sources, the protein from *Octopus vulgaris* has been shown to have delta-5 desaturase activity (Monroig et al., 2012). Thus, D6D(*Ot*) is structurally and functionally related to other desaturases that are safely consumed by humans as food and by animals as feed.

Bioinformatic analysis was used to determine whether the amino acid sequence of D6D(*Ot*) had significant sequence similarity to known protein toxins or antinutrients. D6D(*Ot*) did not show significant homology to proteins that are toxic to humans (40 CFR 725.421, 2016). D6D(*Ot*) did not show significant homology to known antinutrients (OECD, 2002a, b, 2011a, b, 2012b, 2016).

To determine the impact of commercial processing, D6D(*Ot*) structural integrity following heat treatment was evaluated. D6D(*Ot*) underwent aggregation at ≥ 50°C within 5 min. These results demonstrate that D6D(*Ot*) protein is heat-labile. The enzyme activity of D6D(*Ot*) in response to heat was not assessed because enzyme activity was not detectable in LBFLFK PPP.

The digestibility of D6D(*Ot*) in SGF and in SIF was evaluated at 37°C during a 60-min time course. Protein degradation was then analyzed by western blot analysis using a D6D(*Ot*)-specific antibody. It was estimated that ≥ 98.4% of the full-length D6D(*Ot*) was digested within 0.5 min with SGF. It was also estimated that ≥ 98.3% of the full-length D6D(*Ot*) was digested within 0.5 min with SIF. These results demonstrate that D6D(*Ot*) is susceptible to digestion and rapidly degraded in SGF and SIF.

5.4.1.c. Delta-6 elongase (*Tp*) safety assessment

The HOSU of D6E(*Tp*) and its donor organism *Thalassiosira pseudonana* was evaluated. *T. pseudonana* is not known to produce or contain antinutrients. *T. pseudonana* has been reported to produce the neurotoxin beta-N-methylamino-L-alanine (Jiang et al., 2014), which is produced by many species of diatoms as well as by cyanobacteria and dinoflagellates (Lage et al., 2015). Nevertheless, *T. pseudonana* is frequently used as part of aquafeed diets (e.g., for bivalves and crustacean larvae) (Brown, 2002). In addition, *T. pseudonana*-derived biosilica has been successfully used to deliver chemotherapeutic drugs to cancer cells, indicating a non-toxic nature (Delalat et al., 2015). Extracts from *T. pseudonana* displayed no feeding deterrent activity to model copepods in bioassays (Shaw et al., 1994). *T. pseudonana* has not been reported to cause disease in humans or animals. Delta-6 elongases are found in plant and fungal species (Beaudoin et al., 2000a; Beaudoin et al., 2000b; Zank et al., 2000). No reports of adverse effects were identified due to exposure to delta-6 elongases. The lack of adverse findings for D6E(*Tp*) and its donor organism substantiates their HOSU.

The amino acid sequence of D6E(*Tp*) was compared with other elongases present in consumed food or feed. All but one of the identified sequences, a mollusk, came from fish. The proteins found to have the highest sequence identity to D6E(*Tp*) originated from *Notothenia coriiceps* (Black rockcod, 33.2% identity), *Oreochromis niloticus* (Nile tilapia, 33.1% identity), and *Salmo salar* (Atlantic salmon, 32.5% identity). The Nile tilapia and Atlantic salmon are used extensively in aquaculture around the world. The selected sequences from *Salmo salar* and *Oreochromis niloticus* are not the same as the proteins that have been experimentally verified as delta-6-elongases, which are 25.1% and 27.1% identical to D6E(*Tp*), respectively (Hastings et al., 2004; Agaba et al., 2005). Thus, D6E(*Tp*) is structurally and functionally related to other elongases that are safely consumed by humans as food and by animals as feed.

Bioinformatic analysis was used to determine whether the amino acid sequence of D6E(*Tp*) had significant sequence similarity to known protein toxins or antinutrients. D6E(*Tp*) did not show significant homology to proteins that are toxic to humans (40 CFR 725.421, 2016). D6E(*Tp*) did not show significant homology to known antinutrients (OECD, 2002a, b, 2011a, b, 2012b, 2016).

To determine the impact of commercial processing, D6E(*Tp*) enzyme activity and structural integrity following heat treatment were evaluated. The enzyme activity assay cannot distinguish between the individual delta-6 elongase enzymes D6E(*Tp*) and D6E(*Pp*) in LBFLFK PPP. The D6E proteins were largely inactivated at 50°C within 5 min and completely inactivated after longer treatment (20 min). The complete inactivation was also observed for all temperatures tested > 50°C within 5 min. D6E(*Tp*) protein underwent aggregation at ≥ 70°C within 5 min. These results demonstrate that D6E(*Tp*) protein is heat-labile and the enzymatic activity is unlikely to remain after commercial processing.

The digestibility of D6E(*Tp*) in SGF and in SIF was evaluated at 37°C during a 60-min time course. Protein degradation was then analyzed by western blot analysis using a D6E(*Tp*)-specific antibody. Following exposure to SGF, the full-length D6E(*Tp*) protein, along with a degradation fragment that appeared after 0.5 min of SGF digestion, were detectable at most of the time points monitored. However, the signals decreased significantly at the end of the time course (60 min). Following exposure to SIF, it was estimated that ≥ 98.9% of the full-length D6E(*Tp*) was digested within 0.5 min with SIF. Three degradation fragments that appeared after 0.5 min with SIF were digested within 20 min. Due to limited digestion of D6E(*Tp*) by SGF, a sequential digestion with SGF followed by SIF was performed. Also in this experiment, the D6E(*Tp*) protein remained detectable after 30 min incubation with SGF but was degraded rapidly (within 0.5 min) upon incubation with SIF. These results demonstrate that D6E(*Tp*) is susceptible to sequential digestion in SGF followed by SIF.

5.4.1.d. Delta-6 elongase (*Pp*) safety assessment

The HOSU of D6E(*Pp*) and its donor organism *Physcomitrella patens* was evaluated. *P. patens* is not known to produce or contain toxins or antinutrients. Likewise, *P. patens* has not been reported to cause disease in humans or animals. Delta-6 elongases are found in plant and fungal species (Beaudoin et al., 2000a; Beaudoin et al., 2000b; Zank et al., 2000). No reports of adverse effects were identified due to exposure to delta-6 elongases. The lack of adverse findings for D6E(*Pp*) and its donor organism substantiates their HOSU.

The amino acid sequence of D6E(*Pp*) was compared with other elongases present in consumed food or feed. Most of the proteins identified originated from mammals and birds and from fish. However, the proteins found to have the highest sequence identities to D6E(*Pp*) were elongases from *Thraustochytrium* (40.4% identity) and *Mortierella alpina* (36% identity). Several *Thraustochytrium* species are used for industrial production of docosahexaenoic acid (DHA) (Sijtsma and de Swaaf, 2004). *Mortierella alpina* is used to produce arachidonic acid-rich oil for human consumption that is GRAS (FDA, 2001). All other related sequences were found to have identities to D6E(*Pp*) around 32–33% and

include proteins from species such as *Equus asinus* (donkey, 33.8% identity), *Oreochromis niloticus* (Nile tilapia, 33% identity), and *Salmo salar* (Atlantic salmon, 32.3% identity). The Nile tilapia and Atlantic salmon are used extensively in aquaculture around the world. The elongase from *Mortierella alpina* (Parker-Barnes et al., 2000) has been shown to have delta-6 elongase activity. Thus, D6E(*Pp*) is structurally and functionally related to other elongases that are safely consumed by humans as food and by animals as feed.

Bioinformatic analysis was used to determine whether the amino acid sequence of D6E(*Pp*) had significant sequence similarity to known protein toxins or antinutrients. D6E(*Pp*) did not show significant homology to proteins that are toxic to humans (40 CFR 725.421, 2016). D6E(*Pp*) did not show significant homology to known antinutrients (OECD, 2002a, b, 2011a, b, 2012b, 2016).

To determine the impact of commercial processing, D6E enzyme activity following heat treatment was evaluated. The enzyme activity assay cannot distinguish between the individual delta-6 elongase enzymes D6E(*Tp*) and D6E(*Pp*) in LBFLFK PPP. The D6E proteins were largely inactivated at 50°C within 5 min and completely inactivated after longer treatment (20 min). The complete inactivation was also observed for all temperatures tested > 50°C within 5 min. The D6E(*Pp*) protein was not assessed for structural integrity upon heat treatment or digestibility in SGF or SIF because the D6E(*Pp*) protein was not detected in LBFLFK PPP and LBFLFK tissues.

5.4.1.e. Delta-5 desaturase (*Tc*) safety assessment

The HOSU of D5D(*Tc*) and its donor organism *Thraustochytrium* sp. was evaluated. *Thraustochytrium* sp. are not known to produce or contain toxins or antinutrients. Likewise, *Thraustochytrium* sp. have not been reported to cause disease in humans or animals. Delta-5 desaturases are found in algae, protozoa, fungi, plants, and animals including humans (Meesapyodsuk and Qiu, 2012). No reports of adverse effects were identified due to exposure to delta-5 desaturases. The lack of adverse findings for D5D(*Tc*) and its donor organism substantiates their HOSU.

The amino acid sequence of D5D(*Tc*) was compared with other desaturases present in consumed food or feed. Most of the proteins identified originated from plants and mosses and from fish. However, the protein found to have the most identity to D5D(*Tc*) is a desaturase from *Thraustochytrium aureum* (58.1% identity). Several *Thraustochytrium* species are used for industrial production of DHA (Sijtsma and de Swaaf, 2004). The *Thraustochytrium* protein has been shown to be a functional delta-5 desaturase (Kobayashi et al., 2011). All other sequences were found to have identities to D5D(*Tc*) around 26–27%. These sequences include proteins from major crops such as *Brassica napus* (canola, 26.1% identity), *Capsicum annuum* (bell pepper, 26.8% identity), and *Sesamum indicum* (sesame, 26.2% identity) and sequences from farmed fish such as

Salmo salar (Atlantic salmon, 26.2% identity) and *Oreochromis niloticus* (Nile tilapia, 26.2% identity). Plants are not known to possess delta-5 desaturase activity, and the plant proteins mentioned above are predicted to be delta-8 desaturases. The protein from *Salmo salar* was found to have delta-6 desaturase activity (Monroig et al., 2010). Thus, D5D(*Tc*) is structurally and functionally related to other desaturases that are safely consumed by humans as food and by animals as feed.

Bioinformatic analysis was used to determine whether the amino acid sequence of D5D(*Tc*) had significant sequence similarity to known protein toxins or antinutrients. D5D(*Tc*) did not show significant homology to proteins that are toxic to humans (40 CFR 725.421, 2016). D5D(*Tc*) did not show significant homology to known antinutrients (OECD, 2002a, b, 2011a, b, 2012b, 2016).

To determine the impact of commercial processing, D5D(*Tc*) structural integrity following heat treatment was evaluated. D5D(*Tc*) underwent aggregation at ≥ 50°C within 20 min. These results demonstrate that D5D(*Tc*) protein is heat-labile. The enzyme activity of D5D(*Tc*) in response to heat was not assessed because D5D(*Tc*) enzyme activity was not detectable in LBFLFK PPP.

The digestibility of D5D(*Tc*) in SGF and in SIF was evaluated at 37°C during a 60-min time course. Protein degradation was then analyzed by western blot analysis using a D5D(*Tc*)-specific antibody. It was estimated that ≥ 90.4% of the full-length D5D(*Tc*) was digested within 0.5 min with SGF. It was also estimated that ≥ 89.4% of the full-length D5D(*Tc*) was digested within 0.5 min with SIF. These results demonstrate that D5D(*Tc*) is susceptible to digestion and rapidly degraded in SGF and SIF.

5.4.1.f. Omega-3 desaturase (*Pir*) safety assessment

The HOSU of O3D(*Pir*) and its donor organism *Pythium irregulare* was evaluated. *P. irregulare* is not known to produce or contain toxins or antinutrients. Likewise, *P. irregulare* has not been reported to cause disease in humans or animals. Omega-3 desaturases are found in all photosynthetic organisms. Humans and other mammals are dependent on dietary intake of omega-3 fatty acids because of the lack of endogenous enzymes for omega-3 desaturation (Simopoulos, 2016). No reports of adverse effects were identified due to exposure to omega-3 desaturases. The lack of adverse findings for O3D(*Pir*) and its donor organism substantiates their HOSU.

The amino acid sequence of O3D(*Pir*) was compared with other desaturases present in consumed food or feed. The protein found to have the highest sequence identity to O3D(*Pir*) is from the mollusk *Octopus bimaculoides* (California two-spot octopus, 39% identity). All other proteins identified are from major agricultural crops such as *Cucumis melo* (muskmelon, 34.6% identity), *Cicer arietinum* (chickpea, 33.8% identity), and *Linum usitatissimum* (flax seed, 33.5% identity). The protein from *Linum usitatissimum* has been proven to be an omega-3 desaturase (Khadake et al., 2011). Thus, O3D(*Pir*) is structurally and functionally related to other desaturases that are safely consumed by humans as food and by animals as feed.

Bioinformatic analysis was used to determine whether the amino acid sequence of O3D(*Pir*) had significant sequence similarity to known protein toxins or antinutrients. O3D(*Pir*) did not show significant homology to proteins that are toxic to humans (40 CFR 725.421, 2016). O3D(*Pir*) did not show significant homology to known antinutrients (OECD, 2002a, b, 2011a, b, 2012b, 2016).

To determine the impact of commercial processing, O3D(*Pir*) structural integrity following heat treatment was evaluated. O3D(*Pir*) underwent aggregation at ≥ 50°C within 5 min. These results demonstrate that O3D(*Pir*) protein is heat-labile. The enzyme activity of O3D(*Pir*) in response to heat treatment was not assessed because O3D activity was not detectable in LBFLFK PPP.

The digestibility of O3D(*Pir*) in SGF and in SIF was evaluated at 37°C during a 60-min time course. Protein degradation was then analyzed by western blot analysis using a O3D(*Pir*)-specific antibody. It was estimated that ≥ 96.8% of the full-length O3D(*Pir*) was digested within 0.5 min with SGF. A degradation fragment that appeared after 0.5 min of digestion disappeared within 30 min with SGF. It was also estimated that ≥ 96.4% of the full-length O3D(*Pir*) was digested within 0.5 min with SIF. These results demonstrate that O3D(*Pir*) is susceptible to digestion and rapidly degraded in SGF and SIF.

5.4.1.g. Omega-3 desaturase (*Pi*) safety assessment

The HOSU of O3D(*Pi*) and its donor organism *Phytophthora infestans* was evaluated. *P. infestans* is not known to produce or contain toxins or antinutrients. Likewise, *P. infestans* has not been reported to cause disease in humans or animals. Omega-3 desaturases are found in all photosynthetic organisms. Humans and other mammals are dependent on dietary intake of omega-3 fatty acids because of the lack of endogenous enzymes for omega-3 desaturation (Simopoulos, 2016). No reports of adverse effects were identified due to exposure to omega-3 desaturases. The lack of adverse findings for O3D(*Pi*) and its donor organism substantiates their HOSU.

The amino acid sequence of O3D(*Pi*) was compared with other desaturases present in consumed food or feed. The proteins found to have the highest sequence identity to O3D(*Pi*) are from the mollusk *Octopus bimaculoides* (California two-spot octopus, 37.4% identity) and from *Aphanizomenon flos-aquae* (34.6% identity), which is a cyanobacteria found around the world and used to produce dietary supplements (Spolaore et al., 2006). Other proteins identified are from major agricultural crops such as *Solanum lycopersicum* (tomato, 33.4% identity), *Oryza sativa* (rice, 33.2% identity), and *Brassica napus* (canola, 32.2% identity). The *Solanum lycopersicum* protein has been shown to be an omega-3 desaturase (Wang et al., 2014a). Thus, O3D(*Pi*) is structurally and functionally related to other desaturases that are safely consumed by humans as food and by animals as feed.

Bioinformatic analysis was used to determine whether the amino acid sequence of O3D(*Pi*) had significant sequence similarity to known protein toxins or antinutrients. O3D(*Pi*) did not show significant homology to proteins that are toxic to humans (40 CFR 725.421, 2016). O3D(*Pi*) did not show significant homology to known antinutrients (OECD, 2002a, b, 2011a, b, 2012b, 2016).

The enzyme activity of O3D(*Pi*) in response to heat treatment was not assessed because O3D activity was not detectable in LBFLFK PPP. The O3D(*Pi*) protein was also not assessed for structural integrity following heat treatment or digestibility in SGF or SIF because the O3D(*Pi*) protein was not detected in LBFLFK PPP and LBFLFK tissues.

5.4.1.h. Delta-5 elongase (*Ot*) safety assessment

The HOSU of D5E(*Ot*) and its donor organism *Ostreococcus tauri* was evaluated. *O. tauri* is not known to produce or contain toxins or antinutrients. Likewise, *O. tauri* has not been reported to cause disease in humans or animals. Delta-5 elongases are found in animals, including humans (Wang et al., 2014b; Wang et al., 2014c), microalgae (Robert et al., 2009; Petrie et al., 2010), and liverworts (Kajikawa et al., 2006). No reports of adverse effects were identified due to exposure to delta-5 elongases. The lack of adverse findings for D5E(*Ot*) and its donor organism substantiates their HOSU.

The amino acid sequence of D5E(*Ot*) was compared with other elongases present in consumed food or feed. Most of the proteins identified originated from fish. The protein found to have the highest sequence identity to D5E(*Ot*) is from *Cyprinus carpio* (common carp, 32.1% identity). Other species with proteins similar to D5E(*Ot*) included *Salmo salar* (Atlantic salmon, 31.2% identity), *Mortierella alpina* (30.8% identity), which is used to produce arachidonic acid-rich oil for human consumption that is GRAS (FDA, 2001), and *Octopus vulgaris* (30.4% identity). The protein from *Octopus vulgaris* has been shown to have both delta-6 and delta-5 elongase activity (Monroig et al., 2012). Thus, D5E(*Ot*) is structurally and functionally related to other elongases that are safely consumed by humans as food and by animals as feed.

Bioinformatic analysis was used to determine whether the amino acid sequence of D5E(*Ot*) had significant sequence similarity to known protein toxins or antinutrients. D5E(*Ot*) did not show significant homology to proteins that are toxic to humans (40 CFR 725.421, 2016). D5E(*Ot*) did not show significant homology to known antinutrients (OECD, 2002a, b, 2011a, b, 2012b, 2016).

To determine the impact of commercial processing, D5E(*Ot*) enzyme activity and structural integrity following heat treatment were evaluated. The D5E(*Ot*) protein was inactivated to < limit of quantitation (LOQ) at 50°C within 5 min and completely inactivated to < LOD after longer treatment (20 min). The complete inactivation (< LOD) was also observed for all temperatures > 50°C within 5 min. D5E(*Ot*) protein underwent aggregation at ≥ 50°C that was exacerbated with increasing time and/or temperature. These results demonstrate that D5E(*Ot*) protein is heat-labile and the enzymatic activity is unlikely to remain after commercial processing.

The digestibility of D5E(*Ot*) in SGF and in SIF was evaluated at 37°C during a 60-min time course. Protein degradation was then analyzed by western blot analysis using a D5E(*Ot*)-specific antibody. Following exposure to SGF, two degradation fragments appeared in the first 0.5 min of digestion with SGF, suggesting that the D5E(*Ot*) protein was partially digested. All bands including the full-length D5E(*Ot*) protein were faintly detectable throughout the 60-min incubation with SGF. Following exposure to SIF, it was estimated that ≥ 98.9% of the full-length D5E(*Ot*) was digested within 0.5 min with SIF. Due to limited digestion of D5E(*Ot*) by SGF, a sequential digestion with SGF followed by SIF was performed. Also in this experiment, the D5E(*Ot*) protein remained partially intact for 30 min with SGF but was degraded rapidly (within 0.5 min) upon incubation in SIF. These results demonstrate that D5E(*Ot*) is susceptible to sequential digestion in SGF followed by SIF.

5.4.1.i. **Delta-4 desaturase (*Tc*) safety assessment**

The HOSU of D4D(*Tc*) and its donor organism *Thraustochytrium* sp. was evaluated. *Thraustochytrium* sp. are not known to produce or contain toxins or antinutrients. Likewise, *Thraustochytrium* sp. have not been reported to cause disease in humans or animals. Delta-4 desaturases are found in marine microalga and protists (Qiu et al., 2001; Tonon et al., 2003; Pereira et al., 2004; Guo et al., 2013). No reports of adverse effects were identified due to exposure to delta-4 desaturases. The lack of adverse findings for D4D(*Tc*) and its donor organism substantiates their HOSU.

The amino acid sequence of D4D(*Tc*) was compared with other desaturases present in consumed food or feed. Most of the proteins identified originated from mammals and birds. However, the protein found to have the highest sequence identity to D4D(*Tc*) is from *Thraustochytrium aurem* (70.9% identity). Several *Thraustochytrium* species are used for industrial production of DHA (Sijtsma and de Swaaf, 2004). All other related sequences were found to have identities to D4D(*Tc*) of less than 30% and included species such as the fungus *Mortierella alpina* (29.8% identity), which is used to produce arachidonic acid-rich oil for human consumption that is GRAS (FDA, 2001), the plant *Prunus mume* (Chinese plum, 24.2% identity), and the mammal *Bos taurus* (cow, 24% identity). Plants

are not known to possess delta-4 desaturase activity, but the protein from *Mortierella alpina* (Tavares et al., 2011) has been shown to have delta-5 desaturase activity. Thus, D4D(*Tc*) is structurally and functionally related to other desaturases that are safely consumed by humans as food and by animals as feed.

Bioinformatic analysis was used to determine whether the amino acid sequence of D4D(*Tc*) had significant sequence similarity to known protein toxins or antinutrients. D4D(*Tc*) did not show significant homology to proteins that are toxic to humans (40 CFR 725.421, 2016). D4D(*Tc*) did not show significant homology to known antinutrients (OECD, 2002a, b, 2011a, b, 2012b, 2016).

To determine the impact of commercial processing, D4D(*Tc*) enzyme activity and structural integrity following heat treatment were evaluated. The enzyme activity assay cannot distinguish between the individual delta-4 desaturase enzymes D4D(*Tc*), D4D(*Pl*), and D4D(*Pl*) [A102S] in LBFLFK PPP. The D4D proteins retained enzymatic activity at 30°C after 5 and 20 min, but the proteins were largely inactivated (< LOQ) at ≥ 50°C within 5 min and completely inactivated (< LOD) after longer treatment (20 min) at 90°C. D4D(*Tc*) protein underwent aggregation at ≥ 50°C within 20 min. These results demonstrate that D4D(*Tc*) protein is heat-labile and the enzymatic activity is unlikely to remain after commercial processing.

The digestibility of D4D(*Tc*) in SGF and in SIF was evaluated at 37°C during a 60-min time course. Protein degradation was then analyzed by western blot analysis using a D4D(*Tc*)-specific antibody. It was estimated that ≥ 87.9% of the full-length D4D(*Tc*) was digested within 0.5 min with SGF. It was also estimated that ≥ 86.6% of the full-length D4D(*Tc*) was digested within 0.5 min with SIF. These results demonstrate that D4D(*Tc*) is susceptible to digestion and rapidly degraded in SGF and SIF.

5.4.1.j. **Delta-4 desaturase (*Pl*) safety assessment**

The HOSU of D4D(*Pl*) and its donor organism *Pavlova lutheri* was evaluated. *P. lutheri* is not known to produce or contain toxins or antinutrients. Likewise, *P. lutheri* has not been reported to cause disease in humans or animals. Delta-4 desaturases are found in marine microalga and protists (Qiu et al., 2001; Tonon et al., 2003; Pereira et al., 2004; Guo et al., 2013). No reports of adverse effects were identified due to exposure to delta-4 desaturases. The lack of adverse findings for D4D(*Pl*) and its donor organism substantiates their HOSU.

The amino acid sequence of D4D(*Pl*) was compared with other desaturases present in consumed food or feed. For this assessment, only the intended amino acid sequence of D4D(*Pl*) from LBFLFK Insert1, without the single nucleotide change (section 5.2.10), was used. Most of the proteins identified originated from fish and from plants and mosses. However, the proteins found to have the highest sequence identity to D4D(*Pl*) are from the red alga *Pyropia yezoensis* (Japanese nori, 27.9% identity) and from the plants *Zea*

mays (27.8% identity), *Sorghum bicolor* (27.5% identity), and *Setaria italic* (27.2% identity). Plants are not known to possess delta-4 desaturase activity, and the plant proteins mentioned above are predicted to be delta-8 desaturases. All other sequences were found to have identities to D4D(*Pl*) in the range of 24–26%, including a protein from *Dicentrarchus labrax* (European sea bass, 24.2% identity) that has been shown to be an active delta-6 desaturase (González-Rovira et al., 2009). Thus, D4D(*Pl*) is structurally and functionally related to other desaturases that are safely consumed by humans as food and by animals as feed.

Bioinformatic analysis was used to determine whether the amino acid sequence of D4D(*Pl*) had significant sequence similarity to known protein toxins or antinutrients. The amino acid sequences from both LBFLFK Insert1 and LBFLFK Insert2 were used for bioinformatic analysis. For D4D(*Pl*), LBFLFK Insert2 includes a nucleotide change in the coding region, which results in the A102S amino acid substitution (section 5.2.10). D4D(*Pl*) did not show significant homology to proteins that are toxic to humans (40 CFR 725.421, 2016). D4D(*Pl*) did not show significant homology to known antinutrients (OECD, 2002a, b, 2011a, b, 2012b, 2016).

To determine the impact of commercial processing, D4D(*Pl*) enzyme activity and structural integrity following heat treatment were evaluated. The enzyme activity assay cannot distinguish between the individual delta-4 desaturase enzymes D4D(*Tc*), D4D(*Pl*), and D4D(*Pl*) [A102S] in LBFLFK PPP. The D4D proteins retained enzymatic activity at 30°C after 5 and 20 min, but the proteins were largely inactivated (< LOQ) at ≥ 50°C within 5 min and completely inactivated (< LOD) after longer treatment (20 min) at 90°C. D4D(*Pl*) protein underwent aggregation at ≥ 70°C within 5 min. These results demonstrate that D4D(*Pl*) protein is heat-labile and the enzymatic activity is unlikely to remain after commercial processing.

The digestibility of D4D(*Pl*) in SGF and in SIF was evaluated at 37°C during a 60-min time course. Protein degradation was then analyzed by western blot analysis using a D4D(*Pl*)-specific antibody. It was estimated that ≥ 96.8% of the full-length D4D(*Pl*) was digested within 0.5 min with SGF. It was also estimated that ≥ 96.4% of the full-length D4D(*Pl*) was digested within 0.5 min with SIF. These results demonstrate that D4D(*Pl*) is susceptible to digestion and rapidly degraded in SGF and SIF.

5.4.1.k. Acetohydroxy acid synthase [A122TS653N] (*At*) safety assessment

The HOSU of AHAS(*At*) and its donor organism *Arabidopsis thaliana* was evaluated. *A. thaliana* is not known to produce or contain toxins. As is typical of the Brassicaceae family, *A. thaliana* has been described to contain glucosinolates in varying composition and concentration (Kliebenstein et al., 2001). Glucosinolates are a large group of naturally occurring plant defense compounds that occur in all Brassica-originated feeds and fodders. The primary deleterious effects of ingestion of glucosinolates in animals are reduced palatability and decreased growth/production (Tripathi and Mishra, 2007). *A. thaliana* has not been reported to cause disease in humans or animals. AHAS enzymes are ubiquitous in all plants and microbes and have been isolated from numerous organisms (Mazur et al., 1987). Several commercialized crops have herbicide tolerance conferred by alleles of the *ahas* gene (e.g., Clearfield® canola, Clearfield® wheat, Clearfield® sunflower, Clearfield® lentils). The lack of adverse findings for AHAS(*At*) and its donor organism substantiates their HOSU.

The AHAS(*At*) [A122TS653N] protein expressed in LBFLFK harbors two amino acid substitutions, A122T and S653N, that confer tolerance to imidazolinone herbicides (Tan et al., 2005). The safety of mutant, herbicide-tolerant AHAS enzymes expressed in agricultural crops has been extensively investigated and reported (Mathesius et al., 2009; Chukwudebe et al., 2012). None of the AHAS enzyme variants cause any adverse effects due to exposure to the protein.

The amino acid sequence of AHAS(*At*) [A122TS653N] was compared with other AHAS enzymes present in consumed food or feed. The proteins found to have the highest sequence identity to AHAS(*At*) [A122TS653N] originated from plants and mosses and included commonly consumed crops such as *Brassica napus* (canola, 88.2% identity), *Cicer arietinum* (chickpea, 78.2% identity), *Malus domestica* (apple, 78.9% identity), and *Helianthus annuus* (sunflower, 77.6% identity). The protein from *Helianthus annuus* has been shown to be an active AHAS enzyme (Kolkman et al., 2004). Thus, AHAS(*At*) [A122TS653N] is structurally and functionally related to other AHAS enzymes that are safely consumed by humans as food and by animals as feed.

Bioinformatic analysis was used to determine whether the amino acid sequence of AHAS(*At*) [A122TS653N] had significant sequence similarity to known protein toxins or antinutrients. AHAS(*At*) [A122TS653N] did not show significant homology to proteins that are toxic to humans (40 CFR 725.421, 2016). AHAS(*At*) [A122TS653N] did not show significant homology to known antinutrients (OECD, 2002a, b, 2011a, b, 2012b, 2016).

To determine the impact of commercial processing, AHAS(*At*) [A122TS653N] enzyme activity and structural integrity following heat treatment were evaluated. The enzyme activity assay cannot distinguish between the newly expressed AHAS(*At*) [A122TS653N] protein and the endogenous canola AHAS protein AHAS(*Bn*), both present in LBFLFK. The AHAS proteins retained activity at 30°C after 5 min and 20 min. However, the AHAS proteins were largely inactivated (< LOQ) at 50°C within 5 min and completely inactivated (< LOD) after longer treatment (20 min) and/or higher temperatures (≥ 70°C). These results demonstrate that increasing temperatures caused a loss of AHAS enzyme activity. Furthermore, AHAS proteins underwent structural change (in the form of decreasing signal intensity revealed by a western blot) at ≥ 70°C within 5 min. The antibody generated against the mature, full-length AHAS(*At*) [A122TS653N] protein is not expected to distinguish between the AHAS(*At*) [A122TS653N] protein and the endogenous AHAS(*Bn*) protein as a significant portion of the antigen is identical between the Arabidopsis AHAS(*At*) [A122TS653N] and Brassica AHAS(*Bn*) isoforms. These results demonstrate that the AHAS(*At*) [A122TS653N] protein is heat-labile and the enzymatic activity is unlikely to remain after commercial processing.

The digestibility of AHAS(*At*) [A122TS653N] in SGF and in SIF was evaluated at 37°C during a 60-min time course. Protein degradation was then analyzed by western blot analysis using an AHAS-specific antibody. Unlike the other newly expressed proteins in LBFLFK, AHAS(*At*) [A122TS653N] is a soluble protein. Thus, its digestibility in SGF and SIF was assessed in both PPP and leaf tissue. Since the antibody raised against the full-length AHAS protein is not expected to distinguish between the full-length AHAS(*At*) [A122TS653N] protein and the endogenous AHAS(*Bn*) protein, it is not possible to provide a relative amount of each protein in PPP or leaf tissue. Thus, the digestive fate results are presented qualitatively. Following exposure to SGF, the AHAS proteins were digested within 0.5 min regardless of matrix (both LBFLFK PPP and LBFLFK leaf tissue). For SIF digestion, the AHAS proteins responded differently depending on the matrix. Using leaf tissue, the AHAS proteins were digested within 0.5 min whereas in PPP, the AHAS proteins remained stable over the 60-min time course. Consequently, the digestibility of the AHAS proteins in PPP was assessed in a sequential digestion of SGF followed by SIF. Consistent with the results for SGF digestion alone, the AHAS proteins were degraded rapidly (within 0.5 min) with SGF. Thus, no AHAS proteins were remaining for further digestion with SIF. These results demonstrate that the AHAS proteins are susceptible to digestion in SGF and, when leaf tissue is used, also to digestion in SIF.

5.5. Conclusion on the characterization and safety of the newly expressed proteins

The newly expressed proteins in LBFLFK canola were assessed for safety following a weight-of-evidence approach. This included characterization for identity and amino acid sequence, for apparent molecular weight and immunoreactivity, and for potential glycosylation and enzymatic activity.

The expression levels of each of the eleven newly expressed proteins were also assessed in different plant tissues, including young whole plants (rosettes), flowering whole plants, root, leaf, pollen, immature seeds, and mature seeds. The expression data confirmed that the introduced elongases and desaturases, controlled by seed-specific promoters, were expressed only in seed tissue as expected. However, two of the proteins (O3D(*Pi*) and D6E(*Pp*)) were not found at detectable levels in any tissue sample of LBFLFK canola. The introduced herbicide-tolerant AHAS, controlled by a constitutive promoter, was found at highest concentrations in green plant tissues and was not detected in mature seeds.

For each of the proteins, a history of safe use and consumption was established. No adverse findings were identified for the proteins or donor organisms using a systematic literature search. The protein sequence of each newly expressed protein was found to be structurally and functionally related to other proteins that are safely consumed by humans as food and by animals as feed. There were no known protein toxins, antinutrients, and allergens with significant amino acid similarity found using a bioinformatics approach. Assessments of protein digestibility and of protein degradation with exposure to elevated temperatures also demonstrated the safety of the newly expressed proteins that were tested.

In summary, all newly expressed proteins in LBFLFK are considered to behave as any other dietary protein and do not raise any safety concerns with regard to human or animal health or the environment.

6. AGRONOMIC, PHENOTYPIC, AND ENVIRONMENTAL INTERACTIONS ASSESSMENT

As part of the environmental safety assessment, key agronomic, phenotypic, and environmental interaction characteristics of EPA+DHA canola event LBFLFK were compared to the parental control variety Kumily, which has the same genetic background as LBFLFK but does not contain the genes coding for the EPA+DHA and herbicide tolerance traits. The characteristics for comparisons were selected based on those that are typically observed by plant breeders in selecting new plant varieties for commercialization. The characteristics also included features that are associated with weediness and plant pest potential, as well as seed dormancy, susceptibility to biotic and abiotic stress, an assessment of pollen biology, and interactions with other organisms. Conventional, commercially-available reference canola varieties that represented a range of genetic backgrounds from different breeders were included in the trials to establish a range of natural variation for the observed characteristics. The statistical significance of any observed difference between LBFLFK and Kumily was assessed in the context of variability in the reference canola varieties.

Test, control, and reference varieties

LBFLFK, Kumily, and six conventional varieties (Q2, 46A65, IMC105, IMC302, Wizzard, and Orinoco) served as test, control, and reference substances, respectively (Table 12).

Table 12. List of Test, Control, and Reference Varieties including Herbicide Treatments

Treatment Designation	Purpose	Herbicide Treatment	Herbicide Rate (g a.i./ha)
LBFLFK (non-sprayed)	Test	Standard herbicide	Label
LBFLFK (sprayed)	Test	Standard herbicide + Beyond® herbicide	Label + 35 g a.i./ha
Kumily	Control	Standard herbicide	Label
Q2	Reference	Standard herbicide	Label
46A65	Reference	Standard herbicide	Label
IMC105	Reference	Standard herbicide	Label
IMC302	Reference	Standard herbicide	Label
Wizzard	Reference	Standard herbicide	Label
Orinoco	Reference	Standard herbicide	Label

6.1. Field trial locations and design

Agronomic and phenotypic performance was assessed at 14 trial locations over two seasons and covering a range of canola growing regions. The first season (herein referred to as “winter 2014/15”) included six locations in the southern U.S., with sowing in the fall of 2014 and harvest in the spring of 2015. The second season (herein referred to as “spring 2015”) included eight locations in the northern U.S., with sowing in the spring of 2015 and subsequent harvest in the late summer of that year (Figure 9, Table 13).

Figure 9. Map of Locations for Agronomic/Phenotypic Data Collection for LBFLFK during 2014/15 and 2015 Field Trials

Field trials conducted in the winter 2014/15 season are shown as stars. Two sites near Houston, TX appear as a single star. Field trials conducted in the spring 2015 season are shown as balloons.

Table 13. Field Trial Designations, Locations, and Planting Dates for 2014/15 and 2015 Seasons

Field Trial Designation	Nearest Town, State	Planting Date	Trial Season
3SRBLY1	Beasley, TX	20-November-2014	Winter 2014/15
3SRJV	Jeffersonville, GA	03-December-2014	Winter 2014/15
3SRKT	Kendleton, TX	03-December-2014	Winter 2014/15
3SROM	Odem, TX	18-November-2014	Winter 2014/15
3SRRH	Rio Hondo, TX	19-November-2014	Winter 2014/15
3SRWN1	Washington, LA	14-November-2014	Winter 2014/15
3NRLS	Lime Springs, IA	13-May-2015	Spring 2015
3NRGE	Geneva, MN	20-May-2015	Spring 2015
3NRSC	Sartell, MN	21-May-2015	Spring 2015
3NRCB	Campbell, MN	22-May-2015	Spring 2015
3NRNW-1	Northwood, ND	22-May-2015	Spring 2015
3NRMA-2	Malta, MT	23-May-2015	Spring 2015
3NREP	Ephrata, WA	19-May-2015	Spring 2015
3NRBRK	Brookings, SD	02-June-2015	Spring 2015

As Kumily and the reference varieties are spring canola varieties, performance of the plants during spring 2015 was notably better than the winter. Both data sets are presented and discussed in the petition as part of the comparative assessment, but it is noted that the spring data is more representative of the intended growing environment for event LBFLFK.

Trials were prepared as a randomized complete block design (RCBD) with four plot replications at each location. All agronomic practices followed standards for canola production in the region of each field site. Each plot was approximately 5–6 meters (m) long and consisted of 18 rows with a seeding rate of 17–25 seeds per meter. A fallow area approximately 1.6 m wide surrounded each plot. In addition, to minimize cross-pollination between plots, a conventional male sterile canola variety surrounded each plot and the entire trial area at each field location.

Growing conditions and management practices were representative of commercial canola production in the specific regions of the United States. All plots received a standard herbicide treatment program, which was applied equally to all plots at each location. Additionally, as needed to maintain the health of the plots for meaningful data collection, insecticides and/or fungicides were applied evenly to all plots at a given field location.

Plots of LBFLFK sprayed with Beyond® herbicide (active ingredient imazamox, an imidazolinone) were included in the study design at each location during each season. LBFLFK treated with Beyond® herbicide is referred to herein as LBFLFK (sprayed) and untreated as LBFLFK (non-sprayed). Beyond® herbicide was applied at the 3–4 leaf stage for each trial for the sprayed treatments at a rate of 34–37 grams active ingredient/hectare (g a.i./ha). The gathered data confirm that the application of Beyond® herbicide does not have any direct impacts on the phenotype or agronomic performance of LBFLFK. Unless otherwise indicated, all observations of LBFLFK performance apply to both sprayed and non-sprayed treatments.

Assessments were performed at different growth stages on plants that were in the outer three rows of each plot, excluding the outside row to reduce any border effects. During the course of the studies, the following parameters were measured: field emergence, early plant stand, seedling vigor, days to start of flowering, days to end of flowering, days to maturity, plant height, plant lodging, pod shattering, and final plant stand. Pod count was also measured in spring 2015 trials only. Additionally, disease incidence, insect damage, and abiotic stress damage were monitored.

Grain samples from each plot were collected at harvest (at BBCH 99, according to Meier (2001)). Post-harvest phenotypic data collection included an assessment of seed quality, moisture, thousand seed weight, and yield.

Agronomic, phenotypic, and environmental interactions data collection, including measured characteristics and timings of assessments, are summarized in Table 14.

Across-site statistical analyses for the detection of genotype-by-environment (referred to in this document as entry-by-site) interactions and for differences between LBFLFK and Kumily were carried out for all agronomic and phenotypic assessments. Some data were transformed to avoid strong deviations from ANOVA assumptions. Linear mixed model ANOVA methods were used for performing mean comparisons between the LBFLFK entries (sprayed or non-sprayed) and Kumily. A significance level of $\alpha = 0.05$ (confidence level = 95%) was used for all statistical tests. Individual site analyses were performed if a statistically significant entry-by-site interaction was observed. Data meeting at least one of the following three criteria were considered not suitable for ANOVA.

- The characteristic has 6 or less distinct values.
- The mode of the characteristic has more than 40% frequency.
- More than 40% of the site-entry combinations had null variance.

Across-site mean values were compared to the range of means generated from the reference varieties to provide context for the comparative analyses and assess the broader biological relevance of the results. Further details of experimental design, data collection, and statistical analysis methods are presented in Appendix E.

Additional studies to assess seed germination and dormancy as well as pollen characteristics (Table 14) were conducted in controlled conditions. Seed germination and dormancy experiments were carried out with grain harvested from the spring 2015 field trials (six locations). The seed germination and dormancy data are discussed in section 6.2.4 with the details of experimental design presented in Appendix F. As described in section 6.2.5, pollen morphology and viability were determined in LBFLFK, Kumily, and three reference varieties grown under greenhouse conditions. Details of this pollen study are given in Appendix G.

A separate study was performed to further assess for any possible differences of field-grown LBFLFK compared to other canola varieties in terms of field ecological interactions (Table 14). This assessment of ecological interactions, including LBFLFK (sprayed), Kumily, and three reference canola varieties, was performed at three locations and is summarized in section 6.2.6 with further details provided in Appendix H.

Table 14. Agronomic and Phenotypic Characteristics Measured

General Characteristic	Characteristic Measured	Rating Time Point or Condition[1]	Description of Rating
Germination/ Emergence	Field emergence	BBCH 12–13	Percent of emerged plants
	Early plant stand	BBCH 12–13	Seedlings per 2 rows (count)
	Seedling vigor	BBCH 12–13	1 to 5; 1 = healthy, 5 = unhealthy
Vegetative growth parameters	Days to start of flowering	BBCH 60–62	Number of days with 50% of the plants having started anthesis
	Days to end of flowering	BBCH 69	Number of days where only 10% of the plants are still flowering
	Days to maturity	BBCH 85–86	Number of days to physiological maturity
	Plant height	BBCH 85–86	Plant height (cm) at maturity
	Pod count (2015 season only)	BBCH 85	Pods per plant
	Lodging	BBCH 85–86	1 to 10; 1 = 0–10% lodging, 10 = greater than 90% lodging
	Pod Shattering	BBCH 85–86	1 to 10; 1 = 0–10% shattering, 10 = greater than 90% shattering
	Final plant stand	BBCH 85–86	Plants per 2 rows (count)
Stress response	Disease incidence	BBCH 12–16, BBCH 60–62, BBCH 69, BBCH 85–86	1 to 3; 0 = no damage, 3 = severe damage
	Insect and arthropod damage	BBCH 12–16, BBCH 60–62, BBCH 69, BBCH 85–86	1 to 3; 0 = no damage, 3 = severe damage
	Abiotic stress damage	BBCH 12–16, BBCH 60–61, BBCH 69, BBCH 85–86	1 to 3; 0 = no damage, 3 = severe damage
Post-harvest seed characteristics	Seed moisture	BBCH 99	Percent moisture
	Plot yield[2]	BBCH 99	Weight (g) per plot
	Seed quality	BBCH 99	Percent of distinctly green seeds
	Thousand seed weight	BBCH 99	Weight (g) of 1000 seeds

General Characteristic	Characteristic Measured	Rating Time Point or Condition[1]	Description of Rating
Reproductive biology	Pollen viability	BBCH 60–69	Percent of viable pollen
	Pollen morphology	BBCH 60–69	Pollen length and width
	AOSA standard seed germination[3]	Three days at 15/25°C	Percent normal seedlings
		Seven days at 15/25°C	Percent normal seedlings
	Warm seed germination	Three days at 25°C	Percent normal seedlings
		Seven days at 25°C	Percent normal seedlings
	Cold seed germination	Ten days at 10°C	Percent normal seedlings
		Ten days at 10°C, followed by three days at 25°C	Percent normal seedlings
	Secondary/dark dormancy seed germination	Sixteen to 35 days at 18°C after dormancy induction	Percent normal seedlings
		35 days after dormancy induction	Percent dead seeds
		35 days after dormancy induction	Percent viable by tetrazolium assay
Environmental interactions	"Non-target" organism assessment	BBCH 12–16, BBCH 60–61, BBCH 69, BBCH 83–87	Number & diversity of arthropods
		BBCH 60–61, BBCH 83–87	Number and weight of earthworms

[1] Canola plant growth stages are described in (Meier, 2001).
[2] Yield was adjusted to 9% seed moisture.
[3] Methods for testing seed were consistent with AOSA guidelines (AOSA, 2009, 2014).

6.2. Agronomic and phenotypic assessments

The data for the agronomic and phenotypic characteristics obtained from the winter 2014/15 and spring 2015 trials are first described per season (sections 6.2.1 and 6.2.2, respectively) followed by an across-season discussion (section 6.2.3). All genotype-by-environment (here referred to as entry-by-site) interactions analysis data mentioned in the following sections are presented in Appendix E.

6.2.1. Agronomic and phenotypic field trials - Winter 2014/15

A summary of the results of the winter 2014/15 field trials is described below. Table 15 contains a comparison of the measured field data that was suitable for ANOVA. Table 16 shows the data for measured field characteristics that were not suitable for ANOVA, with data summarized in terms of across-site means and ranges.

Early Plant Development – Winter 2014/15

During the 2014/15 winter season, field emergence and early plant stand count showed statistically significant entry-by-site interactions, with LBFLFK generally reduced compared to Kumily and at the lower end of the range of the reference varieties across sites (Table 15). The by-site analysis demonstrated that LBFLFK values were below the range of the reference varieties at most locations. Additionally, the seedlings of LBFLFK were generally less vigorous at the time of evaluation compared to Kumily, though the assessments were within the range of the reference varieties (Table 16).

Vegetative growth parameters – Winter 2014/15

As shown in Table 15, the number of days to start of flowering in LBFLFK was statistically significantly different than Kumily, with LBFLFK flowering about two days later, though within the range of the reference varieties. Days to end of flowering was also statistically significantly delayed (about two days) in LBFLFK with a statistically significant entry-by-site interaction, but within the range of the reference varieties at all locations. Days to maturity also showed a statistically significant entry-by-site interaction, with the average difference between LBFLFK and Kumily reduced to about one day and no longer statistically significantly different across locations. Mean differences for final plant stand were also statistically significantly affected by site, with LBFLFK reduced compared to Kumily and the by-site analysis showing LBFLFK below the range of the reference varieties at four of the six locations. The differences in days to start of flowering, days to end of flowering, and final plant stand are attributed to the reduced field emergence and differences in early plant stand count that are described above. Plant height means of LBFLFK were greater than Kumily, but this comparison was statistically significant only for LBFLFK (sprayed), and means were all within the range of the reference varieties. The mean lodging and pod shattering ratings observed for LBFLFK were similar to Kumily, and all were within the range of the reference varieties (Table 16).

Post-harvest seed characteristics – Winter 2014/15

The mean percent seed moisture was statistically significantly higher in LBFLFK compared to Kumily, though it was within the range of the reference varieties (Table 15). Mean yields of LBFLFK were reduced compared to Kumily, with the difference being statistically significant for LBFLFK (non-sprayed). Yields were within the range of the reference varieties. The means for thousand seed weights of LBFLFK were not statistically different from Kumily and were within the range of of the reference varieties. As shown in Table 16, seed quality (% green seeds) was similar across LBFLFK and Kumily and was within the range of of the reference varieties.

Table 15. Agronomic and Phenotypic Characteristics – Across-Site Summary Statistics – Winter 2014/15

Characteristic	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)	ANOVA p-values[4]
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	Entry-by-site (Entry effect)
Field emergence (%)	62.96 (3.93) 52–68.25	46.17 (3.93) 31.5–59.5	16.79 (3.8) (0.001)*	36.25–80.25	48.46 (3.93) 38.5–59.5	14.5 (3.8) (0.003)*	0.0006* (0.0026*)
Early plant stand (count)	146 (10.55) 97.25–163.5	110.58 (10.55) 75.5–143.25	35.42 (10.38) (0.007)*	87.25–192.25	115.33 (10.55) 91.75–142	30.67 (10.38) (0.014)*	0.0082* (0.0133*)
Days to start of flowering	111.08 (2.18) 104.75–118	113.08 (2.18) 105–120	-2 (0.41) (0.001)*	90–120	112.38 (2.18) 104.75–120	-1.29 (0.41) (0.01)*	0.1112 (0.0019*)
Days to end of flowering	135.88 (4.33) 122–152	137.71 (4.33) 122–152	-1.83 (0.79) (0.043)*	119–152.5	135.96 (4.33) 122-152	-0.083 (0.79) (0.918)	0.0138* (0.0732)
Days to maturity	158.75 (3.24) 149.75–171	159.75 (3.24) 150.5–171	-1 (0.48) (0.064)	138–171	159.04 (3.24) 150.5–171	-0.29 (0.48) (0.557)	0.0103* (0.1511)
Plant height (cm)	117.1 (10.84) 82.95–160.8	123.64 (10.84) 91.05–162.85	-6.54 (1.89) (0.001)*	75.4–234.4	120.38 (10.84) 83.6–159.85	-3.27 (1.89) (0.09)	1.0000 (0.0048*)
Final plant stand (count)	133.29 (10.66) 93.25–161.25	96.96 (10.66) 74.25–138.5	36.33 (9.94) (0.004)*	77.5–189	106.67 (10.66) 82–144	26.62 (9.94) (0.023)*	0.0002* (0.0118*)
Seed moisture (%)	8.23 (0.55) 7.1–10.55	9.09 (0.55) 7.67–11.47	-0.86 (0.052) (< 0.001)*	6.35–10.82	9.05 (0.55) 7.58–11.53	-0.81 (0.052) (< 0.001)*	0.9999 (0.0000*)
Thousand seed weight (g)	2.87 (0.14) 2.39–3.37	2.91 (0.14) 2.42–3.5	-0.039 (0.035) (0.272)	1.86–3.43	2.88 (0.14) 2.41–3.29	-0.009 (0.035) (0.794)	0.9999 (0.5157)
Plot yield (g)	2442.13 (531.65) 447.86–3760.99	2278.94 (531.65) 440.79–3517.38	163.19 (103.78) (0.122)	211.48–4375.18	2068.79 (531.65) 283.68–3236.57	373.35 (103.78) (0.001)*	1.0000 (0.0033*)

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of six locations with four plot replications at each location.
[4] * indicates p≤ 0.05 for entry-by-site interactions or an entry effect.

Table 16. Agronomic and Phenotypic Characteristics Not Suitable for ANOVA – Across-Site Comparison – Winter 2014/15

Characteristic	Statistic	Control (Kumily)	LBFLFK (sprayed)[1]	LBFLFK (non-sprayed)[2]	Reference Varieties
Seedling vigor (rating)[3]	Mean	1.75	2.71	2.63	1.99
	Standard deviation	0.57	0.68	0.38	0.72
	Minimum	1.00	1.50	2.00	1.00
	Maximum	2.25	3.50	3.00	3.25
Plant lodging (rating)[3]	Mean	1.04	1.04	1.17	1.56
	Standard deviation	0.10	0.10	0.41	1.26
	Minimum	1.00	1.00	1.00	1.00
	Maximum	1.25	1.25	2.00	8.00
Pod shattering (rating)[3]	Mean	1.25	1.25	1.17	1.41
	Standard deviation	0.39	0.42	0.30	0.68
	Minimum	1.00	1.00	1.00	1.00
	Maximum	2.00	2.00	1.75	4.50
Seed quality (% green seed)	Mean	0.12	0.42	0.33	0.21
	Standard deviation	0.21	0.66	0.38	0.35
	Minimum	0.00	0.00	0.00	0.00
	Maximum	0.50	1.50	1.00	1.25

Means are averages of six locations with four plot replications at each location.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] See Table 14 for rating scale.

6.2.2. Agronomic and phenotypic field trials – Spring 2015

A summary of the results of the spring 2015 field trials is described below. Table 17 contains a comparison of the measured field data that was suitable for ANOVA. Table 18 shows the data for measured field characteristics that were not suitable for ANOVA, with results summarized in terms of across-site means and ranges.

Early plant development – Spring 2015

Unlike for the winter 2014/15 field trials, early plant development characteristics, including field emergence and early plant stand count, were not statistically significantly different in LBFLFK compared to Kumily (Table 17). Seedling vigor was also similar in LBFLFK versus Kumily (Table 18). For each of these characteristics, the LBFLFK means were within the range of the reference varieties.

Vegetative growth characteristics – Spring 2015

As shown in Table 18, the mean values for days to start of flowering, days to end of flowering, and days to maturity were similar in LBFLFK compared to Kumily and were within the range of the reference varieties. Lodging and pod shattering values observed for LBFLFK were also similar to Kumily and within the range of the reference varieties.

Final plant stand and plant height means of LBFLFK were not statistically significantly different from Kumily and were within the range of the reference varieties (Table 17). Due to statistically significant variance heterogeneity present for plant height, data for this characteristic were also subjected to by-site analysis, which revealed a statistically significant difference between LBFLFK (non-sprayed) and Kumily at one location that was still within the range of the reference varieties.

Pod count was not statistically significantly different between LBFLFK and Kumily, but the ANOVA did exhibit a statistically significant entry-by-site interaction (Table 17). At one location, the mean pod count of LBFLFK (sprayed) was statistically significantly higher than Kumily and above the range of the reference varieties, but at another location, the mean pod count of LBFLFK (non-sprayed) was statistically lower than Kumily and below the range of the reference varieties. At all other sites, the mean pod counts of LBFLFK were not statistically significantly different from Kumily although some measured values for LBFLFK (and also Kumily) fell outside the range of the reference varieties at some locations. These data indicate a high degree of natural biological variability and, therefore, these differences are not considered as biologically relevant (Assefa et al., 2018).

Post-harvest seed characteristics – Spring 2015

As for the winter 2014/15 trials, seed moisture exhibited a statistically significant entry-by-site interaction. At all but one location, the mean seed moisture of LBFLFK was statistically significantly higher than Kumily and, for all sites, was above the range of the reference varieties.

Thousand seed weight means were not statistically significantly different in LBFLFK compared to Kumily and were within the range of the reference varieties (Table 17).

Plot yield (on a per plot basis adjusted to 9% seed moisture) showed a statistically significant entry effect in the across-site analysis (Table 17), with the mean yield of Kumily being statistically significantly higher ($p \leq 0.05$) than LBFLFK. However, LBFLFK mean yields were within the range of the reference varieties.

All entries showed similar seed quality (% green seeds) means that were within the range of the reference varieties (Table 18).

Table 17. Agronomic and Phenotypic Characteristics – Across-Site Summary Statistics – Spring 2015

Characteristic[1]	Control (Kumily)	LBFLFK (sprayed)[2]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[3]	Control minus LBFLFK (non-sprayed)	ANOVA p-values[4]
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE)[5] p-value[6]	min–max	Mean (SE) min–max	Difference (SE)[5] p-value[6]	Entry-by-site (Entry effect)
Field emergence (%)	50.90 (6.00) 30.82–78.50	50.53 (6.00) 36.38–101.00	0.37 (3.24) 0.9102	35.80–91.50	48.79 (6.00) 32.00–82.00	2.11 (3.24) 0.5253	0.0724 (0.7884)
Early plant stand^ (count)	161.51 (0.11) 118.00–322.50	153.74 (0.11) 104.25–266.00	7.77 0.3836	108.50–350.50	154.06 (0.11) 118.25–275.50	7.45 0.4037	0.4375 (0.6070)
Final plant stand^ (count)	151.61 (0.84) 94.50–311.00	143.99 (0.84) 86.50–273.50	7.62 0.2464	94.50–337.25	145.31 (0.84) 94.50–278.50	6.3 0.3356	1.0000 (0.4390)
Plant height (cm)	116.35 (11.56) 52.70–151.15	115.48 (11.56) 51.40–144.80	0.87 (1.50) 0.5722	52.50–170.35	115.89 (11.56) 51.80–149.20	0.46 (1.50) 0.7627	0.8330 (0.8475)
Pod count^ (count)	132.14 (0.11) 79.95–190.90	132.85 (0.11) 91.10–211.50	-0.71 0.9388	72.05–278.95	127.04 (0.11) 74.70–166.90	5.1 0.5768	0.0223* (0.7811)
Plot yield (g)	3513.32 (283.44) 2272.30–4312.21	3145.25 (283.44) 1980.99–4450.58	368.06 (144.38) 0.0231*	675.76–4626.77	3031.88 (283.44) 1886.98–4411.95	481.44 (144.38) 0.0049*	0.8307 (0.0126*)
Moisture (%)	6.76 (0.37) 5.42–8.38	7.81 (0.37) 6.38–9.62	-1.05 (0.13) 0.0000*	5.35–8.47	7.72 (0.37) 6.47–9.10	-0.96 (0.13) 0.0000*	0.0104* (0.0000*)
Thousand seeds weight (g)	3.53 (0.18) 2.90–4.48	3.50 (0.18) 2.90–4.42	0.03 (0.06) 0.6218	2.38–4.60	3.49 (0.18) 2.85–4.66	0.03 (0.06) 0.5671	1.0000 (0.8180)

[1] Data set noted with a ^ were transformed using a square root function to better meet statistical model assumptions.
[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[3] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[4] * indicates p≤ 0.05 for entry-by-site interactions or an entry effect.
[5] SE is not shown for differences estimated using transformed values.
[6] Difference test p-value: * indicates p≤ 0.05; means are averages of eight sites with four plot replications at each location.

Table 18. Agronomic and Phenotypic Characteristics Not Suitable for ANOVA – Across-Site Comparison – Spring 2015

Characteristic	Statistic	Control (Kumily)	LBFLFK (sprayed)[1]	LBFLFK (non-sprayed)[2]	Reference Varieties
Seedling vigor (rating)[3]	Mean	2.03	2.34	2.38	1.86
	Standard deviation	0.59	0.40	0.44	0.59
	Minimum	1.00	1.75	1.75	1.00
	Maximum	2.75	2.75	2.75	3.00
Days to start of flowering (days)	Mean	43.19	43.69	43.84	42.03
	Standard deviation	2.72	2.69	2.58	2.86
	Minimum	39.75	40.00	40.00	36.25
	Maximum	46.75	48.00	47.25	47.00
Days to end of flowering (days)	Mean	67.63	67.81	68.13	66.18
	Standard deviation	5.74	5.09	5.15	5.26
	Minimum	60.50	60.75	62.50	57.75
	Maximum	79.00	77.25	78.50	78.25
Days to maturity (days)	Mean	91.47	91.38	91.97	89.72
	Standard deviation	6.52	6.15	6.02	6.30
	Minimum	84.00	84.75	85.00	80.00
	Maximum	103.75	102.25	102.50	103.00
Plant lodging (rating)[3]	Mean	3.09	2.97	3.46	5.09
	Standard deviation	2.78	2.91	2.62	3.03
	Minimum	0.00	0.00	0.00	0.00
	Maximum	8.00	8.75	8.75	10.00
Pod shattering (rating)[3]	Mean	0.84	0.75	0.75	0.87
	Standard deviation	0.55	0.46	0.46	0.59
	Minimum	0.00	0.00	0.00	0.00
	Maximum	1.50	1.00	1.00	2.50
Seed quality (% green seed)	Mean	0.97	1.22	1.16	0.84
	Standard deviation	0.56	0.63	0.72	0.99
	Minimum	0.25	0.00	0.00	0.00
	Maximum	1.75	2.00	2.25	4.25

Means are averages of eight sites with four plot replications at each location.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] See Table 14 for rating scale.

6.2.3. Agronomic and phenotypic data across seasons

Agronomic and phenotypic data collected and analyzed for LBFLFK, Kumily, and six conventional reference varieties over two seasons showed that LBFLFK performs similarly to other canola regardless of treatment with Beyond® herbicide (in the absence of significant weed pressure). Data from two growing seasons and a total of 14 field sites provided a diverse range of possible canola growing environments in the U.S.

The cold temperatures experienced at many of the winter 2014/15 trial locations had an impact on the performance of the plants. LBFLFK in particular was more sensitive to cold during germination and emergence than Kumily or the reference varieties, which is attributed to the different oil profile of the seed (see section 6.2.4 for further discussion of the impact of the EPA+DHA canola trait on seed germination). As a result, statistically significant ($p \leq 0.05$) entry-by-site interactions were observed in field emergence, early plant stand, days to end of flowering, days to maturity, and final plant stand. Without the cold temperature during early plant development that occurred during the winter 2014/15 field trials, fewer statistically significant differences between LBFLFK and Kumily were observed. In spring 2015 trials, statistically significant ($p \leq 0.05$) entry-by-site interactions were only observed in pod count and seed moisture.

There was a statistically significant entry effect detected for most comparisons of plot yield, with LBFLFK having reduced yield compared to Kumily. A slight reduction in harvest yield may be associated with the production of long-chain fatty acids in LBFLFK (Nelson and Cox, 2017). However, the overall yields of LBFLFK were within the range of the references, and the differences are therefore considered not biologically meaningful.

In addition, there was a small but statistically significant increase of seed moisture (of approximately 1%) in the grain of LBFLFK compared to Kumily, which may be attributable to the intended change in fatty acid composition of the seed. LBFLFK is known to contain more unsaturated and elongated fatty acids than Kumily, and water is a by-product of the desaturation and elongation reactions (Nelson and Cox, 2017). It is possible that this water by-product results in a higher moisture content of the harvested grain. A slightly higher seed water content at harvest does not contribute to any selective advantage to LBFLFK and does not increase any environmental risks associated with the cultivation of canola.

In summary, LBFLFK performed similarly to Kumily when grown as a spring canola in the northern regions of the U.S., except for reduced yield and increased seed moisture. A reduced and delayed seed germination rate of LBFLFK was seen in cold conditions during the winter of 2014/15. This demonstrates that the introduced trait may have altered the sensitivity of LBFLFK compared to conventional canola to colder weather conditions, contributing to site-specific differences during field emergence and early plant development. The average parameters measured for LBFLFK were within the range of the reference varieties.

Overall, the data collected in these studies support the conclusions that LBFLFK, as a spring canola, is similar in agronomic and phenotypic characteristics to other conventional canola varieties and cultivation of LBFLFK does not present a different agronomic impact compared to other canola.

6.2.4. Seed germination evaluations and discussion

Standardized assays are routinely used to measure the germination and dormancy characteristics of canola seed (AOSA, 2014). These measurements provide insight into the quality of a seed for cultivation and may be indicative of changes in weediness potential as part of a comparative assessment. To test for any differences between LBFLFK and Kumily, seed germination was measured under different temperature regimes. Dormancy potential was also assessed. The generated data were analyzed using a mixed model ANOVA. The results for all assays are found in Table 19 and are summarized below. Further details of the materials and methods and the statistical analyses for these assays are presented in Appendix F.

Harvested seed from LBFLFK (sprayed and non-sprayed), Kumily, and six conventional canola reference varieties from the spring 2015 field trials at six locations were subjected to seed germination and dormancy analyses using criteria established by the Association of Official Seed Analysts (AOSA, 2014). Seed germination under three temperature regimes was assessed, including a standard assessment (daily cycle of 25°C for 8 hours in light, 15°C for 16 hours in dark), a warm temperature assessment (25°C with an 8/16 hours light/dark cycle), and a cold temperature assessment (10°C in the dark for 10 days, then 25°C 8/16 hours light/dark cycle for an additional 3 days).

Additionally, a secondary dormancy germination assay based on the method of Schatzki et al. (2013) was performed. For this assay, seeds moistened with a polyethylene glycol solution were first placed into the dark (18°C) for 28 days to induce dormancy, followed by alternating temperature and dark/light conditions (12 hours dark at 5°C, 12 hours light at 25°C) for seven more days. Seeds were evaluated at multiple time points during this procedure for the number of seeds with emerged radicles. A percent of total germination was calculated based on the total number of seeds that emerged. Seeds that had not emerged at the last evaluation were subjected to a tetrazolium (TZ) assay, and a percent viability for each entry was calculated.

In the standard assay, the mean germination rate of LBFLFK seed was statistically significantly lower than that of Kumily (~81% versus ~89%, respectively) (Table 19). The natural variability in this assay was high as the germination rates of the reference varieties varied from 59–97%. The combined mean rate of abnormal and dead seeds (confirmed by TZ assay) was also statistically significantly higher in LBFLFK than Kumily (~16% versus ~10%) though the means observed were within the range of references (~1–43%).

A similar trend was seen in the warm germination test. LBFLFK germinated at a statistically significantly lower mean rate than Kumily (~78% versus ~89%), but again this was within the range of the reference varieties. In addition, the mean rate of abnormal or dead LBFLFK seed in the warm test was again statistically significantly higher than that of Kumily (~18% versus ~10%) but within the range of the reference varieties (~1–34%).

The differences in germination rates between LBFLFK and Kumily were more pronounced in the cold germination assay. The analyses by-site are shown in Appendix F. The mean number of seedlings germinated in cold conditions was lower in LBFLFK than in Kumily and was below the range of the reference varieties at each site. The difference was less pronounced for the 13-day post-sowing assessment compared to the 10-day post-sowing assessment (Appendix F). These results further indicate that LBFLFK seeds tend toward delayed germination, if seeds germinate at all. The statistically significant differences observed for field emergence and final plant stand count for LBFLFK compared to Kumily during the winter 2014/15 field trials (section 6.2.1) are attributed to this delayed and reduced germination.

This phenotype of delayed and reduced germination of LBFLFK in cold conditions is likely associated with the altered fatty acid trait in LBFLFK. The very long chain polyunsaturated fatty acids may impact seed vigor through changes in the properties of the membrane characteristics that arise from an altered fatty acid profile, which includes altered 18-carbon fatty acids and the addition of the very long chain polyunsaturated fatty acids. There may also be an effect on vigor due to the fact that the introduced very long chain polyunsaturated fatty acids in LBFLFK have reduced utility in LBFLFK as an energy source (breakdown by endogenous lipases) for seedling establishment (Shrestha et al., 2016).

Previous research has shown that alterations in the seed fatty acid profile impact seed germination and seedling emergence. Bhattacharya et al. (2015) found that increasing the amount of stearic acid in the seed of *Brassica juncea* decreased oil content and reduced germination. This aligns with results obtained by Knutzon et al. (1992) for *B. napus* and *B. rapa*. Likewise, increased amounts of oleic acid also caused a reduction in germination of 10–15% in *B. napus* (Schierholt and Becker, 2011). In *Arabidopsis thaliana* (a member of the Brassicaceae), Miquel (1994) showed that only a marginal impact on seed

germination at ambient temperatures was present in the oleic acid-accumulating *fad2* mutant. However, a statistically significant delay in seed germination was observed in the *fad2* mutants under low temperatures, suggesting a strong influence of fatty acid changes on the temperature-dependent physiology of the seeds.

To confirm that the reduced germination of LBFLFK is not associated with any increased dormancy, secondary seed dormancy testing of the seeds was performed. Secondary dormancy, a weediness attribute (section 2.2), occurs in canola, which can result in an accumulation of viable seeds in the soil seed bank. In the assay for secondary seed dormancy (Table 19), LBFLFK again had statistically significantly lower germination than Kumily (12–17% lower). The total percent viability of LBFLFK also was 12–17% lower. However, seed dormancy (% viable as measured by the TZ assay) was not increased, with values at the lower end of the reference variety ranges. These data demonstrate that lower seed viability, and not increased dormancy, contribute to the lower total germination rates across all the germination assays performed. Therefore, the reduced germination phenotype of LBFLFK does not result in an increase in seed longevity within the soil seed bank but is rather associated with the reduced fitness of the line. This is likely related to the altered fatty acid profile as discussed above.

In summary, statistically significantly lower mean germination rates were observed for LBFLFK seed, with the difference more pronounced after exposure to cold temperatures. The alterations in the seed fatty acid profile in LBFLFK are likely impacting seed germination and seedling emergence, contributing to delayed and reduced germination rates. However, the reduced germination is a consequence of lower viability and not increased dormancy and, therefore, the reduced germination rate is not expected to contribute to increased weediness potential.

Table 19. Seed Germination, Viability, and Dormancy – Across-Site Summary Statistics

Germination Assay	Characteristic[1]	Control (Kumily)	LBFLFK (sprayed)	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[3]	Control minus LBFLFK (non-sprayed)	ANOVA p-values[4]
		Mean min–max	Mean min–max	Difference (p-value)[5]	min–max	Mean min–max	Difference (p-value)[5]	Entry-by-site (Entry effect)
Standard (AOSA)	% Germinated (7 days)	89.25 72.25–96.75	82.15 61.50–91.25	7.10 (0.008)*	59.00–97.00	80.24 64.00–89.50	9.02 (0.002)*	0.2517 (0.0049*)
	% Abnormal	3.64 2.00–8.25	6.66 5.25–10.00	-3.02 (0.043)*	0.50–14.00	7.76 4.25–11.50	-4.12 (0.013)*	0.1259 (0.0311*)
	% Dead	5.77 1.25–17.00	8.76 3.00–28.00	-2.98 (0.01)*	0.25–28.50	9.11 3.50–22.50	-3.34 (0.005)*	1.000 (0.0081*)
Warm (25°C)	% Germinated (7 days)	88.57 67.75–96.00	78.84 57.75–86.75	9.73 (< 0.001)*	65.75–96.50	76.82 61.00–86.50	11.75 (< 0.001)*	0.5883 (0.0003*)
	% Abnormal	3.09 1.75–5.25	6.09 4.00–8.50	-3.00 (< 0.001)*	0.25–9.75	6.60 4.00–10.25	-3.52 (< 0.001)*	1.000 (0.0002*)
	% Dead	7.18 1.75–26.75	11.46 3.75–27.00	-4.28 (0.015)*	0.75–24.25	11.64 3.50–24.00	-4.46 (0.0121)*	0.4524 (0.0193*)
Cold (10°C)	% Germinated (10 days)	61.04 39.75–82.50	4.60 3.25–8.25	56.44 (< 0.001)*	22.25–68.50	3.41 1.50–12.75	57.63 (< 0.001)*	0.0276* (0.0000*)
	% Germinated (13 days)	86.26 66.75–95.50	64.46 45.75–78.25	21.80 (0.001)*	58.50–94.75	57.23 36.25–76.5	29.02 (< 0.001)*	0.0010* (0.0005*)
Secondary Dormancy	%Total Germination[6]	93.74 79.29–99.01	81.47 58.83–89.44	12.27 (< 0.001)*	64.28–100.00	76.91 52.49–88.58	16.83 (< 0.001)*	0.0320* (0.0002*)
	% Viable by TZ Assay	0.12 (0.21) 0.00–0.50	0.043 (0.1) 0.00–0.26	NA	0.00–21.92	0.00 (0) 0.00–0.00	NA	NA
	Total % Viability[7]	93.87 79.29–99.01	81.52 58.83–89.44	12.35 (< 0.001)*	75.61–100.00	76.91 52.49–88.58	16.96 (< 0.001)*	0.0340* (0.0002)

NA = Not suitable for statistical analysis
[1] Data were arcsine transformed to achieve normality. Means were back transformed, and differences are differences of back transformed means.
[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[3] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[4] * indicates p≤ 0.05 for entry-by-site interactions or an entry effect.
[5] Difference test p-value: * indicates p≤ 0.05; means are averages of six locations with four plot replications at each location.
[6] Number of seeds that had germinated by 35 days after sowing.
[7] Sum of % total germination and % viable by TZ assay.

6.2.5. Data and summary of pollen morphology and viability assessments

An evaluation of the pollen grain characteristics of LBFLFK was performed to assess for any impacts of the conferred fatty acid or herbicide tolerance trait compared to conventional canola.

Pollen morphology and viability of LBFLFK was compared to Kumily and three conventional canola reference varieties. Details of experimental methods and statistical analyses are presented in Appendix G. Briefly, LBFLFK and Kumily pollen was collected from plants cultivated within greenhouses. Pollen viability was measured using fluorescein diacetate fluorescence staining (Heslop-Harrison et al., 1984). Viability was also measured by *in vitro* testing the ability of the pollen grains to form pollen tubes. Additionally, pollen grain morphology was assessed by measurement of the length and width of collected pollen grains.

The mean percent of viable pollen from LBFLFK, as measured by staining, was statistically significantly lower than Kumily (77% versus 86%) and was also below the lower limit of the range of the reference varieties (Table 20). This reduced value of pollen viability does not meaningfully affect seed set as a low threshold of pollen viability will induce seed set (Larden and Triboi-Blondel, 1994; Pline et al., 2002). In addition, the measured mean percent of germinated pollen of LBFLFK was similar to that of Kumily (at ~20%) and was well within the range of the reference varieties of ~16–39%.

Measurement of pollen grain dimensions (length and width) of LBFLFK were not significantly different from Kumily (Table 20). Mean pollen lengths of both LBFLFK and Kumily (~37 µm) were slightly below the lower limit of the range of the references varieties (~38 µm), indicating that the Kumily varietal background may have a shorter pollen grain length than the reference varieties selected for this study.

In summary, pollen morphology in event LBFLFK is similar to Kumily and conventional canola varieties, and though LBFLFK may have a slight reduction of pollen viability as a result of the introduced trait, this is not considered as biologically meaningful.

Table 20. Pollen Germination, Viability, and Morphology – Summary Statistics

Characteristic[1]	Control (Kumily) Mean (SE) min–max	LBFLFK Mean (SE) min–max	Control minus LBFLFK Difference (p-value)[2]	Reference Variety Range min–max
Germinated pollen^ (%)	18.65 0.99–49.37	20.72 1.41–48.14	-2.07 (0.9537)	15.73–38.74
Viable pollen^ (%)	85.79 55.67–96.59	76.93 52.25–92.61	8.86 (0.0357*)	80.13–90.09
Pollen length (µm)	37.05 (0.37) 34.43–39.19	36.64 (0.37) 34.78–39.46	0.41 (0.51) (0.7074)	37.85–38.60
Pollen width (µm)	26.56 (0.17) 25.33–27.44	26.74 (0.17) 25.64–27.79	-0.18 (0.24) (0.7421)	26.42–27.20

[1] A ^ after a characteristic name indicates data were arcsine-transformed data, back-transformed data are reported without standard error (SE).
[2] Difference Tukey HSD test p-value: * indicates p≤ 0.05; means are averages of sixteen replications for viability and morphology and twelve replications for germination assessments.

6.2.6. Data and summary of ecological interactions assessment

While there are no 'target' or 'non-target' species of LBFLFK, assessments of whether exposure to LBFLFK canola might have be differentially impacted or have an impact on pest species or species beneficial to agriculture were performed. An evaluation of differential impacts from abiotic stressors was also performed. During the winter 2014/15 and spring 2015 field trials, plots were monitored for disease and pest damage stressors, as well as damage from any naturally occurring abiotic stresses such as drought, wind, or hail (Table 14). Experimental design and methods are detailed in Appendix E. Additionally, a separate assessment of the ecological interactions of LBFLFK as compared with Kumily and three reference canola varieties was performed at three field locations in 2015. This separate assessment had a different trial design with larger plots, using varied sampling methods to assess the ecological community associated with the field trial plots. The experimental design and detailed results for these ecological interactions study are presented in Appendix H. A summary of the findings is provided below.

The assessment of abiotic stress damage, disease incidence, and insect/arthropod damage observations from the winter 2014/15 and spring 2015 agronomy trials are summarized in Table 21, Table 22, and Table 23, respectively. The abiotic stress factors that were observed were excessive rainfall and wind, moisture stress, drought, heat, and cold/wet weather. As indicated previously, LBFLFK exhibits delayed and reduced germination/emergence compared to conventional varieties as a result of cold stress during early plant development (sections 6.2.1 and 6.2.4). However, in terms of damage to the plants, no differences were observed between LBFLFK and Kumily for their responses to abiotic stress factors at all growth stages measured (Table 21).

Diseases typical to the growing regions (e.g., anthracnose, downy mildew, black leg, black rot, Sclerotinia, and black spot) were also evaluated. For assessment of pest damage, the effects of major crop pests such as aphids, diamondback moths, cabbage loopers, stink bugs, and leaf beetles on the plants were measured. Disease and pest damage were limited and, where present, ranged from none to minimal or minimal to mild stress in LBFLFK and Kumily (Table 22 and Table 23).

For the ecological interactions assessment study, the abundance and diversity of arthropod communities were measured using standard sampling techniques (visual observations, sticky traps, and pitfall traps) at different plant developmental stages throughout the trial period. The Shannon-Weaver index (S-W index), a measure of diversity (Shannon, 1948), was calculated for each location, developmental stage, and sampling method. The S-W index is used to evaluate the number of taxa (species richness) present in test, control, and reference plots and the relative amount (abundance) of each taxon.

The diversity and abundance of enumerated taxa varied greatly at different plant growth stages and field trial locations. A total of 64,413 arthropod organisms representing 13 taxonomic orders, including 16 recognized families, were collected across all locations and canola development stages using multiple sampling methods (visual count, sticky traps, and pitfall traps) (Table 24). Appendix H contains the detailed results of this sampling study.

In summary, there were no consistent differences in the LBFLFK fields compared to Kumily, and the results of the above assessments support the conclusion that LBFLFK is similar to Kumily and is no more likely to be susceptible or resistant to insect pests or diseases typical of canola growing regions. There were no statistically significant, consistent differences in the diversity of invertebrate taxa (pest or beneficial) associated with LBFLFK compared to other canola, and no patterns or trends of biological relevance were observed. These data further support the conclusion that LBFLFK is no more likely to result in the introduction or spread of a pest or disease, to be more susceptible to any pest or disease, or to have adverse impacts on organismal diversity compared to other canola varieties.

Table 21. Abiotic Observations of LBFLFK and Kumily

Abiotic Stressor	Number of Observations[1] across Locations (Rating)[2]		Number of Observations in which Differences were Observed between LBFLFK and the Control		Comments
Season	**2014/15**	**2015**	**2014/15**	**2015**	
Excessive Rainfall	132 (1)	24 (1)	0	0	Minor flooding
Moisture Stress	36 (0)		0		Plots proactively rated, no damage seen
Drought	12 (0)		0		Plots proactively rated, no damage seen
Heat	132 (0)	24 (3,1)	0	0	Heat stress observed at two development stages at one location
Wind	120 (0)	12 (0)	0	0	Plots proactively rated, no damage seen
Cold/Wet Weather	72 (1,3)		0		Frost damage observed at one location during 3 growth stages
Total	504	60	0	0	

Data were not subjected to statistical analysis.
Observational data were collected at four crop development stages: BBCH 12–16, 60–62, 69, and 85–86.
[1] Number of observations included the number of LBFLFK (sprayed and non-sprayed) and Kumily plots assessed for a given stressor (total of 12 plots at each location).
[2] Damage ratings made during observations (0 = none, 1 = mild, 2 = moderate, 3 = severe).

Table 22. Disease Stressor Observations of LBFLFK and Kumily

Biotic Stressor	Number of Observations[1] across Locations (Rating)[2]		Number of Observations in which Differences were Observed between LBFLFK and the Control		Comments
Season	**2014/15**	**2015**	**2014/15**	**2015**	
Alternaria		24 (1)		0	Alternaria observed at two locations
Anthracnose	24 (1,2)		1		One observation of moderate damage (rating 2) for single LBFLFK plot; all other plots had mild damage (rating 1)
Aster Yellows		12 (0,1)		4	Four observations of mild damage (rating 1) in LBFLFK plots with no damage seen in Kumily (rating 0)
Black Leg	132 (0)	24 (0)	0	0	Plots proactively rated, no damage seen
Black Rot	48 (0)		0		Plots proactively rated, no damage seen
Black Spot	96 (0)		0		Plots proactively rated, no damage seen
Downy Mildew	96 (0,1,2)		7		Damage ranging from none (rating 0) to moderate (rating 2) was observed across multiple entries; no trends seen
Root Rot Complex		12 (0)		0	Plots proactively rated, no damage seen
Sclerotinia	60 (0,1)	24 (0,1)	1	2	Mild damage (rating 1) for an LBFLFK plot in 2014/15. Two observations of mild damage (rating 1) in LBFLFK plots in 2015.
Seedling Disease Complex		12 (0)		0	Plots proactively rated, no damage seen
Total	456	108	9	6	

Data were not subjected to statistical analysis.
Observational data were collected at four crop development stages: BBCH 12–16, 60–62, 69, and 85–86.
[1] Number of observations included the number of LBFLFK (sprayed and non-sprayed) and Kumily plots assessed for a given stressor (total of 12 plots per location).
[2] Damage ratings made during observations (0 = none, 1 = mild, 2 = moderate, 3 = severe).

Table 23. Insect Stressor Observations of LBFLFK and Kumily

Biotic Stressor	Number of Observations[1] across Locations (Rating)[2]		Number of Observations in which Differences were Observed between LBFLFK and the Control		Comments
Season	**2014/15**	**2015**	**2014/15**	**2015**	
Aphids	144 (0,1)	12 (1)	5	0	Mild/no damage at two locations at the same growth stage in 2014/15; no trends seen
Armyworm		24 (0)		0	Plots proactively rated, no damage seen
Beet webworm		24 (0)		0	Plots proactively rated, no damage seen
Cutworm		12 (0)		0	Plots proactively rated, no damage seen
Cabbage Looper	84 (0)		0		Plots proactively rated, no damage seen
Cabbage Moth		24 (1)		0	Mild damage at one location at two growth stages
Corn Rootworm	48 (0,1)		0		Mild damage at two locations at the same growth stage
Diamondback Moth	120 (0,1)	24 (0)	0	0	Mild damage at two locations at the same growth stage
Flea Beetle		72 (1)		0	Mild damage at two growth stages for one location and at all four growth stages for another location
Leaf Beetle	36 (0)		0		Plots proactively rated, no damage seen
Looper		12 (0)		0	Plots proactively rated, no damage seen
Seed Pod Weevil		12 (1)		0	Mild damage at one location
Stink Bug	60 (0,1)		0		Plots rated for insect pest; none to mild damage observed in 2014/15
Total	492	216	5	0	

Data were not subjected to statistical analysis.
Observational data were collected at four crop development stages: BBCH 12–16, 60–62, 69, BBCH 85–86.
[1] Number of observations included the number of LBFLFK (sprayed and non-sprayed) and Kumily plots assessed for a given stressor (total of 12 plots per location).
[2] Damage ratings made during observations (0 = none, 1 = mild, 2 = moderate, 3 = severe).

Table 24. Ecological Interactions Study – Summary of Taxonomic Groups Captured

Arthropod Taxonomic Group (Order)	Number of Recognized Families	Total of Organisms Enumerated[1]		
		Pitfall Traps	Visual Observations	Sticky Traps
Coleoptera	4	4910	4521	8920
Lepidoptera	4	94	2501	1067
Diptera	2	518	10	31559
Hemiptera	4	108	380	410
Collembola	NI[2]	4865	–	–
Hymenoptera	1	53	3	593
Neuroptera	NI	–	–	443
Thysanoptera	NI	1431	1	–
Dermaptera	1	1	–	2
Ephemeroptera	NI	–	–	21
Orthoptera	NI	28	–	–
Odonata	NI	–	–	1
Araneae	NI	1622	80	271
Total		**13630**	**7496**	**43287**

[1] Includes adult and immature arthropods
[2] NI = family not identified

6.3. Conclusion on the agronomic, phenotypic, and environmental interactions assessment

EPA+DHA canola event LBFLFK is generally equivalent to the parental control variety Kumily and other canola varieties in terms of agronomic performance and phenotypic properties. Event LBFLFK does not have properties that would cause any additional concerns of environmental impacts or other ecological considerations when compared to other canola. The data collected on LBFLFK does indicate that the EPA+DHA trait may result in reduced germination, especially when exposed to reduced temperatures. Additionally, LBFLFK had a slightly reduced harvest yield and increased moisture level at harvest. These phenotypic differences are attributed to the altered fatty acid profile of the seeds, are not contributing to any increased weediness or other improved fitness of the plants, and will not impact the agronomic practices associated with the production of a canola crop.

The following is a summary of the agronomic and phenotypic assessment of EPA+DHA canola event LBFLFK.

- Statistically significant differences were observed in field emergence, plant stand, and plant development in the winter 2014/15 season, but these were not observed in the 2015 season. These differences are attributed to the extreme cold weather at sites during the winter 2014/15 season, which differentially impacted LBFLFK. Across seasons, seed moisture at harvest was lower by ~1% for LBFLFK. These phenotypic differences are attributed to the altered fatty acid profile of the seeds.

- Seed germination rates of LBFLFK were comparable to conventional reference varieties but statistically significantly delayed and lower than the parental variety Kumily. Especially under cold conditions, LBFLFK germination was statistically significantly reduced compared to Kumily and below the range of the references varieties. This reduced germination is considered the result of diminished viability and not increased dormancy.

- Pollen germination and pollen grain morphology characteristics were not statistically significantly different for event LBFLFK and Kumily. However, the viability of LBFLFK pollen grains as assessed by a staining assay appeared slightly reduced.

- LBFLFK is not different than other canola in regard to pest or disease susceptibility.

- There were no statistically significant differences between LBFLFK and Kumily in the assessment of diversity and abundance of pest or beneficial organisms.

Overall, the results of these assessments indicate LBFLFK is comparable to the conventional parental variety Kumily and does not pose a plant pest risk or an increased weediness potential as compared to conventional canola.

7. COMPOSITIONAL ASSESSMENT

As part of the food, feed, and environmental safety assessment of EPA+DHA canola event LBFLFK, a comparative assessment of grain components of LBFLFK was performed. For this assessment, LBFLFK, along with the parental control variety Kumily and conventional canola (*Brassica napus*) reference varieties, were grown and harvested under the same conditions at multiple locations. The purpose of the comparison, performed following Codex guidelines (Codex Alimentarius Commission, 2009), was to establish that nutritional components were not altered in a manner that would have an adverse impact on human and animal health or the environment.

Canola grain harvested from field-grown plots was used for compositional analysis. This includes plots of LBFLFK sprayed with Beyond® herbicide (sprayed), LBFLFK (non-sprayed), Kumily, and six conventional reference varieties (Q2, 46A65, IMC105, IMC302, Wizzard, and Orinoco). Data from two growing seasons, winter 2014/15 and spring 2015 (as described in section 6.1 of this petition), were generated. Harvested mature seed from five of the winter trials and from seven of the spring trials were used for compositional analysis (Table 25), with comparisons both within and across seasons. All four plot replicates of a field entry at each location were analyzed.

Table 25. Field Trial Locations used for Compositional Analysis of Harvested Seed

Field Trial ID	City, State	Trial Season
3SRBLY1	Beasley, TX	Winter 2014/15
3SRJV	Jeffersonville, GA	Winter 2014/15
3SRKT	Kendleton, TX	Winter 2014/15
3SROM	Odem, TX	Winter 2014/15
3SRRH	Rio Hondo, TX	Winter 2014/15
3NRLS	Lime Springs, IA	Spring 2015
3NRGE	Geneva, MN	Spring 2015
3NRCB	Campbell, MN	Spring 2015
3NRNW-1	Northwood, ND	Spring 2015
3NRMA-2	Malta, MT	Spring 2015
3NREP	Ephrata, WA	Spring 2015
3NRLS	Lime Springs, IA	Spring 2015

The components selected for analysis were based primarily on the guidance provided in the consensus document for canola from the Organisation for Economic Co-operation and Development (OECD, 2011b). A total of 112 components were measured in canola grain (Table 26).

Across-site statistical analyses for the detection of genotype-by-environment (referred to in this document as entry-by-site) interactions and for differences between LBFLFK and Kumily were carried out for all compositional assessments. Some data were transformed to avoid strong deviations from ANOVA assumptions. Linear mixed model ANOVA methods were used for performing mean comparisons between the LBFLFK entries (sprayed or non-sprayed) and Kumily. A significance level of $\alpha = 0.05$ (confidence level = 95%) was used for all statistical tests. Individual site analyses were performed if a statistically significant entry-by-site interaction was observed. Data meeting at least one of the following three criteria were considered not suitable for ANOVA.

- The characteristic has 6 or less distinct values.
- The mode of the characteristic has more than 40% frequency.
- More than 40% of the site-entry combinations had null variance.

Across-site mean values were compared to 1) the range of means generated from the reference varieties, 2) the International Life Sciences Institute (ILSI) Crop Composition Database data (ILSI, 2016), and 3) peer-reviewed scientific literature to provide context for the comparative analyses and assess the broader biological relevance of the results.

Additional details of the materials and methods for compositional analysis, including the statistics used for data comparisons, the results of by-site individual site analysis performed, and a table presenting the reference data from the ILSI Crop Composition database and peer-reviewed scientific literature are provided in Appendix I.

Table 26. Measured Canola Grain Components

Amino Acids[1]		
Alanine	Leucine	Threonine
Arginine	Isoleucine	Tryptophan
Aspartic Acid	Methionine	Tyrosine
Cystine	Phenylalanine	Valine
Glutamic Acid	Proline	Hydroxyproline
Glycine	Serine	Total Lysine
Histidine		
Antinutrients[1]		
Progoitrin	Gluconapoleiferin	4-Hydroxyglucobrassicin
Glucoalyssin	Gluconasturtiin	Phytic Acid
Glucobrassicin	Glucoraphanin	Tannins
Glucobrassicanapin	Neoglucobrassicin	Sinapine
Glucoiberin	Epi-Progoitrin	Coumaric Acid
Gluconapin	Total Glucosinolates[4]	Ferulic Acid
Fatty Acids[2]		
C14:0	C18:2n-9	C20:4n-6
C16:0	C18:2 trans	C20:5n-3
C16:1n-7	C18:3n-3	C22:0
C16:1n-9	C18:3n-6	C22:1n-9
C16:1 trans	C18:4n-3	C22:2n-6
C16:3n-3	C20:0	C22:4n-3
C17:0	C20:1n-9	C22:4n-6
C17:1	C20:2n-6	C22:5n-3
C18:0	C20:2n-9	C22:5n-6
C18:1n-7	C20:3n-3	C22:6n-3
C18:1n-9	C20:3n-6	C24:0
C18:1 trans	C20:3n-9	C24:1n-9
C18:2n-6	C20:4n-3	Total trans fatty acids
Minerals[1]		
Calcium	Phosphorus	Potassium
Copper	Magnesium	Sodium
Iron	Manganese	Zinc
Proximates and Fibers[1]		
Acid detergent fiber	Ash	Moisture[3]
Crude fiber	Crude fat	Protein
Neutral detergent fiber		
Sterols[1]		
24-Methylene cholesterol	Cholesterol	Delta-7 avenasterol
Beta-sitosterol	Clerosterol	Delta-7 stigmastenol
Brassicasterol	Delta-5 avenasterol	Sitostanol
Campestanol	Delta-5,23 stigmastadienol	Stigmasterol
Campesterol	Delta-5,24 stigmastadienol	Total phytosterols
Vitamins[1]		
Vitamin K1	Beta-tocopherol	Delta-tocopherol
Alpha-tocopherol	Gamma-tocopherol	Total tocopherols

[1] Data are reported on a dry weight basis.
[2] Data are reported as percent of total fatty acids.
[3] Data are reported as percent of fresh weight.
[4] Data are obtained by calculation.

7.1. Proximates and fibers

Proximates and fibers are major components of canola grain that impact processing and the application of processed products as a food and animal feed (OECD, 2011b). The

proximates and fiber components analyzed were moisture, crude fat, protein, ash, crude fiber, acid detergent fiber, and neutral detergent fiber. These components were measured on a percent dry weight basis except for moisture, which was measured on a percent fresh weight basis.

7.1.1. Winter 2014/15 season

For all proximate and fiber components across all locations for the 2014/15 season (Table 27), no statistically significant differences were observed between LBFLFK and Kumily. Only neutral detergent fiber had a statistically significant entry-by-site interaction for this season, with statistically significant differences at three locations and LBFLFK sometimes above the range of the references, but with no consistent trend seen across locations. The across-site means for all proximate and fiber components for LBFLFK were within the range of the reference varieties.

7.1.2. Spring 2015 season

For the 2015 season, statistically significant differences, though small in magnitude, were observed for acid detergent fiber, crude fiber, and neutral detergent fiber (Table 28). LBFLFK was statistically significantly lower than Kumily for acid detergent fiber, crude fiber, and neutral detergent fiber. There were no statistically significant entry-by-site interactions for this season. The results for all proximate and fiber components for LBFLFK (sprayed and non-sprayed) were within the range of the reference varieties.

Table 27. Proximates and Fiber – Across-Site Summary Statistics – Winter 2014/15

Component (% dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Acid detergent fiber	13.5 (0.27) 12.85–13.97	13.93 (0.27) 12.82–15.28	-0.43 (0.34) (0.231)	11.09–14.52	13.87 (0.27) 13.45–14.15	-0.37 (0.34) (0.307)
Ash	4.73 (0.21) 4.22–5.09	4.7 (0.21) 4.08–5.52	0.023 (0.13) (0.869)	3.59–5.77	4.62 (0.21) 4.1–5.35	0.11 (0.13) (0.451)
Crude fat	35.11 (1.83) 30.98–42.46	34.46 (1.83) 31.23–40.59	0.65 (0.44) (0.176)	27.82–44.56	35.42 (1.83) 32.41–41.72	-0.31 (0.44) (0.498)
Crude fiber	13.56 (0.28) 13.07–14.05	13.21 (0.28) 12.08–14.12	0.35 (0.4) (0.387)	11.07–14.8	13.03 (0.28) 12.03–13.5	0.54 (0.4) (0.186)
Moisture[4]	8.39 (0.21) 7.77–8.91	8.36 (0.21) 7.92–8.96	0.029 (0.087) (0.742)	7.59–9.45	8.29 (0.21) 7.6–8.83	0.11 (0.087) (0.246)
Neutral detergent fiber	16.92 (0.36) 16.1–18.4	16.84 (0.36) 15.85–18.7	0.07 (0.51) (0.886)	14.97–18.02	17.03 (0.36) 16.77–17.55	-0.11 (0.5) (0.833)
Protein	26.53 (1.43) 20.75–28.34	26.6 (1.43) 21.54–28.88	-0.07 (0.36) (0.849)	21.45–32.03	26.41 (1.43) 20.51–28.45	0.12 (0.36) (0.748)

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of five locations with four plot replications at each location.
[4] Moisture content in fresh weight.

Table 28. Proximates and Fibers – Across-Site Summary Statistics – Spring 2015

Component (% dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Acid detergent fiber	11.73 (0.3) 10.95–13.1	11.32 (0.3) 10.39–13.1	0.41 (0.2) (0.046)*	9.14–11.95	11.29 (0.3) 10.41–12.18	0.43 (0.2) (0.034)*
Ash	4.57 (0.16) 4.13–5.38	4.5 (0.16) 3.93–5.24	0.076 (0.064) (0.238)	3.92–5.53	4.55 (0.16) 4.09–5.15	0.021 (0.064) (0.749)
Crude fat	38.95 (0.96) 34.02–42.17	38.45 (0.96) 31.95–45.08	0.49 (0.64) (0.444)	31.29–44.23	38.6 (0.96) 35.22–41.9	0.34 (0.64) (0.595)
Crude fiber	10.37 (0.19) 9.46–11	9.39 (0.19) 8.9–10.24	0.99 (0.17) (< 0.001)*	7.57–10.95	9.39 (0.19) 8.7–9.83	0.98 (0.17) (< 0.001)*
Moisture[4]	7.33 (0.14) 6.64–7.73	7.3 (0.14) 6.7–7.66	0.03 (0.05) (0.559)	6.37–8.1	7.25 (0.14) 6.57–7.66	0.088 (0.05) (0.108)
Neutral detergent fiber	15.26 (0.26) 14.45–16.3	14.55 (0.26) 13.65–15.4	0.72 (0.29) (0.031)*	12.45–15.7	14.51 (0.26) 13.8–15.72	0.76 (0.29) (0.024)*
Protein	26.21 (0.56) 24.5–28.18	26.17 (0.56) 24.57–27.78	0.042 (0.17) (0.802)	22.53–31.24	26.29 (0.56) 24.13–28.18	-0.075 (0.17) (0.655)

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of seven locations with four plot replications at each location.
[4] Moisture content in fresh weight.

7.1.3. Across seasons and biological relevance

Comparing the results of the 2014/15 and the 2015 seasons, no differences between LBFLFK and Kumily were observed for moisture, crude fat, protein, and ash, and the values for these components were within the ranges of the reference varieties.

Statistically significant differences, small in magnitude, were only observed in the 2015 season for acid detergent fiber, crude fiber, and neutral detergent fiber for LBFLFK compared to Kumily. No trends were observed across seasons for these components. Furthermore, the proximate components of LBFLFK were within the ranges of the reference varieties across sites within a season and were within the range presented by either the peer-reviewed literature or ILSI Crop Composition Database values. Because these LBFLFK proximate and fiber component values were within the range of natural variation, the observed differences are considered not biologically relevant.

7.2. Amino acids

Amino acids are minor components of canola seed that impact the use of canola meal as a feedstock for livestock, poultry, and fish (OECD, 2011b). Nineteen amino acid components were analyzed and reported on a percent dry weight basis (Table 26).

7.2.1. Winter 2014/15 season

For the 2014/15 season, no statistically significant differences were observed between LBFLFK and Kumily (Table 29), and no statistically significant entry-by-site interactions were present. The means for all 19 amino acids for LBFLFK were within the ranges of the reference varieties.

7.2.2. Spring 2015 season

For the 2015 season, alanine, aspartic acid, leucine, methionine, tyrosine, and valine showed statistically significant differences, though small in magnitude. Except for aspartic acid, which was statistically higher in both LBFLFK treatments compared to Kumily (Table 30), these differences were inconsistent. The results for all 19 amino acids measured in LBFLFK were within the ranges of the reference varieties.

7.2.3. Across seasons and biological relevance

Comparing the results of the 2014/15 and the 2015 seasons, no differences between LBFLFK and Kumily were observed for 13 amino acids (arginine, cystine, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, phenylalanine, proline, serine, threonine, total lysine, and tryptophan). The values for these components were also within the ranges of the reference varieties.

Statistically significant differences, small in magnitude, were observed in the 2015 season for alanine, aspartic acid, leucine, methionine, tyrosine, and valine. No across-season trends were observed. Furthermore, all amino acid components of LBFLFK were also within the range presented by the ILSI Crop Composition Database values; therefore, the observed differences are considered not biologically relevant.

Table 29. Amino Acids – Across-Site Summary Statistics – Winter 2014/15

Component (% dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Alanine	1.13 (0.052) 0.91–1.21	1.15 (0.052) 0.99–1.22	-0.017 (0.018) (0.367)	0.94–1.33	1.15 (0.052) 0.92–1.22	-0.01 (0.018) (0.591)
Arginine	1.57 (0.09) 1.2–1.7	1.57 (0.09) 1.25–1.71	0.001 (0.027) (0.971)	1.29–1.96	1.55 (0.09) 1.19–1.71	0.013 (0.027) (0.64)
Aspartic acid	2.09 (0.11) 1.66–2.35	2.14 (0.11) 1.79–2.31	-0.052 (0.034) (0.126)	1.55–2.43	2.15 (0.11) 1.75–2.41	-0.058 (0.034) (0.089)
Cystine	0.62 (0.053) 0.43–0.73	0.62 (0.053) 0.43–0.73	0.0015 (0.011) (0.9)	0.49–0.82	0.62 (0.053) 0.41–0.72	0.0015 (0.011) (0.9)
Glutamic acid	4.44 (0.31) 3.24–4.9	4.47 (0.31) 3.31–5.03	-0.032 (0.071) (0.664)	3.55–5.8	4.47 (0.31) 3.19–4.99	-0.03 (0.071) (0.678)
Glycine	1.28 (0.065) 1.02–1.38	1.28 (0.065) 1.05–1.38	-0.0008 (0.018) (0.966)	1.06–1.56	1.28 (0.065) 1.01–1.39	-0.0006 (0.018) (0.975)
Histidine	0.7 (0.044) 0.52–0.77	0.69 (0.044) 0.53–0.76	0.012 (0.0099) (0.24)	0.57–0.88	0.69 (0.044) 0.52–0.76	0.011 (0.0099) (0.297)
Hydroxyproline	0.21 (0.008) 0.19–0.22	0.2 (0.008) 0.17–0.21	0.0075 (0.01) (0.467)	0.17–0.26	0.21 (0.008) 0.19–0.23	-0.0035 (0.01) (0.734)
Isoleucine	1.04 (0.054) 0.81–1.14	1.03 (0.054) 0.84–1.11	0.0083 (0.015) (0.591)	0.85–1.27	1.02 (0.054) 0.81–1.08	0.02 (0.015) (0.196)

Table 29. Amino Acids – Across-Site Summary Statistics – Winter 2014/15 (continued)

Component (% dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Leucine	1.77 (0.094) 1.38–1.93	1.74 (0.094) 1.4–1.88	0.029 (0.026) (0.254)	1.45–2.21	1.73 (0.094) 1.35–1.89	0.034 (0.026) (0.183)
Methionine	0.53 (0.032) 0.41–0.58	0.52 (0.032) 0.41–0.58	0.005 (0.0077) (0.533)	0.43–0.64	0.52 (0.032) 0.39–0.57	0.008 (0.0077) (0.328)
Phenylalanine	1.03 (0.051) 0.83–1.12	1.02 (0.051) 0.84–1.1	0.0063 (0.015) (0.669)	0.84–1.25	1.02 (0.051) 0.81–1.11	0.01 (0.015) (0.481)
Proline	1.55 (0.1) 1.16–1.67	1.52 (0.1) 1.16–1.7	0.032 (0.024) (0.183)	1.29–1.98	1.52 (0.1) 1.1–1.68	0.035 (0.024) (0.152)
Serine	1.08 (0.052) 0.88–1.17	1.07 (0.052) 0.89–1.15	0.0091 (0.02) (0.659)	0.89–1.34	1.08 (0.052) 0.86–1.2	0.0023 (0.02) (0.911)
Threonine	1.1 (0.051) 0.9–1.18	1.09 (0.051) 0.91–1.17	0.01 (0.016) (0.529)	0.91–1.31	1.1 (0.051) 0.89–1.19	0.0066 (0.016) (0.687)
Total Lysine	1.64 (0.11) 1.22–1.82	1.7 (0.11) 1.31–1.9	-0.059 (0.039) (0.135)	1.34–2.05	1.61 (0.11) 1.17–1.79	0.028 (0.039) (0.482)
Tryptophan	0.39 (0.021) 0.3–0.41	0.38 (0.021) 0.3–0.41	0.0065 (0.0056) (0.282)	0.31–0.48	0.38 (0.021) 0.3–0.4	0.006 (0.0056) (0.318)
Tyrosine	0.73 (0.035) 0.59–0.8	0.73 (0.035) 0.6–0.79	-0.002 (0.011) (0.853)	0.61–0.88	0.74 (0.035) 0.6–0.8	-0.0095 (0.011) (0.379)
Valine	1.35 (0.07) 1.05–1.46	1.34 (0.07) 1.1–1.46	0.005 (0.019) (0.794)	1.09–1.61	1.33 (0.07) 1.06–1.42	0.018 (0.019) (0.35)

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of five locations with four plot replications at each location.

Table 30. Amino Acids – Across-Site Summary Statistics – Spring 2015

Component (% dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Alanine	1.14 (0.025) 1.06–1.22	1.14 (0.025) 1.06–1.21	-0.001 (0.007) (0.867)	0.99–1.33	1.15 (0.025) 1.03–1.23	-0.015 (0.007) (0.033)*
Arginine	1.61 (0.044) 1.49–1.76	1.6 (0.044) 1.48–1.71	0.011 (0.014) (0.431)	1.32–1.95	1.61 (0.044) 1.42–1.76	0.001 (0.014) (0.944)
Aspartic acid	2.06 (0.083) 1.83–2.33	2.14 (0.083) 1.86–2.41	-0.076 (0.017) (< 0.001)*	1.55–2.5	2.17 (0.083) 1.83–2.51	-0.11 (0.017) (< 0.001)*
Cystine	0.58 (0.016) 0.49–0.64	0.57 (0.016) 0.49–0.61	0.012 (0.0091) (0.197)	0.52–0.78	0.57 (0.016) 0.49–0.63	0.013 (0.0091) (0.16)
Glutamic acid	4.44 (0.081) 4.13–4.9	4.42 (0.081) 4.23–4.64	0.027 (0.039) (0.497)	3.87–5.68	4.46 (0.081) 4.13–4.73	-0.01 (0.039) (0.791)
Glycine	1.3 (0.028) 1.22–1.4	1.29 (0.028) 1.21–1.38	0.006 (0.009) (0.515)	1.12–1.54	1.3 (0.028) 1.18–1.4	-0.007 (0.009) (0.428)
Histidine	0.69 (0.012) 0.65–0.74	0.68 (0.012) 0.65–0.72	0.011 (0.0082) (0.181)	0.62–0.87	0.69 (0.012) 0.64–0.72	0.0032 (0.0082) (0.696)
Hydroxyproline	0.19 (0.0039) 0.17–0.2	0.18 (0.0039) 0.17–0.2	0.0036 (0.0054) (0.515)	0.15–0.21	0.18 (0.0039) 0.17–0.19	0.0079 (0.0054) (0.162)
Isoleucine	1.04 (0.025) 0.96–1.13	1.03 (0.025) 0.96–1.1	0.012 (0.011) (0.268)	0.89–1.26	1.04 (0.025) 0.94–1.11	0.0031 (0.011) (0.777)

Table 30. Amino Acids – Across-Site Summary Statistics – Spring 2015 (continued)

Component (% dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Leucine	1.79 (0.042) 1.66–1.96	1.75 (0.042) 1.64–1.88	0.034 (0.012) (0.007)*	1.53–2.18	1.77 (0.042) 1.58–1.92	0.019 (0.012) (0.119)
Methionine	0.51 (0.0092) 0.47–0.55	0.5 (0.0092) 0.48–0.52	0.011 (0.0057) (0.065)	0.46–0.61	0.5 (0.0092) 0.47–0.54	0.012 (0.0057) (0.043)*
Phenylalanine	1.05 (0.029) 0.94–1.15	1.04 (0.029) 0.96–1.12	0.0074 (0.012) (0.526)	0.88–1.27	1.05 (0.029) 0.93–1.15	-0.0012 (0.012) (0.92)
Proline	1.55 (0.029) 1.44–1.72	1.52 (0.029) 1.46–1.62	0.031 (0.02) (0.127)	1.37–1.97	1.53 (0.029) 1.45–1.64	0.017 (0.02) (0.412)
Serine	1.1 (0.023) 1.04–1.19	1.09 (0.023) 1.03–1.16	0.012 (0.009) (0.185)	0.95–1.31	1.1 (0.023) 1–1.19	-0.001 (0.009) (0.95)
Threonine	1.12 (0.023) 1.05–1.2	1.11 (0.023) 1.04–1.17	0.0084 (0.011) (0.459)	0.97–1.3	1.11 (0.023) 1.01–1.19	0.0016 (0.011) (0.887)
Total Lysine	1.55 (0.023) 1.5–1.63	1.55 (0.023) 1.48–1.6	-0.0014 (0.019) (0.942)	1.42–1.92	1.55 (0.023) 1.46–1.66	0.0036 (0.019) (0.855)
Tryptophan	0.38 (0.0083) 0.34–0.4	0.37 (0.0083) 0.35–0.4	0.0061 (0.0052) (0.249)	0.33–0.46	0.37 (0.0083) 0.34–0.4	0.0046 (0.0052) (0.377)
Tyrosine	0.706 (0.018) 0.64–0.76	0.711 (0.018) 0.65–0.77	-0.005 (0.003) (0.134)	0.6–0.85	0.715 (0.018) 0.64–0.77	-0.008 (0.003) (0.018)*
Valine	1.34 (0.03) 1.26–1.45	1.34 (0.03) 1.25–1.41	-0.004 (0.008) (0.586)	1.14–1.62	1.35 (0.03) 1.21–1.46	-0.016 (0.008) (0.042)*

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of seven locations with four plot replications at each location.

7.3. Fatty acid levels

Canola is produced as a crop primarily for its oil, having a low content of saturated fatty acids, a high content of monounsaturated fatty acids, and a low content (< 2% of total fatty acids) of erucic acid (C22:1n-9) (OECD, 2011b). EPA+DHA canola event LBFLFK has an intentional change to the fatty acid profile compared to other canola varieties, producing long-chain polyunsaturated fatty acids, including EPA (C20:5n-3) and DHA (C22:6n-3), as a consequence of the introduced metabolic pathway. Therefore, an expanded panel of fatty acids was assessed to account for this intentional change. In total, 39 fatty acid components were analyzed (Table 26). Comparisons of fatty acids were performed using relative percent data values (percentage of the total fatty acid content of an individual sample), which is the standard approach for assessing fatty acid levels in vegetable oils (OECD, 2011b).

7.3.1. Winter 2014/15 season

No comparative statistical analysis could be performed for the fatty acids C16:3n-3[6], C18:2 trans, C22:1n-9 (erucic acid), and C22:2n-6, which were below the LOQ across all sample entries.

In addition, no comparative statistical analysis could be performed for the fatty acids C16:1 trans, C18:2n-9, C18:3n-6, C18:4n-3, C20:2n-9, C20:3n-3, C20:3n-6, C20:3n-9, C20:4n-3, C20:4n-6, C20:5n-3 (EPA), C22:4n-3, C22:4n-6, C22:5n-3, C22:5n-6, and C22:6n-3 (DHA), which were measured in LBFLFK samples but were generally below the LOQ in Kumily and reference variety samples. The presence of these fatty acids in LBFLFK is attributable to the EPA+DHA trait (Table 31).

The fatty acids that could be subjected to statistical analysis to determine the difference in composition for LBFLFK as compared to Kumily were C16:0, C16:1n-7, C18:0, C18:1n-7, C18:1n-9, C18:2n-6, C18:3n-3, C20:0, C20:1n-9, C22:0, C24:0, C24:1n-9, and total trans fatty acids (Table 32). Of these, the fatty acids C16:1n-7, C18:0, C18:1n-9, C18:2n-6, C18:3n-3, C20:0, C20:1n-9, C22:0, C24:0, C24:1n-9, and total trans fatty acids showed statistically significant differences when compared to Kumily. Except for C20:0 and C24:0, statistically significant entry-by-site interactions were observed for all the fatty acids listed. The mean values for C16:1n-7, C18:3n-3, C22:0, and C24:1n-9 in LBFLFK were within the range of the reference varieties.

[6] C16:3n-3 was detected at a very low level in one sample of LBFLFK (sprayed), which was likely a false signal – data not shown

7.3.2. Spring 2015 season

No comparative statistical analysis could be performed for the fatty acids C16:3n-3, C18:2 trans, C22:1n-9 (erucic acid), and C22:2n-6 as they were again below the LOQ across all sample entries.

In addition, no comparative statistical analysis could be performed for the fatty acids C16:1 trans, C18:1 trans, C18:2n-9, C18:3n-6, C18:4n-3, C20:2n-9, C20:3n-3, C20:3n-6, C20:3n-9, C20:4n-3, C20:4n-6, C20:5n-3 (EPA), C22:4n-3, C22:4n-6, C22:5n-3, C22:5n-6, and C22:6n-3 (DHA), which were measured in LBFLFK samples but were generally below the LOQ in Kumily and reference variety samples. The presence of these fatty acids in LBFLFK is attributable to the EPA+DHA trait (Table 33).

The fatty acids that could be subjected to statistical analysis to determine the difference in composition for LBFLFK as compared to Kumily were C16:0, C16:1n-7, C18:0, C18:1n-7, C18:1n-9, C18:2n-6, C18:3n-3, C20:0, C20:1n-9, C22:0, C24:0, C24:1n-9, and total trans fatty acids (Table 34). The fatty acids C16:1n-7, C18:0, C18:1n-7, C18:1n-9, C18:2n-6, C18:3n-3, C20:0, C20:1n-9, C22:0, C24:0, C24:1n-9, and total trans fatty acids showed statistically significant differences in LBFLFK compared to Kumily. Except for C20:0 and total trans fatty acids, statistically significant entry-by-site interactions were observed for all the fatty acids listed. The mean values for C16:1n-7, C18:1n-7, C18:3n-3, C20:0, and C22:0 in LBFLFK were within the range of the reference varieties. The values of C18:1n-7 in LBFLFK were both higher and lower than those of Kumily at different sites, and so there was not a clear trend for this analyte.

7.3.3. Across seasons and biological relevance

The introduction of the EPA+DHA trait and the associated enzymatic pathway in LBFLFK results in the presence of fatty acids not normally found in conventional canola, as expected. In addition, the enzymatic pathway uses endogenous fatty acids as substrates to produce EPA and DHA, which impacts the content of some fatty acids that are normally present in conventional canola varieties. Therefore, the across-season comparison of fatty acids provided here is discussed below in three parts: canola endogenous fatty acids not impacted by the trait, canola endogenous fatty acids impacted by the trait, and EPA+DHA trait-associated fatty acids.

Canola endogenous fatty acids not impacted by the trait

Among the fatty acids that were above the LOQ in both LBFLFK and Kumily and therefore suitable for statistical analysis, only C16:0 did not show any statistically significant differences in either season. The means of C16:0 for both LBFLFK and Kumily were slightly above the upper limit of the reference range but within the ranges of natural variation based on the ILSI Crop Composition Database and peer-reviewed literature values. The fatty acid C18:1n-7 only showed statistically significant differences in the spring 2015 data set, and a by-site analysis demonstrated both higher and lower values compared to the control at different sites. Therefore, the differences seen with these two fatty acids are not considered to be biologically relevant.

Values for the fatty acids C14:0, C16:1n-9, C17:0, C17:1, and C20:2n-6 in LBFLFK in both seasons and for C18:1 trans in one season were not suitable for statistical comparative analysis. However, mean values for these fatty acids were generally within the range of the reference varieties. These components represent a relatively minor fraction of the fatty acid profile, and means were within the ranges of natural variation based on the peer-reviewed literature and the ILSI Crop Composition Database, hence LBFLFK is considered equivalent to conventional canola for these fatty acids.

It is noted that the fatty acid C22:1n-9 (erucic acid) was consistently below the LOQ across all samples for LBFLFK and Kumily for both seasons. Canola varieties must have levels of this antinutrient below 2% (OECD, 2011b).

Canola endogenous fatty acids impacted by the trait

Statistically significant differences were observed across both seasons when comparing LBFLFK with Kumily for C16:1n-7, C18:0, C18:1n-9, C18:2n-6, C18:3n-3, C20:0, C20:1n-9, C22:0, C24:0, C24:1n-9, and total trans fatty acids (Table 32 and Table 34). These differences are attributed to the introduction of the EPA+DHA trait in LBFLFK. In both seasons, LBFLFK had statistically significantly lower C16:1n-7, C18:1n-9, C18:3n-3, C20:0, C20:1n-9, C22:0, C24:0, and C24:1n-9 compared to Kumily. LBFLFK had statistically significantly higher C18:0, C18:2n-6, and total trans fatty acids than Kumily. The mean values for C16:1n-7, C18:3n-3, C20:0, C22:0, and C24:1n-9 were still generally within the reference ranges and within the range of natural variation based on the ILSI Crop Composition Database and/or peer-reviewed literature. The measurement for C24:0 was outside the respective reference range but within the range of natural variation based on the peer reviewed literature and ILSI Crop Composition Database values.

In LBFLFK across both seasons, the mean values for C18:1n-9 (oleic acid, decreased), C18:2n-6 (linoleic acid, increased), and total trans fatty acids (slightly increased) were consistently outside of the reference ranges and outside the range of natural variation based on the peer-reviewed literature and the ILSI Crop Composition Database values.

Oleic acid and linoleic acid are primary precursors for the production of the long-chain polyunsaturated fatty acids in EPA+DHA canola. Oleic acid, the starting substrate fatty acid for the newly introduced fatty acid synthesis pathway, is statistically significantly lower in LBFLFK across treatments and seasons relative to Kumily. The conversion of oleic acid into longer chain and more highly unsaturated fatty acids also likely has a secondary effect on the overall levels of C18:0, which were higher in LBFLFK treatments across seasons than Kumily and the reference ranges. Additionally, the higher relative linoleic acid content is attributable to the newly expressed delta-12 desaturase from *Phytophthora sojae* (D12D(*Ps*)) that produces this fatty acid from oleic acid (Yilmaz et al., 2017).

The trend of increased total trans fatty acids across seasons in LBFLFK, primarily in the form of C18:1 trans, represents only a marginal and low amount of the total fatty acids (0.3%). This increase in trans fatty acids is minor compared to the amount of trans fatty acids produced as a result of conventional commercial processing of canola seeds to refined, bleached, and deodorized oil, with the introduction of trans isomers coming primarily from deodorization (Unger, 2015). Additionally, this minor increase in trans fats is not suprising as isomerization of fatty acids to trans fats occurs spontaneously and at a faster rate with fatty acids with higher degrees of unsaturation like those produced in LBFLFK (Wolff, 1993; Chardingy, 1996).

EPA+DHA trait-associated fatty acids

The fatty acids C16:1 trans, C18:2n-9, C18:3n-6, C18:4n-3, C20:2n-9, C20:3n-3, C20:3n-6, C20:3n-9, C20:4n-3, C20:4n-6, C20:5n-3 (EPA), C22:4n-3, C22:4n-6, C22:5n-3, C22:5n-6, and C22:6n-3 (DHA) were consistently below the LOQ in Kumily and the reference varieties across both seasons (Table 31 and Table 33). The presence of these fatty acids in LBFLFK but not in Kumily and reference varieties is expected and attributed to the introduction of the EPA+DHA trait in LBFLFK (Yilmaz et al., 2017).

Table 31. Fatty Acids Consistently Quantified only in LBFLFK – Across-Site Comparison – Winter 2014/15

Component (% of total fatty acids)	LBFLFK (sprayed)[1]	LBFLFK (non-sprayed)[2]
	Mean (SE) min–max	Mean (SE) min–max
C16:1 trans	0.068 (0.0031) 0.065–0.073	0.066 (0.0038) 0.06–0.07
C18:2n-9	0.9 (0.089) 0.82–1.03	0.91 (0.071) 0.84–1.01
C18:3n-6	1.75 (0.42) 1.12–2.17	1.7 (0.44) 0.97–2.09
C18:4n-3	0.26 (0.039) 0.2–0.29	0.25 (0.044) 0.18–0.29
C20:2n-9	0.22 (0.042) 0.17–0.28	0.23 (0.038) 0.19–0.26
C20:3n-3	0.064 (0.0063) 0.057–0.073	0.062 (0.0076) 0.052–0.073
C20:3n-6	3.56 (0.79) 2.25–4.19	3.56 (0.77) 2.29–4.19
C20:3n-9	0.062 (0.012) 0.048–0.07	0.064 (0.0099) 0.052–0.077
C20:4n-3	1.77 (0.39) 1.15–2.11	1.8 (0.37) 1.27–2.12
C20:4n-6	2.26 (0.36) 1.89–2.72	2.19 (0.39) 1.66–2.62
C20:5n-3	7.21 (1.26) 4.98–7.94	7.21 (1.34) 4.83–7.96
C22:4n-3	0.51 (0.12) 0.32–0.64	0.51 (0.1) 0.36–0.61
C22:4n-6	0.46 (0.11) 0.29–0.56	0.44 (0.11) 0.27–0.54
C22:5n-3	2.94 (0.53) 2.05–3.44	2.93 (0.46) 2.16–3.38
C22:5n-6	0.089 (0.027) 0.051–0.12	0.085 (0.022) 0.048–0.11
C22:6n-3	1.02 (0.18) 0.73–1.18	1.02 (0.18) 0.71–1.15

Means are averages of five locations with four plot replications at each location.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

Table 32. Fatty Acids – Across-Site Summary Statistics – Winter 2014/15

Component (% of total fatty acids)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
C14:0	0.063 (0.0072) 0.052–0.073	0.071 (0.0027) 0.068–0.075	NA	0.04–0.08	0.067 (0.0054) 0.06–0.073	NA
C16:0	4.87 (0.091) 4.49–5.16	4.84 (0.091) 4.61–5.06	0.032 (0.036) (0.41)	3.04–4.72	4.81 (0.091) 4.59–5.04	0.059 (0.036) (0.142)
C16:1n-7	0.31 (0.0075) 0.28–0.34	0.21 (0.0075) 0.2–0.22	0.098 (0.0085) (< 0.001)*	0.2–0.33	0.2 (0.0075) 0.19–0.22	0.1 (0.0085) (< 0.001)*
C16:1n-9	0.053 (0.01) 0.037–0.065	0.06 (0.012) 0.042–0.075	NA	0.03–0.087	0.058 (0.0086) 0.045–0.068	NA
C17:0	0.047 (0.0021) 0.045–0.05	0.046 (0.0042) 0.04–0.05	NA	< LOQ–0.048	0.048 (0.0045) 0.042–0.052	NA
C17:1	0.05 (0.0021) 0.048–0.052	< LOQ	NA	< LOQ–0.06	< LOQ	NA
C18:0	1.97 (0.043) 1.86–2.06	2.54 (0.043) 2.37–2.68	-0.58 (0.038) (< 0.001)*	1.78–2.22	2.49 (0.043) 2.38–2.62	-0.53 (0.038) (< 0.001)*
C18:1n-7	3.34 (0.054) 3.16–3.5	3.4 (0.054) 3.31–3.56	-0.052 (0.033) (0.154)	2.77–3.56	3.35 (0.054) 3.16–3.48	-0.012 (0.033) (0.728)
C18:1n-9	54.61 (1.29) 53.64–55.5	25.5 (1.29) 22.53–31.04	29.11 (1.17) (< 0.001)*	55.59–76.02	25.94 (1.29) 22.62–32.26	28.67 (1.17) (< 0.001)*

Table 32. Fatty Acids – Across-Site Summary Statistics – Winter 2014/15 (continued)

Component (% of total fatty acids)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
C18:1 trans	< LOQ < LOQ–0.08	0.16 (0.054) 0.07–0.2	NA	< LOQ–0.07	0.17 (0.041) 0.1–0.2	NA
C18:2n-6	20.07 (0.52) 19.03–21	28.79 (0.52) 27.35–30.28	-8.72 (0.38) (< 0.001)*	5.68–23.45	28.39 (0.52) 27.21–29.86	-8.32 (0.38) (< 0.001)*
C18:3n-3	7.49 (0.33) 6.55–8.38	4.83 (0.33) 4.12–5.65	2.67 (0.11) (< 0.001)*	1.69–8.39	4.91 (0.33) 4.14–6.16	2.58 (0.11) (< 0.001)*
C20:0	0.66 (0.031) 0.59–0.69	0.52 (0.031) 0.44–0.63	0.13 (0.039) (0.001)*	0.55–0.79	0.6 (0.031) 0.56–0.63	0.052 (0.039) (0.183)
C20:1n-9	0.97 (0.021) 0.93–1	0.64 (0.021) 0.59–0.72	0.33 (0.022) (< 0.001)*	0.93–1.34	0.65 (0.021) 0.59–0.73	0.33 (0.022) (< 0.001)*
C20:2n-6	0.056 (0.0033) 0.052–0.06	0.1 (0.0011) 0.098–0.1	NA	0.034–0.08	0.099 (0.0014) 0.098–0.1	NA
C22:0	0.34 (0.0074) 0.3–0.37	0.26 (0.0074) 0.24–0.26	0.086 (0.0081) (< 0.001)*	0.24–0.44	0.25 (0.0074) 0.25–0.26	0.088 (0.0081) (< 0.001)*
C24:0	0.2 (0.013) 0.13–0.24	0.093 (0.013) 0.061–0.12	0.11 (0.012) (< 0.001)*	0.13–0.38	0.093 (0.013) 0.074–0.12	0.11 (0.012) (< 0.001)*
C24:1n-9	0.13 (0.0045) 0.1–0.15	0.086 (0.0045) 0.078–0.091	0.045 (0.0044) (< 0.001)*	0.084–0.16	0.086 (0.0045) 0.08–0.091	0.045 (0.0044) (< 0.001)*
Total trans fatty acids	0.11 (0.018) 0.093–0.14	0.32 (0.018) 0.24–0.36	-0.21 (0.019) (< 0.001)*	0.077–0.13	0.34 (0.018) 0.26–0.37	-0.23 (0.019) (< 0.001)*

NA indicates not suitable for statistical analysis.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of five locations with four plot replications at each location.

Table 33. Fatty Acids Consistently Quantified only in LBFLFK – Across-Site Comparison – Spring 2015

Component (% of total fatty acids)	LBFLFK (sprayed)[1] Mean (SE) Min–Max	LBFLFK (non-sprayed)[2] Mean (SE) Min–Max
C16:1 trans	0.057 (0.0043) 0.05–0.06	0.053 (0.0022) 0.05–0.055
C18:1 trans	0.12 (0.019) 0.1–0.15	0.13 (0.022) 0.1–0.15
C18:2n-9	1.12 (0.15) 0.96–1.38	1.12 (0.15) 0.96–1.4
C18:3n-6	1.6 (0.16) 1.44–1.82	1.62 (0.16) 1.46–1.85
C18:4n-3	0.26 (0.038) 0.21–0.33	0.26 (0.039) 0.22–0.34
C20:2n-9	0.33 (0.06) 0.27–0.43	0.33 (0.062) 0.26–0.44
C20:3n-3	0.067 (0.0093) 0.06–0.082	0.066 (0.0089) 0.06–0.08
C20:3n-6	4.06 (0.38) 3.65–4.53	4.08 (0.31) 3.74–4.5
C20:3n-9	0.079 (0.016) 0.06–0.1	0.077 (0.016) 0.057–0.1
C20:4n-3	1.92 (0.27) 1.54–2.37	1.92 (0.25) 1.55–2.35
C20:4n-6	1.87 (0.25) 1.62–2.19	1.87 (0.25) 1.57–2.23
C20:5n-3	6.27 (0.46) 5.47–6.98	6.26 (0.49) 5.32–6.93
C22:4n-3	0.68 (0.12) 0.54–0.9	0.72 (0.1) 0.6–0.91
C22:4n-6	0.45 (0.042) 0.38–0.5	0.45 (0.046) 0.38–0.51
C22:5n-3	2.75 (0.15) 2.51–3	2.74 (0.17) 2.44–2.97
C22:5n-6	0.072 (0.017) 0.05–0.1	0.072 (0.015) 0.055–0.098
C22:6n-3	0.77 (0.12) 0.59–0.96	0.76 (0.11) 0.61–0.95

Means are averages of seven locations with four plot replications at each location.

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

Table 34. Fatty Acids – Across-Site Summary Statistics – Spring 2015

Component (% of total fatty acids)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
C14:0	0.061 (0.0069) 0.052–0.075	0.064 (0.0038) 0.06–0.07	NA	0.04–0.07	0.064 (0.0049) 0.06–0.073	NA
C16:0	4.54 (0.11) 4.12–5.13	4.57 (0.11) 4.2–5.12	-0.031 (0.018) (0.114)	2.91–4.46	4.56 (0.11) 4.2–5.07	-0.022 (0.018) (0.248)
C16:1n-7	0.29 (0.011) 0.25–0.38	0.19 (0.011) 0.17–0.23	0.1 (0.007) (< 0.001)*	0.18–0.28	0.19 (0.011) 0.17–0.23	0.1 (0.007) (< 0.001)*
C16:1n-9	0.04 (0.0057) 0.032–0.05	0.048 (0.0049) 0.04–0.055	NA	0.03–0.062	0.047 (0.0044) 0.04–0.052	NA
C17:0	0.048 (0.0028) 0.042–0.05	0.048 (0.0037) 0.042–0.052	NA	0.032–0.048	0.049 (0.0028) 0.042–0.05	NA
C17:1	0.053 (0.0035) 0.05–0.06	< LOQ	NA	0.04–0.06	< LOQ	NA
C18:0	2.18 (0.061) 1.95–2.33	2.77 (0.061) 2.52–3.06	-0.59 (0.021) (< 0.001)*	1.73–2.23	2.74 (0.061) 2.52–3	-0.56 (0.021) (< 0.001)*
C18:1n-7	3.5 (0.1) 3.28–4.13	3.46 (0.1) 3.21–3.98	0.042 (0.021) (0.047)*	2.57–3.47	3.44 (0.1) 3.21–3.98	0.057 (0.021) (0.007)*
C18:1n-9	54.83 (0.74) 49.59–56.69	26.41 (0.74) 23.31–28.18	28.43 (0.42) (< 0.001)*	55.21–76.44	26.27 (0.74) 23.01–27.98	28.56 (0.42) (< 0.001)*

Table 34. Fatty Acids – Across-Site Summary Statistics – Spring 2015 (continued)

Component (% of total fatty acids)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
C18:2n-6	19.29 (0.47) 17.98–21.88	27.89 (0.47) 26.09–29.61	-8.6 (0.6) (< 0.001)*	5.81–23.23	28.07 (0.47) 25.97–29.86	-8.78 (0.6) (< 0.001)*
C18:3n-3	8.01 (0.21) 7.17–9.08	5.37 (0.21) 4.88–6.08	2.64 (0.1) (< 0.001)*	1.97–8.52	5.37 (0.21) 4.9–6.06	2.64 (0.1) (< 0.001)*
C20:0	0.7 (0.017) 0.64–0.78	0.66 (0.017) 0.6–0.73	0.044 (0.005) (< 0.001)*	0.57–0.8	0.65 (0.017) 0.59–0.72	0.048 (0.005) (< 0.001)*
C20:1n-9[4]	1.03[4] 1–1.08	0.7[4] 0.68–0.73	1.47[4] (< 0.001)*	1–1.45	0.7[4] 0.68–0.73	1.47[4] (< 0.001)*
C20:2n-6	0.071 (0.019) 0.05–0.1	0.1 (0) 0.1–0.1	NA	0.045–0.12	0.1 (0) 0.1–0.1	NA
C22:0	0.34 (0.011) 0.29–0.41	0.26 (0.011) 0.22–0.3	0.085 (0.0046) (< 0.001)*	0.23–0.45	0.26 (0.011) 0.22–0.3	0.085 (0.0046) (< 0.001)*
C24:0	0.19 (0.0078) 0.16–0.24	0.13 (0.0078) 0.11–0.15	0.067 (0.0043) (< 0.001)*	0.15–0.31	0.12 (0.0078) 0.1–0.15	0.069 (0.0043) (< 0.001)*
C24:1n-9	0.13 (0.0079) 0.094–0.19	0.082 (0.0079) 0.066–0.11	0.046 (0.005) (< 0.001)*	0.084–0.18	0.083 (0.0079) 0.064–0.11	0.045 (0.005) (< 0.001)*
Total trans fatty acids	0.062 (0.0065) 0.055–0.07	0.27 (0.0065) 0.25–0.32	-0.21 (0.0091) (< 0.001)*	< LOQ–0.1	0.28 (0.0065) 0.26–0.3	-0.22 (0.0091) (< 0.001)*

NA indicates not suitable for statistical analysis.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of seven locations with four plot replications at each location.
[4] Data were log-transformed. Means and differences were back-transformed. Difference column data are the ratio, rather than the difference, of the two means. Back-transformed SE is not provided.

7.4. Vitamins and minerals

Vitamins and minerals are minor components of canola that primarily impact the utility of canola meal. The use of pre-mixes in least cost rations lessens the importance of these components, except for phosphorous bound in phytic acid (OECD, 2011b). Fat-soluble vitamin K1 and tocopherols (including vitamin E) are found in processed edible oils and contribute to human health and nutrition. Tocopherols (alpha-, beta-, gamma-, delta-, and total tocopherols), vitamin K1, and nine mineral components in LBFLFK were compared to Kumily and conventional canola varieties.

7.4.1. Winter 2014/15 season

For the vitamins analyzed in the 2014/15 season (Table 35), delta-tocopherol was statistically significantly lower and vitamin K1 was statistically significantly higher in LBFLFK (sprayed only) compared to the Kumily, though the differences were small in magnitude. No statistically significant differences were observed for any of the other vitamins for LBFLFK compared to Kumily. There were no statistically significant entry-by-site interactions. All vitamin components in grain from LBFLFK were within the ranges of the reference varieties.

For minerals analyzed in the 2014/15 season (Table 36), calcium and magnesium were statistically significantly lower in LBFLFK compared to Kumily, though the differences were small in magnitude.

Copper, manganese, and zinc did not meet the criteria required for statistical analysis. There were no statistically significant entry-by-site interactions for this season. Mineral components measured for LBFLFK were within or slightly outside the range of the reference varieties.

7.4.2. Spring 2015 season

For the vitamins analyzed in the 2015 season (Table 37), vitamin K1 was statistically significantly higher in LBFLFK compared to Kumily, with the differences small in magnitude. No statistically significant differences were observed for any of the other vitamins for LBFLFK compared to Kumily, and all measured values were within or close to the range of the reference varieties.

For the minerals analyzed in the 2015 season (Table 38), calcium and magnesium were again statistically significantly lower in LBFLFK compared to Kumily, with the differences small in magnitude. Phosphorus was statistically significantly higher in LBFLFK (non-sprayed) compared to Kumily, but this difference was also small in magnitude.

Copper, iron, manganese, sodium, and zinc did not meet the criteria required for statistical analysis. The calcium content in LBFLFK was only slightly lower than the lower limit of the reference variety range. All other minerals measured for LBFLFK were within the range of the reference varieties.

7.4.3. Across seasons and biological relevance

Comparing the vitamin and mineral results of the 2014/15 and the 2015 seasons, no differences were observed between LBFLFK and Kumily for alpha-tocopherol, beta-tocopherol, gamma-tocopherol, total tocopherols, copper, iron, manganese, potassium, sodium, and zinc. The values for these components were all within the range of the reference varieties.

A statistically significantly lower delta-tocopherol content was observed for LBFLFK (sprayed) compared to Kumily only in the 2014/15 season (Table 35). A statistically significantly higher phosphorous content was only observed for LBFLFK (non-sprayed) compared to Kumily in the 2015 season (Table 36). For both delta-tocopherol and phosphorous, no trends were observed across seasons, and all values were within the range of the reference varieties. Therefore, these differences in values for these components are considered not biologically relevant.

A statistically significant but slight increase in vitamin K1 content was observed for LBFLFK compared to Kumily (Table 35 and Table 37). However, these increased vitamin K1 values were well within the range of reference varieties for each season and within ILSI Crop Composition Database and peer-reviewed literature values. Therefore, these observed differences are considered not biologically relevant.

Though differences were small in magnitude, calcium and magnesium were both statistically significantly lower for LBFLFK compared to Kumily across both seasons. Though these minerals were marginally lower than the reference range in one season, the values for both were well within the range of the ILSI Crop Composition Database values; therefore, the differences in values for these components are considered not biologically relevant.

In summary, vitamin and mineral values in LBFLFK were within the range of natural variation for canola, and all observed differences are therefore considered not biologically relevant.

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Table 35. Vitamins – Across-Site Summary Statistics – Winter 2014/15

Analytical Component (mg/100 g dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Alpha-tocopherol	16.84 (1.45) 10.5–19.8	15.94 (1.45) 11.12–17.45	0.9 (0.69) (0.2)	11.05–25.3	15.88 (1.45) 10.59–17.77	0.97 (0.7) (0.177)
Beta-tocopherol	0.31 (0.045) 0.13–0.42	0.32 (0.045) 0.17–0.4	-0.0068 (0.024) (0.783)	0.13–0.55	0.32 (0.045) 0.14–0.4	-0.009 (0.024) (0.716)
Delta-tocopherol	0.73 (0.045) 0.61–0.84	0.63 (0.045) 0.52–0.73	0.1 (0.034) (0.017)*	0.41–1.07	0.67 (0.045) 0.57–0.79	0.064 (0.034) (0.099)
Gamma-tocopherol	26.96 (0.8) 25.93–28.15	27.01 (0.8) 23.75–29.75	-0.05 (0.92) (0.958)	19.1–27.93	26.98 (0.8) 25.45–29.75	-0.015 (0.92) (0.987)
Total tocopherols	44.88 (1.71) 38–49.27	43.91 (1.71) 40.75–48.17	0.98 (1.43) (0.514)	31.27–49.18	43.88 (1.71) 38.27–48.7	1.01 (1.45) (0.506)
Vitamin K1	0.088 (0.01) 0.067–0.11	0.097 (0.01) 0.075–0.13	-0.0091 (0.0034) (0.027)*	0.038–0.11	0.091 (0.01) 0.075–0.12	-0.0039 (0.0034) (0.28)

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of five locations with four plot replications at each location.

Table 36. Minerals – Across-Site Summary Statistics – Winter 2014/15

Component (% dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Calcium	0.33 (0.022) 0.25–0.4	0.3 (0.022) 0.22–0.36	0.027 (0.0081) (0.01)*	0.27–0.47	0.29 (0.022) 0.24–0.34	0.037 (0.0081) (0.002)*
Copper	0.00031 (7.4e-05) 0.0002–0.0004	0.0003 (7.1e-05) 0.0002–0.0004	NA	0.0002–0.0004	0.00028 (3.4e-05) 0.00022–0.0003	NA
Iron	0.01 (0.0034) 0.0046–0.019	0.01 (0.0034) 0.0053–0.028	-0.0002 (0.0028) (0.953)	0.0034–0.024	0.01 (0.0034) 0.0053–0.022	0.0002 (0.0028) (0.955)
Magnesium	0.33 (0.0081) 0.31–0.35	0.31 (0.0081) 0.28–0.33	0.016 (0.0038) (0.002)*	0.32–0.38	0.31 (0.0081) 0.29–0.33	0.016 (0.0038) (0.003)*
Manganese	0.0052 (0.00047) 0.0047–0.0057	0.0064 (0.0033) 0.004–0.012	NA	0.003–0.0062	0.0052 (0.00057) 0.0045–0.006	NA
Phosphorus	0.73 (0.043) 0.61–0.86	0.74 (0.043) 0.61–0.89	-0.006 (0.015) (0.694)	0.6–0.94	0.75 (0.043) 0.63–0.88	-0.013 (0.015) (0.389)
Potassium	1.08 (0.05) 0.96–1.28	1.07 (0.05) 0.98–1.23	0.0088 (0.021) (0.676)	0.68–1.41	1.06 (0.05) 0.99–1.19	0.017 (0.021) (0.41)
Sodium	0.0044 (0.0011) < LOQ–0.0055	0.0047 (0.0011) < LOQ–0.0096	-0.0002 (0.0009) (0.799)	< LOQ–0.015	0.0042 (0.0011) < LOQ–0.0062	0.0002 (0.0009) (0.824)
Zinc	0.0032 (0.00035) 0.003–0.0037	0.0036 (0.00042) 0.003–0.004	NA	0.003–0.004	0.0036 (0.00037) 0.003–0.004	NA

NA indicates not suitable for statistical analysis.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of five locations with four plot replications at each location.

Table 37. Vitamins – Across-Site Summary Statistics – Spring 2015

Component (mg/100 g dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Alpha-tocopherol[4]	9.98[4] 9.14–11.11	10.42[4] 9.23–13.25	0.96[4] (0.142)	9.33–16.32	10.12[4] 8.82–12.42	0.99[4] (0.629)
Beta-tocopherol[4]	0.2[4] 0.12–0.37	0.2[4] 0.11–0.43	0.99[4] (0.970)	< LOQ–0.55	0.16[4] 0.12–0.55	1.21[4] (0.343)
Delta-tocopherol[4]	0.61[4] 0.49–0.77	0.63[4] 0.52–0.79	0.97[4] (0.759)	0.34–0.8	0.62[4] 0.41–1	0.98[4] (0.859)
Gamma-tocopherol	23.42 (1.21) 19.75–25.45	24.01 (1.21) 19.52–30.7	-0.59 (0.94) (0.545)	13.9–23.93	22.7 (1.21) 17.8–27	0.72 (0.94) (0.460)
Total tocopherols	34.34 (1.6) 29.51–37.25	35.38 (1.6) 31.14–45	-1.04 (1.21) (0.408)	23.85–41.94	33.82 (1.6) 27.8–40.76	0.52 (1.21) (0.677)
Vitamin K1[4]	0.11[4] 0.091–0.21	0.12[4] 0.099–0.18	0.92[4] (0.01)*	0.059–0.15	0.13[4] 0.11–0.19	0.9[4] (0.002)*

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of seven locations with four plot replications at each location.
[4] Data were log-transformed. Means and differences were back-transformed. Difference column data are the ratio, rather than the difference, of the two means. Back-transformed SE is not provided.

Table 38. Minerals – Across-Site Summary Statistics – Spring 2015

Component (% dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Calcium	0.32 (0.018) 0.25–0.42	0.29 (0.018) 0.23–0.37	0.03 (0.0057) (< 0.001)*	0.3–0.52	0.29 (0.018) 0.22–0.35	0.034 (0.0057) (< 0.001)*
Copper	0.00027 (5.4e-05) 0.0002–0.00035	0.00028 (4e-05) 0.0002–0.00032	NA	0.00017–0.0005	0.00027 (3.5e-05) 0.0002–0.0003	NA
Iron	0.008 (0.0011) 0.007–0.01	0.0075 (0.00092) 0.006–0.0085	NA	0.004–0.014	0.0079 (0.0011) 0.0062–0.0092	NA
Magnesium	0.34 (0.0075) 0.31–0.38	0.32 (0.0075) 0.3–0.35	0.018 (0.0023) (< 0.001)*	0.32–0.4	0.33 (0.0075) 0.3–0.37	0.012 (0.0023) (< 0.001)*
Manganese	0.0044 (0.00072) 0.003–0.0052	0.0044 (0.00079) 0.003–0.0052	NA	0.002–0.005	0.0044 (0.00072) 0.003–0.005	NA
Phosphorus	0.8 (0.032) 0.69–0.92	0.81 (0.032) 0.71–0.9	-0.018 (0.011) (0.129)	0.73–1.07	0.82 (0.032) 0.73–0.92	-0.026 (0.011) (0.035)*
Potassium	0.98 (0.049) 0.88–1.25	0.98 (0.049) 0.85–1.26	0.0034 (0.0097) (0.727)	0.65–1.18	1 (0.049) 0.87–1.27	-0.014 (0.0097) (0.15)
Sodium	0.0021 (0.0015) < LOQ–0.005	< LOQ < LOQ–0.0047	NA	< LOQ–0.0065	< LOQ < LOQ–0.0042	NA
Zinc	0.0036 (0.00045) 0.003–0.0042	0.0039 (0.00028) 0.0032–0.004	NA	0.003–0.0042	0.004 (0.00038) 0.0032–0.0045	NA

NA indicates not suitable for statistical analysis.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of seven locations with four plot replications at each location.

7.5. Antinutrients

Antinutrients are those components present in canola that may have a negative impact on either animal or human health (OECD, 2011b). Glucosinolates (twelve individual analytes and a calculated total glucosinolate value), phytic acid, tannins, and representative phenolic acids (sinapine as a derivative of sinapic acid, coumaric acid, and ferulic acid) in LBFLFK were compared to Kumily and conventional canola varieties. Though not specifically recommended by the OECD consensus document, coumaric acid and ferulic acid were included in the analysis as additional representatives of phenolic acid occurring in canola grain.

7.5.1. Winter 2014/15 season

For antinutrients in the 2014/15 season (Table 39), statistically significant differences were observed for glucoalyssin, glucobrassicin, glucobrassicanapin, gluconapin (sprayed only), and sinapine for LBFLFK compared to Kumily. Total glucosinolates were statistically significantly higher for LBFLFK (non-sprayed) compared to Kumily. Only glucobrassicin had a statistically significant entry-by-site interaction for this season. The glucosinolates epi-progoitrin, glucoiberin, gluconapoleiferin, and glucoraphanin did not meet the criteria required for statistical analysis.

Coumaric acid content was below LOQ in grain from LBFLFK, hence the values for this component were not suitable for statistical analysis. Tannin content was below the LOQ across all samples.

For all antinutrient components, the mean LBFLFK values were within the range of the reference varieties except for neoglucobrassicin, where the mean values were slightly higher than the upper limit of the range of the reference varieties, and coumaric acid, where the mean values (below LOQ) were below the lower limit of the range of the reference varieties.

7.5.2. Spring 2015 season

For antinutrients in the 2015 season (Table 40), statistically significant differences were observed for glucobrassicin, gluconapin, total glucosinolates, and sinapine for LBFLFK compared to Kumily.

Coumaric acid was statistically significantly lower in LBFLFK (non-sprayed) and was the only component in this data set to have a statistically significant entry-by-site interaction for this season. The glucosinolates epi-progoitrin, glucoiberin, gluconapoleiferin, glucoraphanin, and neoglucobrassicin did not meet the criteria required for statistical analysis. Tannin content was below the LOQ across all samples.

For all antinutrient components, the mean values of LBFLFK were within the range of the reference varieties except for coumaric acid, where the mean value (below LOQ) for LBFLFK (sprayed) was below the range of the reference varieties.

7.5.3. Across seasons and biological relevance

Comparing the antinutrient results of the 2014/15 and the 2015 seasons, no differences were observed between LBFLFK and Kumily for phytic acid, ferulic acid, and the glucosinolates 4-hydroxyglucobrassicin, epi-progoitrin, gluconapoleiferin, gluconasturtiin, neoglucobrassicin, and progoitrin. In both seasons, glucoiberin, glucoraphanin, and tannins were consistently below LOQ for all measurements.

Coumaric acid showed a statistically significant difference only for LBFLFK (non-sprayed) in the 2015 season whereas the other values were below LOQ for LBFLFK across both seasons and could not be subjected to statistical analysis. Therefore, any differences in values for coumaric acid in LBFLFK as compared to Kumily are considered not biologically relevant.

Sinapine was statistically significantly lower for LBFLFK compared to Kumily in both the 2014/15 and 2015 seasons. However, because mean values of sinapine were within the range of the reference varieties and ILSI Crop Composition Database values, these differences are within the range of natural variability and are considered not biologically relevant.

Glucoalyssin and glucobrassicanapin were statistically significantly lower in LBFLFK as compared to Kumily in the 2014/15 season only. No trends were observed across seasons, and the means were within the range of the reference varieties. Therefore, differences in values for these components in LBFLFK are considered not biologically relevant.

Gluconapin content across seasons was statistically significantly higher, though the difference was small in magnitude, for LBFLFK as compared to Kumily, except for LBFLFK (non-sprayed) in the 2014/15 season. No trends were observed across seasons, and the means were within the range of the reference varieties. Therefore, differences in values for this component in LBFLFK are considered not biologically relevant.

Glucobrassicin was statistically significantly higher in LBFLFK compared to Kumily consistently across both the 2014/15 and 2015 seasons. Mean values of glucobrassicin, however, were within the within-season range represented by the reference varieties and within the range of ILSI Crop Composition Database values. Furthermore, the contribution of glucobrassicin to the total glucosinolates content in canola is small, and as the values were within the range of natural variation, the observed differences are considered not biologically relevant.

The calculated measure of total glucosinolates in LBFLFK was statistically significantly higher than Kumily in the 2015 season, and a similar difference was seen in the comparison of LBFLFK (non-sprayed) to Kumily in the 2014/15 season. However, the

mean values for LBFLFK were within the range of the reference varieties. Additionally the measured total glucosinolates values for LBFLFK meet the quality standards for canola (OECD, 2011b) and are within the range of values found in peer-reviewed literature and the ILSI Crop Composition Database values. Therefore, these differences are within the range of natural variation, considered not biologically relevant, and do not introduce any new safety concerns compared to conventional canola.

In summary, all antinutrient values for LBFLFK were within the range of natural variation, and observed differences are considered not biologically relevant.

Table 39. Antinutrients – Across-Site Summary Statistics – Winter 2014/15

Component (unit)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Phytic acid (% dry weight)	2.02 (0.13) 1.53–2.46	1.98 (0.13) 1.6–2.38	0.044 (0.064) (0.492)	1.52–2.77	2.07 (0.13) 1.73–2.36	-0.047 (0.064) (0.468)
Tannins (% dry weight)	< LOQ	< LOQ	NA	< LOQ	< LOQ	NA
4-hydroxyglucobrassicin (µmol/g dry weight)	2.14 (0.19) 1.76–2.5	2.05 (0.19) 1.48–2.72	0.091 (0.15) (0.559)	1.53–4.48	2.3 (0.19) 1.84–2.68	-0.17 (0.15) (0.295)
Epi-progoitrin (µmol/g dry weight)	< LOQ < LOQ–0.055	< LOQ < LOQ–0.055	NA	< LOQ–0.092	< LOQ < LOQ–0.068	NA
Glucoalyssin (µmol/g dry weight)	0.69 (0.076) 0.45–0.89	0.62 (0.076) 0.45–0.81	0.07 (0.03) (0.027)*	0.077–0.71	0.62 (0.076) 0.39–0.82	0.077 (0.03) (0.015)*
Glucobrassicin (µmol/g dry weight)	0.26 (0.11) 0.11–0.37	0.59 (0.11) 0.22–0.96	-0.33 (0.072) (0.002)*	< LOQ–0.9	0.56 (0.11) 0.18–0.83	-0.3 (0.072) (0.003)*
Glucobrassicanapin (µmol/g dry weight)	0.24 (0.034) 0.13–0.33	0.21 (0.034) 0.11–0.29	0.032 (0.012) (0.028)*	0.053–0.32	0.19 (0.034) 0.099–0.29	0.047 (0.012) (0.004)*
Glucoiberin (µmol/g dry weight)	< LOQ	< LOQ	NA	< LOQ	< LOQ	NA
Gluconapin (µmol/g dry weight)	1.4 (0.14) 0.94–1.79	1.72 (0.14) 1.53–1.92	-0.32 (0.088) (0.006)*	0.96–3.51	1.55 (0.14) 1.02–1.95	-0.15 (0.088) (0.121)

Table 39. Antinutrients – Across-Site Summary Statistics – Winter 2014/15 (continued)

Component (unit)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Gluconapoleiferin (µmol/g dry weight)	0.051 (0.03) < LOQ–0.095	< LOQ < LOQ–0.073	NA	< LOQ–0.54	< LOQ < LOQ–0.085	NA
Gluconasturtiin (µmol/g dry weight)	0.32 (0.061) 0.12–0.44	0.27 (0.061) 0.12–0.44	0.05 (0.04) (0.217)	< LOQ–0.62	0.27 (0.061) 0.12–0.41	0.049 (0.04) (0.222)
Glucoraphanin (µmol/g dry weight)	< LOQ	< LOQ	NA	< LOQ	< LOQ	NA
Neoglucobrassicin (µmol/g dry weight)	0.25 (0.072) < LOQ–0.41	0.24 (0.072) < LOQ–0.41	0.011 (0.023) (0.637)	< LOQ–0.22	0.28 (0.072) < LOQ–0.47	-0.034 (0.023) (0.135)
Progoitrin (µmol/g dry weight)	2.83 (0.2) 2.41–3.36	2.85 (0.2) 2.27–3.37	-0.014 (0.13) (0.915)	0.87–5.39	2.84 (0.2) 2.27–3.38	-0.004 (0.13) (0.976)
Total glucosinolates[4] (µmol/g dry weight)	6.95 (0.41) 5.81–7.8	7.44 (0.41) 6.39–8.54	-0.49 (0.28) (0.084)	4.55–11.57	7.54 (0.41) 6.1–8.44	-0.59 (0.28) (0.041)*
Coumaric acid (µg/g dry weight)	13.09 (4.56) < LOQ–17.61	< LOQ	NA	10.44–57.51	< LOQ	NA
Ferulic acid (µg/g dry weight)	177.82 (9.98) 160.9–208.83	163.62 (9.98) 137.07–178.28	14.2 (12.64) (0.294)	111.83–248.33	159.66 (9.98) 127.1–203.72	18.16 (12.64) (0.189)
Sinapine (% dry weight)	1 (0.03) 0.93–1.11	0.89 (0.03) 0.85–0.96	0.11 (0.019) (< 0.001)*	0.76–1.08	0.91 (0.03) 0.84–0.97	0.096 (0.019) (< 0.001)*

NA indicates not suitable for statistical analysis.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of five locations with four plot replications at each location.
[4] Obtained by calculation; sum of 4-hydroxyglucobrassicin through progoitrin.

Table 40. Antinutrients – Across-Site Summary Statistics – Spring 2015

Component (unit)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference[4] (SE) (p-value)[3]
Phytic acid (% dry weight)	2.28 (0.1) 1.99–2.58	2.31 (0.1) 2.03–2.57	-0.026 (0.043) (0.537)	1.99–3.24	2.33 (0.1) 1.96–2.63	-0.046 (0.043) (0.284)
Tannins (% dry weight)	< LOQ	< LOQ	NA	< LOQ	< LOQ	NA
4-hydroxyglucobrassicin (µmol/g dry weight)	3.47 (0.21) 2.71–4.14	3.51 (0.21) 2.65–4.27	-0.042 (0.11) (0.711)	2.14–5.21	3.33 (0.21) 2.45–3.96	0.14 (0.11) (0.223)
Epi-progoitrin (µmol/g dry weight)	0.068 (0.026) < LOQ–0.1	0.083 (0.026) < LOQ–0.12	NA	< LOQ–0.3	0.081 (0.028) < LOQ–0.12	NA
Glucoalyssin[4] (µmol/g dry weight)	1.11[4] 0.61–1.8	1.15[4] 0.68–1.82	0.97[4] (0.45)	0.093–2.65	1.11[4] 0.57–1.73	1[4] (0.9828)
Glucobrassicin[4] (µmol/g dry weight)	0.31[4] 0.16–0.8	0.67[4] 0.39–1.52	0.46[4] (< 0.001)*	0.07–1.3	0.67[4] 0.42–1.6	0.46[4] (< 0.001)*
Glucobrassicanapin[4] (µmol/g dry weight)	0.39[4] 0.19–0.63	0.4[4] 0.19–0.66	0.99[4] (0.803)	0.053–0.73	0.39[4] 0.16–0.6	1.01[4] (0.798)
Glucoiberin (µmol/g dry weight)	< LOQ	< LOQ	NA	< LOQ	< LOQ	NA
Gluconapin (µmol/g dry weight)	2.3 (0.22) 1.44–2.71	2.45 (0.22) 1.61–3.57	-0.15 (0.047) (0.002)*	0.88–5.87	2.5 (0.22) 1.25–3.61	-0.2 (0.047) (< 0.001)*

Table 40. Antinutrients – Across-Site Summary Statistics – Spring 2015 (continued)

Component (unit)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference[4] (SE) (p-value)[3]
Gluconapoleiferin (µmol/g dry weight)	0.08 (0.054) < LOQ–0.17	0.083 (0.063) < LOQ–0.2	NA	< LOQ–0.57	0.089 (0.058) < LOQ–0.19	NA
Gluconasturtiin (µmol/g dry weight)	0.29 (0.023) 0.17–0.34	0.28 (0.023) 0.17–0.35	0.0096 (0.012) (0.43)	< LOQ–0.6	0.29 (0.023) 0.18–0.37	0.0061 (0.012) (0.619)
Glucoraphanin (µmol/g dry weight)	< LOQ	< LOQ	NA	< LOQ	< LOQ	NA
Neoglucobrassicin (µmol/g dry weight)	0.066 (0.025) < LOQ–0.098	0.08 (0.046) < LOQ–0.18	NA	< LOQ–0.095	0.065 (0.021) < LOQ–0.095	NA
Progoitrin[4] (µmol/g dry weight)	4.66[4] 2.6–8.03	4.99[4] 2.78–8.13	0.93[4] (0.129)	0.82–15.5	4.79[4] 2.31–8.23	0.97[4] (0.538)
Total glucosinolates (µmol/g dry weight)	11.62 (1.19) 7.16–15.48	12.78 (1.19) 7.69–17.25	-1.17 (0.34) (0.001)*	4.21–25.57	12.45 (1.19) 6.65–17.08	-0.84 (0.34) (0.019)*
Coumaric acid (µg/g dry weight)	16.89 (1.27) 13.81–21.03	< LOQ < LOQ–14.17	NA	10.22–56.5	11.85 (1.27) < LOQ–15.41	5.03 (1.42) (0.004)*
Ferulic acid (µg/g dry weight)	127.39 (3.77) 115.8–137.9	122.62 (3.77) 108.67–140.1	4.76 (2.91) (0.127)	108.97–177.6	121.14 (3.77) 102.97–130.07	6.24 (2.91) (0.053)
Sinapine (% dry weight)	1.02 (0.031) 0.9–1.2	0.95 (0.031) 0.87–1.1	0.071 (0.013) (< 0.001)*	0.79–1.09	0.95 (0.031) 0.87–1.09	0.069 (0.013) (< 0.001)*

NA indicates not suitable for statistical analysis.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of seven locations with four plot replications at each location.
[4] Data were log-transformed. Means and differences were back-transformed. Difference column data are the ratio, rather than the difference, of the two means. Back-transformed SE is not provided.

7.6. Phytosterols

Phytosterols are minor components of canola and are not included in the list of recommended analytes as part of a comparative assessment as per the OECD consensus document (OECD, 2011b). However, canola has approximately twice the phytosterol content of sunflower or soybean oil, and phytosterol content has been shown to correlate to the iodine value of oil (Vlahakis and Hazebroek, 2000), a cumulative measure of the degree of unsaturation of fatty acids in oil (Unger, 2015). Therefore, phytosterol (fourteen individual species and total phytosterols) components were analyzed. Of these, beta-sitosterol is predominant, with campesterol and brassicasterol being the other major species contributing to the total phytosterol content (Unger, 2015).

7.6.1. Winter 2014/15 season

The phytosterols brassicasterol, delta-5 avenasterol, delta-7 stigmastenol, stigmasterol, and the total phytosterols measurement showed statistically significant differences in the 2014/15 season for LBFLFK when compared to Kumily (Table 41). Delta-5 avenasterol and delta-7 stigmastenol also showed statistically significant entry-by-site interactions.

The phytosterols 24-methylene cholesterol, campestanol, cholesterol, clerosterol, delta-5,23 stigmastadienol, delta-5,24 stigmastadienol, delta-7 avenasterol, and sitostanol did not meet the criteria required for statistical analysis. All mean values of phytosterols in LBFLFK were within the reference ranges.

7.6.2. Spring 2015 season

The phytosterols beta-sitosterol, brassicasterol, campesterol, and total phytosterols showed statistically significant differences in the 2015 season for LBFLFK when compared to Kumily (Table 42). Brassicasterol and total phytosterols also showed statistically significant entry-by-site interactions.

Data for 24-methylene cholesterol, campestanol, cholesterol, clerosterol, delta-5 avenasterol, delta-5,23 stigmastadienol, delta-5,24 stigmastadienol, delta-7 avenasterol, delta-7 stigmastenol, sitostanol, and stigmasterol did not meet the criteria required for statistical analysis.

All mean values of phytosterols for LBFLFK were within the range of the reference varieties, except for delta-7 stigmastenol, which was slightly higher than the upper limit of the range of the reference varieties in LBFLFK (non-sprayed).

7.6.3. Across seasons and biological relevance

Comparing the phytosterol results of the 2014/15 and 2015 seasons, statistically significant differences were observed in 2015 between LBFLFK and Kumily for beta-sitosterol and for campesterol that were not present in 2014/15; however, the mean values for these components were within the range of the reference varieties.

Only in the 2014/15 season were delta-5 avenasterol and stigmasterol statistically significantly lower and delta-7 stigmastenol statistically significantly higher than Kumily. Only in the 2015 season were beta-sitosterol and campesterol statistically significantly lower in LBFLFK compared to Kumily. As no trends were observed across seasons for these components and all values were within the range of the reference varieties, the observed differences in the values for these components are considered not biologically relevant.

Brassicasterol and total phytosterols were statistically significantly lower for LBFLFK compared to Kumily across both seasons. However, the means were within the range of the reference varieties and, therefore, these differences are considered not biologically relevant.

It is noted that some measurements of phytosterols across all samples did not compare well with peer-reviewed literature and ILSI Crop Composition Database values. The values for beta-sitosterol, campesterol, cholesterol, and total phytosterols were approximately 2 to 3-fold higher than the ILSI Crop Composition Database values, and brassicasterol was 1 to 2-fold higher. As all varieties tested had similar elevated results, these differences are most likely attributed to differences in the method of analysis used. The results within each season are internally consistent, and the mean contents for all measured phytosterol components and total phytosterols of LBFLFK were within the range of the reference varieties (except for one value of the minor component delta-7 stigmastenol). Additionally, the results are also consistent with available peer-reviewed literature observations that beta-sitosterol, campesterol, and brassicasterol are the predominant phytosterols in canola, making up over 90% of the relative total sterol content in this species (Reina et al., 1999).

The results across seasons for phytosterol content measurements support the conclusion that LBFLFK is compositionally equivalent to conventional canola varieties for these components and the observed differences in values are considered not biologically relevant.

Table 41. Phytosterols – Across-Site Summary Statistics – Winter 2014/15

Component (% dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
24-methylene cholesterol	0.0038 (0.0016) 0.0027–0.0067	0.0022 (0.00041) 0.0018–0.0027	NA	0.0022–0.01	0.0022 (0.0006) 0.0018–0.0032	NA
Beta-sitosterol	0.5 (0.028) 0.43–0.58	0.49 (0.028) 0.41–0.58	0.0095 (0.0081) (0.253)	0.43–0.68	0.49 (0.028) 0.42–0.57	0.011 (0.0081) (0.173)
Brassicasterol	0.12 (0.0043) 0.11–0.13	0.078 (0.0043) 0.067–0.087	0.042 (0.0021) (< 0.001)*	0.054–0.11	0.078 (0.0043) 0.066–0.087	0.043 (0.0021) (< 0.001)*
Campestanol	< LOQ	< LOQ	NA	< LOQ–0.0014	< LOQ	NA
Campesterol	0.25 (0.0092) 0.22–0.27	0.25 (0.0092) 0.22–0.28	0.0006 (0.0058) (0.926)	0.22–0.34	0.26 (0.0092) 0.23–0.28	-0.0028 (0.0058) (0.646)
Cholesterol	0.0038 (0.00073) 0.003–0.0047	0.0046 (0.0031) 0.0027–0.01	NA	0.002–0.006	0.0032 (0.00053) 0.0025–0.0037	NA
Clerosterol	0.0047 (0.00061) 0.004–0.0055	0.0047 (0.00054) 0.004–0.0052	NA	0.004–0.0065	0.0046 (0.00045) 0.004–0.005	NA
Delta-5 avenasterol	0.01 (0.0015) 0.007–0.02	0.0065 (0.0015) 0.0055–0.009	0.0039 (0.0014) (0.022)*	0.0042–0.03	0.0068 (0.0015) 0.0055–0.01	0.0036 (0.0014) (0.032)*

Table 41. Phytosterols – Across-Site Summary Statistics – Winter 2014/15 (continued)

Component (% dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Delta-5,23 stigmastadienol	< LOQ < LOQ–0.0005	< LOQ	NA	< LOQ	< LOQ	NA
Delta-5,24 stigmastadienol	0.004 (0.001) 0.003–0.0057	0.0044 (0.00038) 0.004–0.005	NA	0.0027–0.007	0.0044 (0.00058) 0.0037–0.0052	NA
Delta-7 avenasterol	0.0011 (0.0005) 0.00082–0.002	0.002 (0.00011) 0.0018–0.002	NA	0.0008–0.003	0.0018 (0.00021) 0.0015–0.002	NA
Delta-7 stigmastenol	0.0025 (0.0008) 0.0018–0.0035	0.0068 (0.0008) 0.0042–0.0097	-0.0043 (0.0006) (< 0.001)*	0.0018–0.01	0.0064 (0.0008) 0.0047–0.01	-0.004 (0.0006) (< 0.001)*
Sitostanol	0.0009 (0.00017) 0.0006–0.001	0.0008 (0.00027) < LOQ–0.001	NA	0.00045–0.0018	0.00084 (0.00016) 0.00057–0.00097	NA
Stigmasterol	0.0051 (0.0005) 0.0027–0.006	0.0041 (0.0005) 0.0022–0.0055	0.0011 (0.0003) (0.003)*	0.0015–0.005	0.0037 (0.0005) 0.0022–0.0047	0.0015 (0.0003) (< 0.001)*
Total phytosterols	0.93 (0.039) 0.83–1.03	0.88 (0.039) 0.77–1.01	0.05 (0.015) (0.01)*	0.78–1.18	0.87 (0.039) 0.79–0.99	0.052 (0.015) (0.008)*

NA indicates not suitable for statistical analysis.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of five locations with four plot replications at each location.

Table 42. Phytosterols – Across-Site Summary Statistics – Spring 2015

Component (% dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
24-methylene cholesterol	0.004 (0.002) 0.002–0.007	0.0019 (0.001) 0.00092–0.0032	NA	0.0015–0.015	0.0018 (0.00085) 0.00095–0.003	NA
Beta-sitosterol	0.47 (0.013) 0.42–0.53	0.44 (0.013) 0.4–0.49	0.026 (0.0056) (< 0.001)*	0.4–0.57	0.44 (0.013) 0.4–0.48	0.025 (0.0056) (0.001)*
Brassicasterol	0.12 (0.002) 0.1–0.13	0.072 (0.002) 0.065–0.076	0.048 (0.001) (< 0.001)*	0.052–0.11	0.072 (0.002) 0.064–0.076	0.048 (0.001) (< 0.001)*
Campestanol	< LOQ	< LOQ	NA	< LOQ–0.00057	< LOQ	NA
Campesterol	0.25 (0.012) 0.22–0.31	0.24 (0.012) 0.21–0.3	0.013 (0.0029) (0.001)*	0.21–0.35	0.24 (0.012) 0.21–0.29	0.012 (0.0029) (0.001)*
Cholesterol	0.0036 (0.00093) 0.0027–0.0055	0.0027 (0.00044) 0.0022–0.0032	NA	0.002–0.005	0.0028 (0.00044) 0.002–0.0032	NA
Clerosterol	0.0046 (0.0004) 0.004–0.005	0.0042 (0.00037) 0.004–0.005	NA	0.004–0.0052	0.0043 (0.0003) 0.004–0.0047	NA
Delta-5 avenasterol	0.011 (0.0041) 0.0072–0.02	0.0076 (0.002) 0.005–0.01	NA	0.005–0.032	0.0076 (0.0019) 0.0052–0.01	NA

Table 42. Phytosterols – Across-Site Summary Statistics – Spring 2015 (continued)

Component (% dry weight)	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
Delta-5,23 stigmastadienol	< LOQ	< LOQ	NA	< LOQ	< LOQ	NA
Delta-5,24 stigmastadienol	0.0042 (0.00051) 0.0032–0.0047	0.0042 (0.00043) 0.0037–0.0047	NA	0.003–0.0055	0.0042 (0.00049) 0.0035–0.005	NA
Delta-7 avenasterol	0.0011 (0.00028) 0.001–0.0018	0.0019 (0.00012) 0.0018–0.002	NA	0.00085–0.0022	0.002 (9.4e-05) 0.0018–0.002	NA
Delta-7 stigmastenol	0.0023 (0.00037) 0.002–0.003	0.0064 (0.00069) 0.0055–0.0075	NA	0.001–0.0065	0.0066 (0.00067) 0.0057–0.0075	NA
Sitostanol	0.0006 (0.00013) 0.00042–0.00075	0.00051 (0.00012) < LOQ–0.00065	NA	< LOQ–0.00095	0.00055 (6.2e-05) 0.0005–0.00065	NA
Stigmasterol	0.0047 (0.00087) 0.0035–0.006	0.0036 (0.00066) 0.0027–0.0045	NA	0.002–0.005	0.0037 (0.00047) 0.003–0.0042	NA
Total phytosterols	0.89 (0.027) 0.78–1.02	0.8 (0.027) 0.71–0.91	0.089 (0.0098) (< 0.001)*	0.74–1.08	0.8 (0.027) 0.71–0.89	0.087 (0.0098) (< 0.001)*

NA indicates not suitable for statistical analysis.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05; means are averages of seven locations with four plot replications at each location.

7.7. Conclusion on the compositional assessment

As part of the food, feed, and environmental safety assessment of EPA+DHA canola event LBFLFK, a comparative assessment was performed with the parental control variety Kumily and other conventional reference canola varieties. Key compositional components were assessed for LBFLFK grain (oilseed) grown and harvested during two seasons in the United States: during the winter of 2014/15 and in the spring of 2015 as part of two randomized complete block design studies. As event LBFLFK contains a trait that confers tolerance to Beyond® herbicide (active ingredient imazamox), plots treated with or without Beyond® herbicide were included. Grain samples were harvested and analyzed for composition, including proximates, fibers, amino acids, fatty acids, vitamins, minerals, antinutrients, and phytosterols. A total of 112 components were measured.

The EPA+DHA trait of event LBFLFK impacted the overall fatty acid composition in harvested grain as intended. For the other grain components measured, the results of the comparative approach demonstrated that EPA+DHA canola event LBFLFK (either sprayed or non-sprayed with Beyond® herbicide) is compositionally equivalent to commercially available canola varieties based on comparisons to Kumily, the ranges presented for conventional reference varieties and referred to in peer-reviewed literature, as well as in the ILSI Crop Composition Database.

8. ENVIRONMENTAL CONSIDERATIONS FOR THE PRODUCTION OF EPA+DHA CANOLA EVENT LBFLFK

As part of the plant pest assessment required by 7 CFR § 340.6(c)(4), impacts to agricultural and cultivation practices must be considered. This section includes a summary of the current agronomic practices in the U.S. for producing canola and is provided as a baseline for assessing possible impacts to agronomic practices due to the cultivation of EPA+DHA canola event LBFLFK.

8.1. Canola production in the U.S.

Since 1989, canola acreage in the U.S. has experienced an impressive development, growing from virtually zero to more than 1.7 million acres (Table 43). At the peak of 1.7 million acres of harvested area, canola production was worth $630.2 million (USDA-NASS, 2016).

Spring and winter canola varieties have been developed to enable production in various regions of the United States. Spring varieties are grown in the northern states during spring and summer months. Winter varieties are grown in the Pacific Northwest, southern Great Plains, Midwest, and Southeast where they are planted in the fall, overwinter, and are harvested the following summer (U.S. Canola Association, 2009). Of the 2.87 million pounds (1301.81 metric tons) of canola produced in 2015, most was produced in North Dakota (Table 44).

Table 43. Canola Planted Area, Harvested Area, and Yield in the U.S. (2005–2016)

Year	Acres Planted (1000)	Acres Harvested (1000)	Yield per Acre (pounds)
2005	1159.0	1114.0	1419
2006	1044.0	1021.0	1366
2007	1176.0	1155.5	1238
2008	1011.0	989.0	1461
2009	820.0	808.0	1813
2010	1448.8	1430.7	1711
2011	1061.5	1033.0	1479
2012	1754.4	1717.9	1392
2013	1348.0	1264.5	1748
2014	1715.0	1556.7	1614
2015	1777.0	1713.5	1680
2016	1714.0	1685.7	1824

Source: UDSA-NASS (2016)

Table 44. Canola Planted Area, Harvested Area, and Yield in Key U.S. States (2016)

State	Acres Planted (1000)	Acres Harvested (1000)	Yield per Acre (pounds)
Idaho	21.0	20.5	2100
Kansas	25.0	23.0	1940
Minnesota	29.0	27.5	1700
Montana	62.0	60.0	1670
North Dakota	1460.0	1445.0	1840
Oklahoma	80.0	75.0	1520
Oregon	4.0	3.7	2400
Washington	33.0	31.0	1900
TOTAL	1714.0	1685.7	1884[1]

[1] Total yield per acre value was calculated as the average value for the key states.

8.1.1. Canola production management

8.1.1.a. Canola crop management

Crop rotation and land preparation

Prior to planting, growers make a series of decisions on crop rotation, soil fertility management, and variety selection to ensure profitable production. Generally, the crop rotation decision is part of a long-term plan to manage soil moisture, to break disease and insect cycles, to avoid residual herbicide damage, to maintain herbicide efficacy, and to take advantage of economic and market opportunities. It is generally recommended to plant canola only once every four years in the same field to avoid yield loss due to potential build-up of soil-borne disease like Sclerotinia stem rot, blackleg, or club root from the previous canola crop (U.S. Canola Association, 2009; NDSU, 2015). It is also recommended to avoid planting canola after crops that are susceptible to Sclerotinia stem rot or Rhizoctonia and Fusarium root rot (such as pea, bean, lentil, chickpea, and soybean). In the U.S., canola is most commonly grown following small grain cereal crops (wheat, barley, sorghum, or maize).

Canola can grow in a wide range of soil types. The ideal soil pH ranges from 6.0–7.0. Well-drained, silt loam soils less susceptible to crusting are preferred to achieve highest grain yields. Canola is very susceptible to water-saturated soil, and planting in poorly drained fields or fields with a known history of flooding is not recommended.

In North America, production of canola is conducted under minimal or no-till systems to protect the soil from erosion. Reduced tillage cultivation systems require pre- and post-emergent weed control systems. Most canola production in the U.S. and Canada uses at least one of three main groups of herbicide-tolerant traits: tolerance to glyphosate, tolerance to glufosinate (both derived from biotechnology), and tolerance to imidazolinone herbicides (derived from mutagenesis) (Canola Council of Canada, 2016b). Herbicide tolerant (HT) canola makes farming easier by facilitating the adoption of direct seeding, allowing simpler and more effective herbicide application (less tank mixes and separate applications, no soil incorporation needed), and by the implementation of better weed management systems (Hartman, 2005). Understanding field herbicide residue history is also essential as canola is sensitive to herbicide carryover in the soil. It is critical to observe plant back restriction requirements following certain herbicide history or soil residue level to avoid crop injury and yield loss. Alternatively, it is possible to mitigate risks and avoid plant back restrictions by selecting appropriate HT varieties (NDSU, 2015).

Land preparation decisions prior to planting depend on local conditions. Achieving good seed-to-soil contact is important for good seed germination, uniform emergence, and full cover establishment. In addition, quick emergence and establishment will give the canola crop a better chance to compete with weeds and achieve higher grain yields.

Conventional tillage or conservation tillage can be used for canola crop production. Conventional tillage allows for easier fertilizer application and seeding operations. It also helps control weeds that have already germinated and creates a finer seed bed for good seed-to-soil contact. However, conventional tillage increases soil erosion, soil compaction, soil moisture loss, and results in decreased organic matter. Conservation tillage, including direct seeding, no-tillage, and minimum tillage systems, maintains residues from previous crops that can help to improve soil structure and organic matter content as well as soil moisture availability. Conservation tillage can also benefit the following crop if appropriate measures to facilitate crop establishment are taken.

Soil fertility and management

It is also important to maintain good soil fertility to achieve high yield and high-quality canola. Soil nutrient needs are assessed through a soil test. Depending on the region and the soil test results, nitrogen, phosphorus, potassium, sulfur, and/or boron are typically the limiting nutrients for successful canola production. In most cases, nitrogen and sulfur are the key elements needed for high yields and quality in canola (U.S. Canola Association, 2009; Franzen and Lukach, 2013). No other micronutrients have shown yield response in canola (U.S. Canola Association, 2009).

Nitrogen is the most limiting of all nutrients, and sufficient supply must be available at all developmental stages. Determining the required nitrogen rate depends on the expected canola yield and the nitrogen availability to achieve such yield. Research studies have developed methods to estimate the expected yield based on the previous crop, rainfall potential, soil type and depth, and planting date. Models to estimate the required nitrogen availability per 100 lb/A of harvested canola grain have been developed (Table 45). Similar but slightly different relationships between canola grain yield and nitrogen availability have been found in different U.S. regions (U.S. Canola Association, 2009).

Table 45. Nitrogen Requirement per Grain Yield Potential in Canola

Expected Grain Yield		Spring Canola Nitrogen Requirement (lb/A)	Winter Canola Nitrogen Requirement (lb/A)
Low	1500 lb/A	98	140
Moderate	2500 lb/A	175	175
High	3500 lb/A	263	210

Source: U.S. Canola Association (2009)

As canola requires a relatively large amount of sulfur, it is considered the second most limiting nutrient after nitrogen for the crop. For example, a 2000 lb/A canola crop contains 12 lb/A of sulfur in the straw and 15 lb/A of sulfur in the seed. This sulfur content is much higher than that of a 40 bu/A wheat crop (~2400 lb/A) that contains only 7 lb/A of sulfur in the straw and 5 lb/A in the seed. Sulfur deficiency in canola results in pale plants, poor growth, and reduced yield. To determine the appropriate level of sulfur application, a soil test is recommended (Table 46). In general, sulfur application rate should not exceed 25 lb/A since it is highly prone to leaching in the soil. However, higher application rates have been recommended with fields having higher yield potential history. In such a case, ~1 lb of sulfur is required for each expected 100 lb/A of grain yield (U.S. Canola Association, 2009).

Table 46. Sulfur Application Rates Based on Sodium Bicarbonate Soil Test Results

Sulfur Soil Test Sulfate-Sulfur SO_4-S (ppm)	Sulfur Application Rate (lb/A)
0 to 5	20 to 40
6 to 10	10 to 20
Over 10	0 to 10

Source: U.S. Canola Association (2009)

8.1.1.b. Planting and in-season management

The planting date has a major impact on the final yield of a canola crop. For spring canola varieties, planting is usually in April and May, but planting as early in the spring as possible is generally recommended to achieve the highest yield. For optimum seed germination, planting should only proceed when soil temperature exceeds 10°C (49°F). Early planting also reduces the risk of heat and drought stress during flowering, which can lead to yield loss. For winter canola varieties, multiple factors should be considered before making a planting date decision, including determining a planting date that enables the canola plants to have at least 45 days of growth (four to six fully-opened leaves) before the onset of winter and associated risk of crop injuries (U.S. Canola Association, 2009).

The seeding rate decision also has an important economic impact as it determines the upfront cost as well as can influence the final yield. An optimum established stand is 8 to 14 plants per square foot for spring canola and 10 to 16 plants per square foot for winter canola. Under typical seeding conditions, between 40 and 60% of the seeds planted will develop into mature plants (U.S. Canola Association, 2009).

As the canola plants develop, proper timing of the field management activities is key to success. Young canola plants are very sensitive to weed competition and only become more competitive when approaching the late rosette and bolting stage. Therefore, effective weed control early in the season is important to minimize yield loss. When planted at the appropriate date and with proper pre-planting seed bed preparation and/or herbicide treatment, the canola plant will emerge and establish faster than most weeds. Chemical weed control in canola relies heavily on selective post-emergent herbicide. However, there is a relatively small list of registered herbicides in canola in comparison to other major row crops (U.S. Canola Association, 2009). Adoption of HT canola varieties has not only enabled the use of broad spectrum post-emergent herbicides such as glyphosate, glufosinate, and imazamox but has also led to environmental and economic benefits (Fernandez-Cornejo et al., 2016).

The water requirement for canola depends on variety, target yield, and crop management. Canola consumes up to 20 inches of water during a growing season and will use as much as 0.3 inches per day during peak periods, similar to most grain crops. In general, the earlier the crop is planted while still avoiding the potential of early frost damage, the less water it will use. The most critical times for irrigating canola are during late vegetation/spiking and throughout the flowering period. Moisture stress during these periods can cause major yield reductions. In general, soil moisture levels should be maintained above 50% available moisture in the active root zone throughout the growing season (Montana State University, 2017).

During the pre-emergence and germination stage, canola is also susceptible to many seed and soil-borne pathogens. It is recommended to plant certified seeds that are free of diseases to avoid the spread of blackleg, Sclerotinia stem rot, and Alternaria black spot. In addition, seed treatments containing appropriate insecticides and fungicides are also common and economically beneficial. A fungicide seed treatment can provide control for damping off, seedling blight, and seed and root rot caused by *Pythium* spp., *Fusarium* spp., and *Rhizoctonia* spp., respectively. Insecticide seed treatment will protect against feeding insects like flea beetles (U.S. Canola Association, 2009).

Seedlings typically emerge 4–10 days after planting. After the first true leaves are developed around 4–8 days after emergence, canola plants quickly establish a rosette. During this rosette stage, rapid and robust vegetative growth takes place that is critical for dry matter production and yield potential. This stage ranges from 40–60 days depending on the environmental conditions. Bud formation is triggered as the days become longer and temperatures rise. Flowering begins with the opening of the lowest bud on the main stem and continues for 14–21 days. During the flowering stage, canola is sensitive to heat and drought stress, which can have a severe adverse impact on grain yield. Seed filling is complete around 35–45 days after flower initiation (U.S. Canola Association, 2009; Canola Council of Canada, 2014).

8.1.1.c. Insect management

Insect infestation can also be a challenge to successful canola production (U.S. Canola Association, 2009). Various insects feed on canola plants, and when an infestation exceeds economic threshold levels, foliar insecticide treatments become necessary (NDSU, 2017a). Table 47 lists the main insect pests found in the primary U.S. canola growing region of North Dakota. The flea beetle is the most damaging canola insect pest in North Dakota (NDSU, 2017a). Overwintering insect populations can cause severe damage by feeding on the emerging cotyledons and the first true leaves of the young plant in the spring. The injuries may lead to plant death or reduced vigor and growth, causing yield loss and/or reduction in oil content. Damage is more limited once the canola plants grow beyond the 4–6 true leaf stage as they can outpace beetle defoliation. The decision on the appropriate insect control method is based on the typical infestation period, available tools, and economic threshold. The key to insect control is frequent field monitoring during the susceptible stage (NDSU, 2017a).

Table 47. Canola Insect Pests in North Dakota

Common Name	Scientific Name
Aphids	*Brevicoryne brassicae*
Aster leafhopper	*Macrosteles quadrilineatus*
Bertha armyworm	*Mamestra configurata*
Blister beetles	*Lytta mutalli, Epicauta fabricii, Epicauta ferruginea*
Cutworms	*Noctudea* spp.
Diamondback moth	*Plutella xylostella*
Flea beetles	*Phyllotreta cruciferae, P. striolata*
Grasshoppers	*Acrididae* spp.
Lygus bugs	*Lygus* spp.
Wireworms	Various species

Source: NDSU (2017a)

Cultural methods can also be used to control insects. For example, a firm and adequately fertilized seedbed will help the canola seedlings to stay ahead of beetle damage during the susceptible stage in the spring. Research studies have also reported the effectiveness of increased seeding rates, seed size, row spacing, and later planting dates (Canola Council of Canada, 2014).

There are fewer registered insecticides for canola in the U.S. compared to Canada and Europe. Seed treatment insecticides are labeled for control of early season insects like flea beetles. They include clothianidin, cyantraniliprole, and thiamethoxan and have a narrower range of insect control. Insecticides for foliar application include *Bacillus thuringiensis*, bifenthrin, chloranthraniliprole, chloranthraniliprole + lambda-cyhalothrin, deltamethrin, gamma-cyhalothrin, imidacloprid, lambda-cyhalothrin sulfoxalor, and zeta-cypermethrin. The range of insects controlled by these insecticides can be rather broad or very specific. For example, bifenthrin, deltamethrin, and gamma-cyhalothrin control a wide range of canola insects while sulfoxalor controls aphids only and *Bacillus thuringiensis* controls first and second instar bertha armyworm only. In general, foliar application is only used when an economical threshold is reached (NDSU, 2017a).

8.1.1.d. Disease management

Damage from disease is another limiting factor to canola production. Primary diseases reported include Sclerotinia stem rot and blackleg. Less-reported canola diseases include white rust or staghead, downy mildew, alternaria blackspot, aster yellows, and club root (NDSU, 2015).

Blackleg is a seed-borne disease caused by *Leptosphaeria maculans* and *Leptosphaeria biglobosa*. This disease is typically introduced to an area by planting infected seeds and can cause serious crop loss in susceptible varieties in the following year. The pathogen survives many years on crop residues, and spores can travel long distances (up to three miles). To reduce blackleg disease risk, planting certified and inspected seeds and use of resistant canola varieties is recommended. Additionally, several fungicides labeled for control of blackleg are available (NDSU, 2015).

Sclerotinia stem rot (also called white mold) is caused by the soil-borne fungus *Sclerotinia sclerotinium*. During wet weather, it can cause serious problems to broadleaf crops. A proactive decision to spray fungicide to treat stem rot is made when the yield loss potential is above normal, the field has a history of Sclerotinia infestation, and field conditions are generally favorable for the pathogen (high moisture).

Crop rotation is also an important practice to keep disease incidence and levels low. A minimum three-year rotation is recommended, avoiding other susceptible crops. A five-year study in western Canada showed yield increases from 0.20–0.36 metric tons per hectare for each increase in the number of crops between canola plantings. Greater canola yields were associated with decreased disease damage as the number of crops in the rotation increased (Harker et al., 2014).

8.1.1.e. **Harvest**

Canola harvest is performed either by swathing and combining or by direct combining of standing canola. Multiple factors are considered in determining the appropriate harvest method, including crop canopy, pod shattering, disease, hail, and frost risks. Since canola is an indeterminate crop, achieving uniform maturity at harvest is important. A common practice for harvesting a commercial canola crop in the U.S. and Canada is to use a swathing and combining method. The canola plants are first cut at the main stem (swathing) and then allowed to cure and finish ripening in the field for a minimum of 7–14 days before harvest (combining). Having a flexible time interval before harvest provides an advantage for growers with large operations; however, the timing of swathing is critical for grain quality and yield. Research studies established correlations between seed color change and many harvest parameters including grain yield, percent green seed, and oil content (Table 48). For practical and economic reasons, seed color change is now commonly used as an indicator of optimal swathing time. Swathing can start as early as 15% and up to 60% of seed color change (NDSU, 2013). Later swathing at 60–80% seed color change has also been recommended to achieve higher yield (Canola Digest, 2014).

Table 48. Correlation Between Seed Color Change and Harvest Parameters

Seed Color Change at Swathing (% change)	Grain Yield (lb/A)	Green Seed (%)	Oil (%)
0 to 5	1603	3.5	39.5
15 to 20	1785	1.4	40.0
30 to 35	1795	1.1	40.1
LSD[1] 0.05	130	1.4	0.4

[1] Least significant difference.

Less than 20% of the canola produced in North Dakota is direct combined. Direct combining is suitable for evenly mature crops and simplifies the harvest operations when appropriately managed. In addition, it can save time and money and potentially improve grain yield and oil content. However, the risk of yield loss due to pod shattering makes direct combining a less preferred method for most growers. More recently, it has become an attractive option because of improved genetics that make plants less prone to pod shattering (NDSU, 2013). Also, harvest aids like desiccants and pod sealants can reduce pod shattering (NDSU, 2017b).

8.1.1.f. Management of weeds during canola production

Weeds generally compete for water, nutrients, and sunlight, suppressing canola growth and productivity. Common weeds found in U.S. canola fields are listed in Table 49. Weed management decisions are generally complex because the crop rotation and the environment may have a significant impact on the weed population (Bullied et al., 2006; Harker et al., 2014). Growers need to decide on the most economical means to control weeds without decreasing crop quality or yields. Appropriate weed control measures are dependent on the types of weeds present in each field. Research studies have shown that early weed control is critical as canola seedlings are poor competitors with weeds that emerge before or with the crop. A successful weed control program will include cultural, mechanical, and chemical methods. Mechanical weed control includes tillage in combination with crop rotation. Pre-plant tillage reduces weed competition by disrupting growth and allowing the canola plants to gain vigor and become more competitive. Soil-applied selective pre-plant herbicides such as trifluralin and ethalfluralin (NDSU, 2015) can also be used. Weed-free canola is desirable between the three- to six-leaf stage to avoid significant yield reduction (Harker et al., 2003). In practice, the first post-emergent herbicide application should be applied prior to the four-leaf stage during which the benefits of weed control are the most beneficial (Canola Council of Canada, 2016a). Other selective herbicides like clopyralid, quizalofop, sethoxydim, and clethodim are labeled in the U.S. for post-emergence weed control in canola (NDSU, 2015, 2017b).

Table 49. Common Weed Species in Canola

Common Name	Scientific Name	Common Name	Scientific Name
Barnyard grass	*Echinochloa crus-galli*	Lanceleaf Sage	*Salvia reflexa*
Biennial wormwood	*Artemisia biennis*	Marshelder	*Iva xanthifolia*
Black nightshade	*Solanum nigrum,*	Pigweed species	*Amaranthus* spp.
Common cocklebur	*Xanthium strumarium*	Prickly lettuce	*Lactuca serriola*
Common mallow	*Malva neglecta*	Common ragweed	*Ambrosia artemisiifolia*
Green foxtail	*Setaria viridis*	Sunflower	*Helianthus annuus*
Hairy nightshade	*Solanum physalifolium*	Russian thistle	*Salsola tragus*
Horseweed	*Erigeron canadensis, Conyza canadiensis*	Volunteer cereals	*Poaceae* spp.
Kochia	*Kochia scoparia*	Wild buckwheat	*Polygonum convolvulus*
Lambsquarters	*Chenopodium album*	Wild oat	*Avena fatua*

Source: NSDU (2011a)

HT canola varieties provide growers with an additional tool to improve weed control and maintain crop safety. The introduction of HT varieties in the canola production systems provides superior weed control, improved crop safety, and time and fuel savings from easier weed control and improved canola grade (Fernandez-Cornejo et al., 2016). In the U.S., there are currently four types of HT canola varieties including imidazolinone-tolerant (Clearfield®), sulfonylurea-tolerant (SU canola), biotechnology-derived glyphosate-tolerant (Roundup Ready®), and biotechnology-derived glufosinate-tolerant (LibertyLink®). In 2013, 95% of the canola acres harvested in the U.S. was planted using the biotechnology-derived HT varieties (Fernandez-Cornejo et al., 2016).

8.1.2. Weediness and volunteer potential of canola

Consensus documents on canola biology describe the crop as a non-weedy species (CFIA, 2012; OECD, 2012a). Cultivated oilseed rape (*B. napus*) is of relatively recent origin and is thought to have first emerged in the Mediterranean coastal region where both its progenitor species are found. In Europe, the first record of cultivation dates back to the Middle Ages. In North and South America, the earliest varieties were introduced in the 18th century and only in 1936 to Canada. In the 1970s, Canadian breeders produced varieties with reduced erucic acid and glucosinolate levels, and the subsequent cultivars are referred to as canola (Canadian oil, low acid).

As canola is a recently domesticated crop, it still retains some characteristics of the wild ancestors that are associated with weedier species, such as pod shattering and secondary dormancy (Hall et al., 2005), though these characteristics have been greatly reduced with domestication.

When left on the ground, seed can be dispersed by birds and other animals as well as wind and water. However, dispersal via human activities is more relevant, in particular via long distance transport along roadsides or railways. Viability of seed lost during harvest is an important factor in determining the presence and number of volunteer plants and populations in subsequent crops. Harvest losses can be substantial, and the survival and persistence of this seed is greatly influenced by environment, seed dormancy, and crop and field management. Most seeds of cultivated Brassica crops, if left on or near the soil surface, will germinate and be killed by frost or cultivation practices or be eaten by rodents, birds, and insects.

The potential for volunteer plants, which interfere with production of succeeding crops, can be controlled by management practices used in the production of the crop. These management practices include the setting of the harvest equipment and the speed of the harvesting operation to contain the loss of seed, the type of post-harvest tillage used, and management of volunteers by herbicide applications or mechanical means.

Canola, like other members of the Brassicaceae family, has the potential to produce a large amount of volunteer plants due to its small seed size and large number of seeds produced. Agronomic management practices and environmental conditions play a significant role in terms of the severity of the problem. Agronomic practices should also limit the development and spread of volunteers by using clean crop seed (e.g., certified seed), preventing crop trait out-crossing, controlling weed influx from field borders, and managing weed seed at harvest / post-harvest to minimize the carryover weed seed-bank into the following crop.

Volunteer canola that grows in the field during the production of a subsequent rotational crop is considered a weed. High seeding rate, stress-induced seed dormancy, or dry conditions causing pod shattering may contribute to an increase of volunteer canola in the following season. Appropriate volunteer control is required to mitigate the risks of growth reduction and yield loss in the following rotational crop. Although canola can volunteer for several years following a canola crop, problems managing canola volunteers are not common (U.S. Canola Association, 2009). At harvest, seed loss during swathing and combining operations should be reduced. Mechanical control or broad spectrum herbicide treatment prior to the six-leaf stage are the best means to control volunteer canola (U.S. Canola Association, 2009).

8.1.2.a. Pollen-mediated gene flow of canola

Pollen-mediated gene flow of *B. napus* and its relevance for assessing the potential of biotechnology-derived canola varieties for increased weediness or invasiveness of the resultant progeny is presented in various official *B. napus* biology consensus documents (e.g., CFIA, 2012; OECD, 2012a; OGTR, 2016). The following information is based on these consensus documents.

Brassica pollen grains, although heavy and slightly sticky, can still become airborne and float on the wind due to their minute size. In addition to wind, which is generally the greatest pollen outflow from Brassica species, pollen can be transferred by insects. Pollen is produced in large quantities, with more than 9 kg emitted per ha per day over a period of 4–5 weeks. Physical contact between neighboring plants also results in pollen dispersal while animals, including humans, passing through flowering canola fields can act as pollen vectors. Under typical conditions, viability of pollen will decrease over 4–5 days with a viability rate of 20% measured 72 hours after emission. The vast majority of pollen travels less than 10 m although pollen has been shown to travel longer distances of about 400 m. At 50 m from the pollen source, the level of outcrossing is reduced to less than 0.5%.

Although *B. napus* is self-compatible, pollen from neighboring and distant *B. napus* plants compete to effect fertilization. No genetic or morphological barriers to cross-pollination exist in *B. napus*, and crossing between proximate fields does occur. Most of the outcrossing occurs between neighboring plants, but long-distance pollen transfer can occur by both wind and insects. Pollen dispersal profiles are highly dependent on topographical and environmental conditions.

In related Brassicaceae species, many conditions must be met for gene transfer and introgression events to occur. Crossing success depends on a series of preconditions that include physical proximity of the parents, pollen movement and longevity, synchrony of flowering, breeding system of the parents, flower characteristics, pollen-style compatibility, and competitiveness of foreign pollen. Further, hybrid fertility and viability under natural conditions are of relevance. *Raphanus raphanistrum*, *Sinapis arvensis*, and *Hirschfeldia incana* are recognized as major weeds of commercial Brassica crops and have been described as potentially compatible with *B. napus*. However, crosses between male sterile *B. napus* and *B. juncea*, *B. nigra*, *H. incana*, and *R. raphanistrum* were found to produce only small seed, resulting in poor seedling establishment of the hybrids under field conditions.

Such crosses between related species have required extensive human intervention. The rates of natural hybridization between *B. napus* and weedy relatives resulting in fertile offspring are very low. *B. juncea* and *B. rapa* each cross more readily with *B. napus* than other Brassicaceae species. The potential of interspecific crossing into species such as *B. nigra* and *H. incana* is much lower, and extremely low for other species such as *Erucastrum gallicum*, *R. raphanistrum*, and *S. arvensis*. Resulting hybrids for any of these crosses also generally have decreased environmental fitness.

8.1.3. Speciality canola production

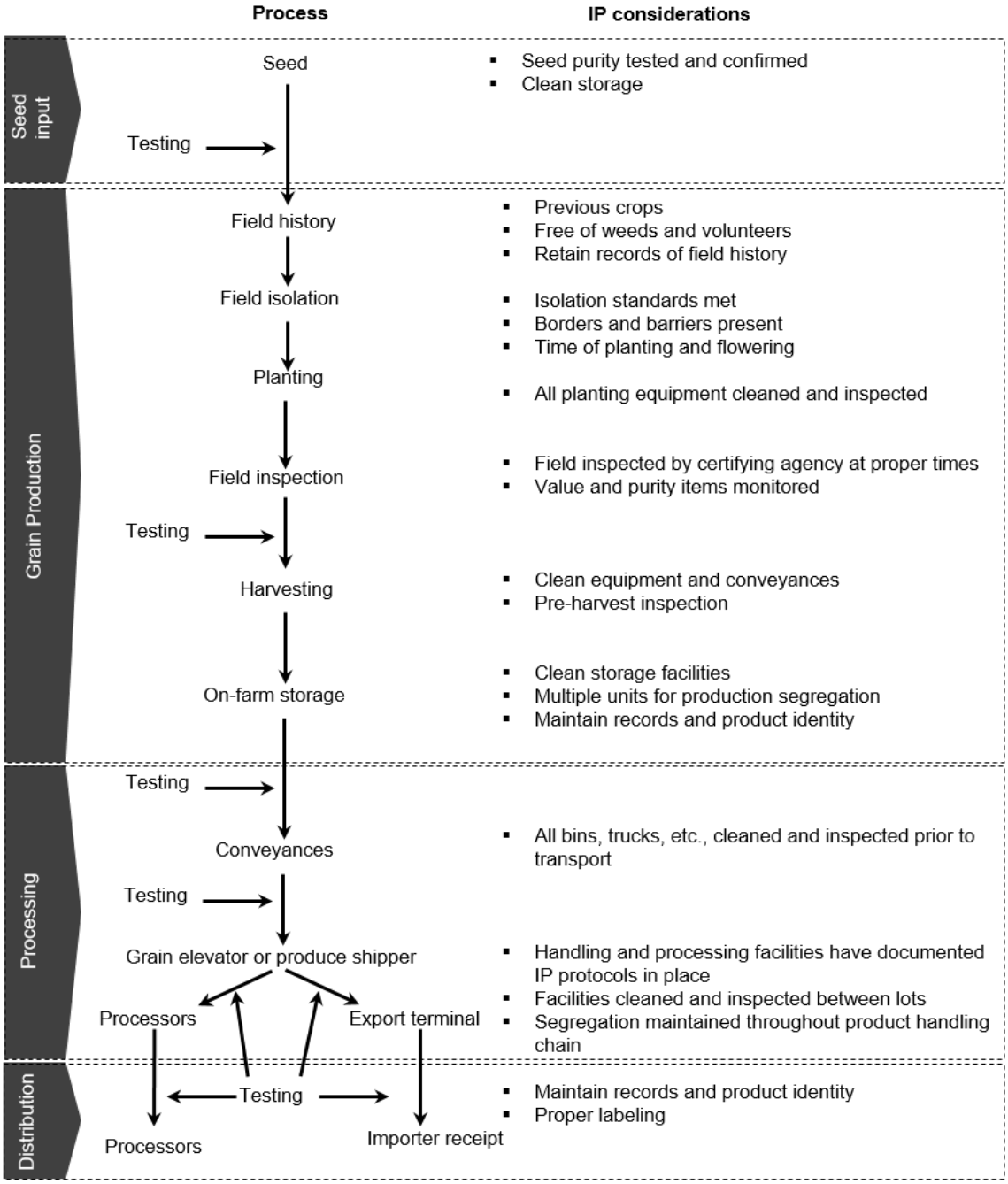
8.1.3.a. Identity Preservation

An Identity Preservation System (IDP) refers to a production and product handling system that ensures the integrity and purity of an agricultural commodity, starting from the breeder seed to as far as the processed products on a retail shelf (Sundstrom et al., 2002). Such a “closed-loop” system aims to deliver grains and/or processed products with specific, defined quality standards required by the manufacturers or the end users. In its simplest form, IDP has been practiced for a long time in agriculture since seeds and grains were traded separately and valued differently. IDP practices have further developed over the years to allow for the differentiation between food and feed grain or conventional and organic production (Massey, 2002). A well-known and often cited IDP model is the seed certification programs used by the Association of Official Seed Certifying Agencies (AOSCA). Established in the 1920s and 1930s, these programs established standards and certification processes to verify the genetic purity of seeds available to growers. In recent decades, the development of crops with unique output traits such as high oil corn, high oleic canola, and high oleic sunflower has led to an increased demand for IDP systems (Sundstrom et al., 2002).

The heart of modern IDP success is a contractual agreement that binds all the stakeholders in the food supply chain. The contract precisely defines the product specifications and includes the production, harvest, storage, and delivery practices required by the buyer. Growers are paid premiums for the additional operations and risk that they have assumed through the contract agreement. Premiums further motivate the growers and handlers to maintain grain purity and identity. Failure to comply with or meet required specifications can lead to price discounts or cargo rejection by the buyer (Massey, 2002). In practice, IDP relies on standards, testing, and record-keeping to preserve the desired product quality. The IDP system also facilitates the flow of information along four key processes of the food supply chain including seed supply, grain production, grain processing, and final product distribution. In this system, the product flows downstream from input supply to distribution as premiums are paid to farmers and grain handlers. IDP is successful because it enables all the stakeholders in the supply chain to share the value created by the segregation (Sundstrom et al., 2002).

Although each IDP arrangement depends on the specifications of the final product to be delivered, there are shared elements (Figure 10). The most critical element is the identity and the purity of the starting seeds. Most IDPs require the planting of specific varieties and the use of certified seeds that are supplied by accredited seed producers. Most often, buyers of IDP grains are food or feed manufacturers, paying third-party handlers to manage the grain production and delivery to appropriate processing facilities. Field isolation, agronomic practices, and equipment and facility cleaning are documented by the growers and inspected by the third-party handlers. If required, samples at various stages of the production are collected and analyzed to confirm identity, purity, and quality. All along the production process, identity labeling and record-keeping ensure that IDP products are segregated in the supply chain. Additional record-keeping may cover the field designation, the harvest amounts, the storage bin location, and the product movements (Sundstrom et al., 2002).

Figure 10. Identity Preservation Flow Chart



Adapted from Sundstrom et al. (2002)

8.1.3.b. **Seed production and certification in an IDP system**

For the certification of canola seeds, quality standards have also been developed and used by accredited national and/or state agencies for inspection (Table 50). The purity of any commercial downstream product propagated by seed begins with the purity of the starting seeds. Typically, seeds used in IDP are produced under certification. The purpose of seed certification is to preserve genetic purity and varietal identity. Once seed has been certified, it meets state, federal, and international seed law requirements (Table 51). Requirements for producing certified seed include special land requirements, planting eligible stock, field inspections, proper seed labeling, and meeting standards based on complete lab analysis (Massey, 2002; Sundstrom et al., 2002).

Table 50. U.S. Seed Classes and Relations with Seed Classes Recognized by the OECD

U.S. Seed Classes	OECD Seed Classes	OECD Color Label
Breeder Seed	Pre-Basic	White with diagonal violet stripe
Foundation Seed	Basic Seed	White
Certified seed (= first generation increase from Foundation or Registered seed)	First generation Certified Seed	Blue
NA	Second or successive generation of Certified Seed	Red
NA	Not finally Certified	Gray
NA	Mixes of herbage	Green

Source: USDA-AMS (2016)
NA = not applicable

Certified fields must maintain a minimum number of years that elapse between crops of the same species, a minimum number of plants of another variety per field, a maximum number of off-types in the cleaned seed, and a minimum isolation distance from fields of any other varieties or fields of the same variety not meeting the varietal purity requirements for certification. In the U.S., federal regulations established minimum standards per crop. In the case of canola/rapeseed, the minimum isolation distance from other canola/rapeseed for cross-pollinated foundation seed is 1,320 feet and 660 feet for self-pollinated varieties (Electronic Code of Federal Regulations, 2017). For certified seed of both types of varieties, the minimum isolation distance is 330 feet (Electronic Code of Federal Regulations, 2017).

In addition, accredited state seed certifying agencies have developed specific minimum requirements for canola. For example, the Montana Seed Growers' Association (MSGA) established minimal isolation distances for foundation, registered, and certified canola seed production in Montana (see Table 51). In North Dakota, there are established requirements for both hybrid and nonhybrid canola/rapeseed (North Dakota Seed Department, 2002, 2012).

Table 51. Minimum Isolation Distances for Canola Seed Production in Montana

Class	Fields of Cross-Pollinated Varieties	Field of Self-Pollinated Varieties	Certified Field of the Same Varieties
Foundation	1320 feet	660 feet	15 feet
Registered	1320 feet	660 feet	15 feet
Certified	660 feet	330 feet	15 feet

8.1.3.c. Grain Production and Processing in an IDP System

Using certified seed as starting material, an IDP system aims to produce and supply grains or processed products to a specific quality standard. The grains or processed products are identified and quality checked from the starting seed material to the consumer product on a retail shelf or equivalent. To achieve this goal, growers are required by contractual agreement to adhere to a well-defined program that specifies production standards, provides for sampling, and ensures appropriate documentation to enable auditing of the products (Smyth and Phillips, 2003). Growers participating in any IDP program must have the ability and the capability to produce grains that create and preserve grain quality. Specific practices that preserve or enhance the quality of the product are often prescribed in the contract (Massey, 2002).

Quality control measures are implemented along the entire grain production chain (Figure 10). In selecting the field, it is necessary to gain access and keep documentation of previous crop and herbicide rotations as well as weed and volunteer history. Before planting, it is necessary to confirm that spatial and/or temporal isolation of the IDP grain production from other compatible crops is in place.

In-season field inspection may also be required to ensure the harvest will meet the contractual requirements. Depending on the product specifications, monitoring of the production purity may also be requested. Such inspections may identify actions that are needed (i.e. removal of weeds or volunteers) prior to harvest (Massey, 2002).

Once production has been conducted per requirements, growers may be required to follow specific harvest and storage requirements to maintain the quality of the crop. For example, some IDP contracts require that growers do not begin the harvest until the grains are at or below some specific moisture level (Massey, 2002). Regular inspection of field equipment is also important, and detailed clean-out procedures may be prescribed. In some cases, dedicated handling equipment may be used to segregate the IDP grains from commodity ones (Massey, 2002).

In general, IDP contracts require growers to keep records of the crop production and the grain storage conditions until the grains are finally delivered to the processing facility. Upon IDP grain delivery, additional tests may be conducted to evaluate the grain quality. Premiums paid to growers will often be based on purity and quality of the grains according to agreed upon specifications (Massey, 2002).

8.2. Production of EPA+DHA canola event LBFLFK

8.2.1. Certified Seed and Grain Production of EPA+DHA canola

BASF Plant Science, L.P. (BASF) is a founding member of the Excellence Through Stewardship® (ETS) program and is committed to the responsible management of biotechnology-derived crops through each stage of the product life cycle – from product concept to discontinuation (Excellence Through Stewardship, 2015). BASF's commercial partner Cargill Inc. (Cargill) is also a member of the ETS program. As an integral part of responsible product stewardship and quality management practices, BASF and Cargill are committed to excellence in all activities relating to assuring strict adherence to all applicable regulations globally and to promote responsible management and trade of any plant biotechnology product.

Cargill, in coordination with BASF, will conduct activities to support variety development, grain production, oil manufacturing, and other commercial activities to prepare EPA+DHA canola event LBFLFK for the marketplace as an alternative source of long-chain polyunsaturated omega-3 fatty acids (LC-PUFA). EPA+DHA canola event LBFLFK production and processing will be conducted under an IDP system controlled by Cargill. Cargill has more than 20 years of experience handling specialty oil under IDP systems from seed production to final product delivery to customers globally (Cargill, 2012). Production of EPA+DHA canola will utilize all the typical measures for the management of a specialty agricultural product under an IDP program to assure a high-quality product and to avoid unintended mixing with other products (see section 8.1.3).

Through breeding, Cargill will develop the commercial hybrid varieties that will be grown under an IDP system to produce EPA+DHA canola oil. As with all IDP programs, the IDP system for EPA+DHA canola event LBFLFK will start with certified seed production, using breeder and foundation seeds. Cargill Specialty Seeds and Oils Division has a long history of successfully producing breeder, foundation, and certified seeds and has trained personnel and dedicated facilities to conduct these activities (Cargill, 2015).

The new specialty oil from event LBFLFK will be produced from proprietary spring canola varieties developed by Cargill through breeding with progeny of the event. Seed and grain production will be carried out within the United States in full compliance with the applicable seed certification standards in the region of production. To preserve the quality of EPA+DHA canola, certified seeds will be supplied to growers who are either under contract with Cargill directly or through selected retailers who will adhere to the same quality parameters implemented by Cargill. The seeds will only be available for cultivation under contract to maintain IDP standards.

For the production and processing of EPA+DHA canola event LBFLFK and derived products, the IDP system implemented by Cargill will oversee the value chain from certified seed to final use of the product. This will maintain product quality and value and ensure segregation from other commodity canola products (Cargill, 2012). The IDP will involve: i) physical separation from different canola lines, ii) extensive quality testing, iii) restricted seed sales (not sold on the open market), iv) commercial grain produced under contract and delivered to a Cargill facility per delivery schedules, v) canola grain crushed and refined separately from other products in Cargill-operated or contracted facilities, and vi) management of oil sales to the final customer.

Through the operations of the IDP, after EPA+DHA canola grain is purchased from the grower by Cargill and delivered to a Cargill-operated receiving point, it will be directed to a Cargill-owned or -contracted oilseed crush facility in the U.S. or Canada. The crush facility will have separate storage tanks for all EPA+DHA canola oil that is produced. For further refinement of the oil, a Cargill-owned or -contracted refinery, also with separated storage, equipment cleaning, quality testing, and inventory management specific to the EPA+DHA canola oil, will be used to maintain the integrity and value of the product.

8.2.2. Management of weeds during EPA+DHA canola production

The presence of a modified acetohydroxy acid synthase (AHAS) enables EPA+DHA canola to be treated with Beyond® herbicide. This allows broad-spectrum weed control with high biological efficacy at low application rates. Imazamox has been in wide agricultural use for canola and other crops for many years as an established weed control option (Tan et al., 2005), allowing growers to target both grassy and broadleaf weeds.

Imidazolinone herbicides possess several environmentally beneficial characteristics compared to other herbicide classes and have been registered for use on various crops globally for many years (Tan et al., 2005). They control a wide spectrum of grass and broadleaf weeds (Shaner and Singh, 1998) and are effective at low application rates. Imazamox is very safe for humans and has a very low environmental impact (Electronic Code of Federal Regulations, 2017).

Beyond® herbicide is a Group 2 herbicide based on its mode of action. This group includes those classed as ALS/AHAS inhibitors. Group 2 herbicides work by inhibiting acetolactate synthase (ALS), an enzyme that is required to produce the amino acids leucine, isoleucine, and valine in plants. Herbicides with the ALS/AHAS enzyme-inhibiting mode of action include sulfonylureas, triazolopyrimidine sulfoanilides, sulfonylaminocarbonyl triazolinones, imidazolinones, and pyrimidyl benzoates.

Beyond® herbicide is approved for use as part of the Clearfield® production system for Clearfield® canola, Clearfield® lentil, Clearfield® rice, Clearfield® sunflower, and Clearfield® wheat (U.S. EPA, 2010). EPA+DHA canola is an imidazolinone-tolerant canola produced by biotechnology techniques whereas the canola sold under the Clearfield® brand name (also imidazolinone-tolerant) was produced by mutagenesis and has been on the market for many years.

Beyond® herbicide is applied when weeds are small and actively growing. Its absorption occurs primarily through foliage and secondarily through roots and translocates in both the xylem and phloem of the plant, accumulating in areas of active growth. Susceptible weeds will stop growing and either die or not be competitive with the crop. Beyond® herbicide primarily controls emerged broadleaf and grass weeds when applied post-emergence with some soil activity. EPA+DHA canola event LBFLFK will be grown under a weed management program following all label directions for Beyond® herbicide as per U.S. EPA registration for EPA+DHA canola. The label will indicate the rate and weed growth stage to apply the herbicide as well as recommended adjuvants, spray volume, and proper nozzle and pressure to ensure effective coverage.

The goal of a weed management program is to prevent or delay the onset of weed resistance, which can be achieved by good management practices including chemical control, cultural practices, and crop management. In general, crop (and herbicide) rotation is effective in managing weed resistance where herbicides of different modes of action are used. Specific key management practices include: i) rotation among herbicide groups for both grass and broadleaf weed control to avoid repetitive use of one or more than one herbicide from the same herbicide group, ii) following the herbicide label recommendations, iii) using pre-emergence non-selective herbicides, iv) using crop rotations and planting crops that can compete with weeds, v) planting quality seed at competitive seeding rates, and vi) combining tillage and/or timely cultivation with herbicide treatments.

While weed resistance to group 2 herbicides is common in a number of weed species, these herbicides remain an important component of successful weed control programs. The International Resistant Weed Survey (Heap, 2017) lists more than 20 weed species as resistant to the active ingredient imazamox (see Table 52). Resistance management should be part a diversified weed control strategy that integrates multiple options including chemical, cultural, mechanical, and biological control tactics. Cultural control tactics include agronomic practices that improve the competitive ability of the crop via rotation, variety/cultivar selection, precision fertilizer placement, and optimum crop plant density. Agronomic practices should also limit the development and spread of weeds by using clean crop seed (e.g., certified seed), preventing crop trait out-crossing, controlling weed influx from field borders, and managing weed seed at harvest / post-harvest to minimize the carryover weed seed bank in the following crop. Mechanical control tactics include timely tillage where practical, equipment cleaning to avoid weed spread, and minimization of harvest crop seed losses in the field through close attention to timeliness of harvesting, correct setup of harvest equipment, and covering crop seed loads during harvest and transport to avoid dispersing seed.

Table 52. Weed Species with Confirmed Resistance to Active Ingredient Imazamox

Common Name	Scientific Name	Common Name	Scientific Name
Barnyard grass	*Echinochloa crus-galli var. crus-galli*	Marshelder	*Iva xanthifolia*
Brome, downy	*Bromus tectorum*	Nightshade, eastern black	*Solanum ptychanthum*
Brome, Japanese	*Bromus japonicus*	Pigweed, redroot	*Amaranthus retroflexus*
Cheat	*Bromus secalinus*	Ragweed, common	*Ambrosia artemisiifolia*
Flatsedge, rice	*Cyperus iria*	Ragweed, giant	*Ambrosia trifida*
Flixweed	*Descurainia sophia*	Rice, red	*Oryza sativa var. sylvatica*
Foxtail, giant	*Setaria faberi*	Ryegrass, Italian	*Lolium perenne ssp. multiflorum*
Foxtail, green	*Setaria viridis*	Shattercane	*Sorghum bicolor*
Goatgrass, jointed	*Aegilops cylindrica*	Sowthistle, spiny	*Sonchus asper*
Kochia	*Kochia scoparia*	Wallflower, bushy	*Erysimum repandum*
Lambsquarter, common	*Chenopodium album*	Waterhemp	*Amaranthus tuberculatus* (= *A. rudis*)

Source: Heap (2017)

In summary, even with the presence of resistant weeds, imazamox remains an important component of a successful weed control program by effectively targeting non-resistant populations. Weed resistance management is part of a diversified weed control strategy that integrates multiple options. These options include using herbicide(s) from a different group plus cultural, mechanical, or biological control practices during harvesting, storage, and transport. Together, these strategies play a major role in the successful management of volunteers.

8.3. Management of EPA+DHA canola volunteers

Controlling EPA+DHA canola volunteers after the crop has been grown relies on herbicides other than those in group 2, such as group 4 herbicides (e.g., benzoic acids, carboxylic acids, phenoxy herbicides), group 5 (triazines, phenylcarbamates), and group 6 (benzothiadiazoles, nitriles). Another effective strategy for managing volunteers from EPA+DHA canola fields is to rotate with a crop having a different (not group 2) herbicide-tolerance trait, such as glyphosate or glufosinolate-tolerant corn or soybean. Other conventional methods for weed control can also be used to manage canola volunteers such as tilling or other mechanical means.

8.4. Assessment of weediness potential of EPA+DHA canola

To assess the weediness potential of LBFLFK, it is relevant to determine changes in characteristics as compared to conventional canola varieties like the proportion of seeds that survive in the soil, the proportion of seedlings emerging following germination, the timing of emergence, and seedling vigor.

As shown in sections 6.2.1 and 6.2.2, studies conducted across the U.S. in the 2014/15 and 2015 seasons found no difference in the pod shattering rate of LBFLFK compared to the control Kumily (Table 16 and Table 18).

As noted in Section 6.2.4, secondary seed dormancy was also examined from LBFLFK seed harvested across six field sites in 2015. LBFLFK had relatively few viable seeds after dormancy induction compared to Kumily and six other canola varieties (Table 19). Seedling emergence in field studies (see section 6.2) and general germination rates in laboratory studies (section 6.2.4) of LBFLFK were also lower than its commercial comparator Kumily, especially under cold temperature conditions.

Therefore, the comparative assessment performed confirmed that LBFLFK will not be more persistent, or otherwise weedier, compared to other canola varieties.

8.5. Gene flow assessment of EPA+DHA canola

LBFLFK is expected to show similar pollen-mediated gene flow properties as any conventional canola variety with no increased potential for out-crossing. Pollen viability of LBFLFK was comparatively assessed (section 6.2.5) and was found to have similar or reduced viability, suggesting there is no increase in gene flow potential compared to other canola cultivars. Any hybrids resulting from outcrossing between event LBFLFK and *B. rapa* or *B. juncea* could be controlled using herbicides other than imidazolinone or by mechanical means. If imidazolinone-tolerant individuals arose through interspecific or intergeneric hybridization, the HT trait would confer no competitive advantage to these plants unless challenged by imidazolinone herbicides, which would only occur in managed ecosystems where imidazolinone herbicides are used for weed control. As with imidazolinone-tolerant event LBFLFK volunteers, these HT hybrid individuals, should they arise, could be controlled using mechanical means or herbicides other than imidazolinone herbicides.

Therefore, all currently applicable crop management practices that manage and minimize any potential outcrossing to related species would also be applied for cultivation of LBFLFK to minimize outcrossing into compatible species.

8.6. Product handling of EPA+DHA canola

To maintain the quality and ensure the segregation of LBFLFK canola seeds, grains, and processed products, an Identity Preservation System (IDP) will be implemented at every step of production and handling. The grains from EPA+DHA canola event LBFLFK will be processed by Cargill at facilities in the U.S. or Canada that are able to maintain separation from conventional canola or other oilseed grains. Depending on end-use demands and the quality specifications of the product, Cargill either will produce pressed oil and crude oil, or refined, bleached, and deodorized (RBD) oil for use as an aquafeed ingredient or a food ingredient, respectively. All along the oil processing steps, oil product quality will be inspected by a Cargill Food Safety Team to ensure that it meets the required specifications.

EPA+DHA canola grain production will be carried out by growers that are either under contract with Cargill directly or through selected retailers. Quality control measures will be implemented before planting, in-season, and during harvest to preserve and guarantee grain quality. Measures to ensure segregation during grain movement and on-farm storage will also be in place. Growers will be required to deliver all the harvested grains to facilities designated by Cargill. Additional testing will be conducted at delivery to evaluate grain quality.

Once the EPA+DHA canola grain is purchased by Cargill from the grower and delivered to a Cargill-operated receiving point, it will be directed either to a Cargill-owned crush facility or to a Cargill-contracted facility capable of maintaining product quality and separation. Quality testing will be applied during oil processing to confirm that the EPA+DHA canola oil meets product specifications for LC-PUFA. Inventory management will be applied to keep track of the EPA+DHA canola grain and oil throughout the process. The crush facility will have separate storage tanks for EPA+DHA canola oil, and all equipment will be flushed with enough non-EPA+DHA canola oil to ensure that supply chains are not compromised. Further, any non-EPA+DHA canola oil produced at the same facility will be tested to confirm the absence of LC-PUFA associated with EPA+DHA canola oil.

If required by customers, the oil may be shipped to either a Cargill-owned and operated refinery or a contracted refinery where the same separate storage, equipment cleaning, quality testing, and inventory management conditions will be applied.

A by-product of canola oil production is defatted canola meal. The defatted meal produced from event LBFLFK will not be treated a specialty product and will be distributed similarly to other conventionally produced defatted canola meal.

EPA+DHA canola grain that does not meet quality specifications will still be accepted at a Cargill-owned or contracted facility but will instead be directed to an appropriate alternate end-use such as the production of bio-fuel. This will ensure that the conventional canola supply chain is not affected by the products of EPA+DHA canola being redirected away from specialty applications.

Further on the supply chain, oil from EPA+DHA canola will be shipped to a Cargill-approved oil packager. The oil products will be packaged and shipped per customer specifications. Given that the oil products from EPA+DHA canola are specialty oils, the label will appropriately differentiate them from conventional canola oil that does not contain the omega-3 fatty acids EPA and DHA.

8.7. Summary on the impact of the introduction of EPA+DHA canola event LBFLFK on agricultural practices

While EPA+DHA canola event LBFLFK will be sold and managed as a specialty canola, typical cultivation and management practices are expected to be followed. The typical agronomic practices suitable for growing other varieties of canola will be used to grow canola event LBFLFK, including the use of Beyond® herbicide with the active ingredient imazamox.

Imazamox has been in wide agricultural use on canola and other crops for many years as an established weed control option, allowing growers to target both grassy and broadleaf weeds (Tan et al., 2005). Management practices (i.e. crop rotation) for optimal production and stewardship of the EPA+DHA canola trait will be similar to those in place for canola varieties containing a similar herbicide tolerance trait (Clearfield®). In more than 20 years, since the first cultivation of imidazolinone-tolerant crops in North America, none of the crops have demonstrated any different environmental characteristics compared to their conventional counterparts, other than the intended tolerance to imidazolinone herbicides.

The data collected in field trials and controlled environments over two seasons support that LBFLFK is not substantially different than its parental control and conventional comparator canola varieties. Data also show that LBFLFK is not anticipated to increase the plant pest or weediness potential in production systems or impact plant diseases, pests, or abiotic stressors. In addition, agronomic and phenotypic performance of LBFLFK is similar to conventional canola, so no significant changes to agricultural or cultivation practices (pesticide application, tillage, harvesting, rotation, and management of volunteers) would be necessary. It is possible that the reduced and delayed germination of LBFLFK under cold weather conditions compared to the parental control Kumily may require an adjustment of the minimal seeding rate for early spring planting.

Taken together, the cultivation of LBFLFK canola is not expected to affect current agricultural practices including cultivation, weed control, volunteer management, and herbicide resistance management for canola production.

8.8. Adverse consequences of introduction

BASF Plant Science, L.P. is not aware of any unfavorable information that would have a bearing on a decision by USDA APHIS to deregulate EPA+DHA canola event LBFLFK. The development and testing of EPA+DHA canola event LBFLFK has not revealed any data or observations indicating that deregulation of this new cultivar would pose a greater plant pest risk to U.S. agriculture or the environment than conventional canola and, therefore, it should not be considered a regulated article under 7 CFR part 340.

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APPENDICES

Appendix A. Regulated Field Releases in the United States

Field trials of LBFLFK have been conducted in the U.S. since 2014. The purpose of these trials was to collect data for agronomic performance, seed production, phenotypic observations on plant disease, insect infestation, and abiotic stressors. In addition, trials were conducted to generate field materials and data required for this petition.

Table A.1. USDA Notifications Approved for LBFLFK and Status of Trials Planted

Field Trial Year	USDA No.	Company	Effective Date	Type	Number of Locations	Release States	Trial Status
2014	14-071-102n	BASF	1-Apr-2014	Field release	6	IA, ID, MN, ND	Completed
	14-181-103n	BASF	21-Jul-2014	Field release	1	HI	Completed
2014/2015	14-255-102n	BASF	15-Oct-2014	Field release	10	GA, LA, TX	Completed
2015	15-075-102n	BASF	13-Apr-2015	Field release	12	IA, ID, HI, MN, MT, ND, SD, WA	Completed
	15-086-104n	BASF	24-Apr-2015	Field release	NA	NA	Not Planted
	15-114-102n	BASF	4-May-2015	Field release	1	ID	Completed
2015/2016	15-267-101n	BASF	13-Oct-2015	Field release	3	HI, TX	Completed
2016	16-061-103n	BASF	1-Apr-2016	Field release	21	IA, ID, MN, MT, ND, NE, SD, WA	Completed
	16-063-101n	BASF	1-Apr-2016	Field release	1	ID	Completed
	16-250-101n	BASF	10-Oct-2016	Field release	NA	NA	Not Planted
2017	17-060-102n	Cargill Global Edible Oil Solutions - Specialties	1-Apr-2017	Field release	3	MT	In progress

NA = Not applicable

Appendix B. Molecular Characterization of EPA+DHA Canola Event LBFLFK – Materials and Methods

B.1. Materials

B.1.1. Test, control, and reference substances for DNA sequence analysis

The test and control substances used for DNA isolation are listed in Table B.1. The DNA isolated from these materials was used for next-generation sequencing (NGS) or for locus-specific sequencing of polymerase chain reaction (PCR) products (Sanger sequencing).

Genomic DNA (gDNA) isolated from seed was used for NGS and subsequent analysis. Genomic DNA isolated from leaf tissue of event LBFLFK seedlings was used to generate a Bacterial Artificial Chromosome (BAC) library, which was screened using insert/locus-specific PCR. Two BAC clones were identified that contained LBFLFK Insert1 and LBFLBK Insert2 along with flanking gDNA. DNA was isolated from these two BAC clones and was used for PCR and Sanger sequencing.

Six *B. napus* endogenous single copy genes were used as reference genes in the bioinformatic analysis of NGS data (see Table B.2). Certificates of analysis were generated for all seed lots of LBFLFK and the parental control variety Kumily that were used in the DNA sequencing studies.

B.1.2. Test and reference substances for Mendelian inheritance analysis

Test substances for Mendelian inheritance assessments were single seed samples from individual seed lots produced after backcrossing the T3 generation of LBFLFK with Kumily.

Reference materials for molecular analysis of segregation included various canola gDNA preparations using LBFLFK and Kumily.

Table B.1. Test and Control Substances for DNA Sequencing Studies

Substance Descriptor (DNA source)	Species	Plant Line/ Variety	Generation	Analysis Type	Analysis Endpoint
Test Substance Seed	*B. napus*	LBFLFK	T3	NGS	• Number of T-DNA inserts • Absence of plasmid backbone • Number of insertion sites • Stability of the introduced gene elements over multiple generations
Test Substance Seed	*B. napus*	LBFLFK	T4		
Test Substance Seed	*B. napus*	LBFLFK	T5		
Control Substance Seed	*B. napus*	Kumily	S2		
Technical Control Substance Seed, spiked with LTM593 (1:1)	*B. napus*	Kumily	S2		NA
Technical Control Substance Seed, spiked with LTM593 (1: 0.1)	*B. napus*	Kumily	S2		NA
Technical Control (Plasmid)	NA	NA	NA	NGS	NA
Analytical Test Substance BAC Clone LBFLFK Insert1	NA	NA	T3	Sanger sequencing and PCR	• Sequences of T-DNA inserts and flanking genomic regions • Organization and integrity of T-DNA inserts
Analytical Test Substance BAC Clone LBFLFK Insert2	NA	NA	T3		
Control Substance Seed	*B. napus*	Kumily	S2		• Rearrangement at the insertion sites

NA = Not Applicable

Table B.2. *B. napus* Endogenous Genes used as Single Copy Sequence References for the Bioinformatics Analytical Pipeline

Gene Name[1]	GenBank Accession	Chromosome[2]	Length (bp)[1]
LOC106447038	NC_027760.1	Chromosome A04	976
LOC106364346	NC_027765.1	Chromosome A09	987
LOC106451380	NC_027761.1	Chromosome A05	1,083
LOC106358142	NC_027763.1	Chromosome A07	1,240
LOC106373669	NC_027767.1	Chromosome C01	998
LOC106402890	NC_027772.1	Chromosome C06	2,069

[1] Gene names and lengths were obtained from NCBI (2017).
[2] Chromosome associations were based on NCBI data and confirmed against the Darmor-*bzh* v4.1 reference genome (Chalhoub et al., 2014). Version v4.1 of the genome sequence was downloaded from Genoscope (Centre National de Séquençage, 2017).

B.2. Methods

B.2.1. DNA preparations

B.2.1.1. DNA preparations for DNA sequencing studies

Genomic DNA was isolated from seeds of LBFLFK and Kumily. DNA was also isolated from BAC clones RS-2183 and RS-2184, which were derived from T3 leaf tissue of LBFLFK and contained Insert1 and Insert2, together with genomic flanking regions, respectively. The plant transformation vector LTM593 plasmid DNA was used to demonstrate that the NGS method was suitable for the detection of LTM593.

B.2.1.1.a. DNA isolation from seeds

Starting materials were approximately 6–8 g dry seed of Kumily and the T3, T4, and T5 generations of LBFLFK. A two-step protocol (extraction and purification) was used to isolate gDNA from the canola seed. Total gDNA was extracted following a modified cetyltrimethylammonium bromide (CTAB) method (Porebski et al., 1997). The extracted gDNA was further purified by using a DNeasy®[1] Plant Maxi kit.

B.2.1.1.b. DNA isolation from BAC clones

500-ml overnight bacterial cultures were used for plasmid DNA extraction of the BAC clones using QIAGEN®[2] Plasmid Maxi kit.

B.2.1.1.c. Spiked control DNA samples

LTM593 plasmid DNA was spiked into samples of Kumily gDNA at two concentrations. One sample was spiked with one haploid canola genome equivalent of plasmid LTM593 DNA. The other was spiked with one-tenth haploid canola genome equivalent of plasmid LTM593 DNA. The formula below was used to calculate how much LTM593 DNA to spike into the Kumily gDNA:

$$Wl = \frac{Sl}{Sc} \times Wc \times m \times 1000$$

Where:

Wl: Weight of LTM593 in ng
Sl: Size of LTM593 in base pairs (bp) (60,074)
Sc: Size of canola haploid genome in bp (1.15×10^9)
Wc: Weight of canola gDNA in µg
m: Intended genome ratio of LTM593/canola in the spiked DNA

[1] DNeasy is a registered trademark of Qiagen GmbH.
[2] QIAGEN is a registered trademark of Qiagen GmbH.

To demonstrate the sensitivity of the NGS analysis, the spiked samples were used as controls in NGS to validate the method.

B.2.1.2. DNA preparations for inheritance studies

Genomic DNA from F2 and F3 seeds was extracted using a NucleoMag®[3] Plant DNA extraction kit.

B.2.2. Next generation sequencing and bioinformatic analysis

B.2.2.1. Method synopsis

A combination of NGS and bioinformatics was used to determine the number of transfer DNA (T-DNA) insertion sites, the copy number of inserted sequences, the absence of vector backbone, and the stability of the inserted material over three generations.

Genomic DNA from the test and control substances was sequenced using an HiSeq®[4] 2500 system to produce large numbers of short sequence reads approximately 125 bp in length. Bioinformatics search tools were then used to select all sequence reads that had sequences similar to the LTM593 transformation plasmid, which were used in further bioinformatics analysis to characterize all junction sequences and to detect for any plasmid backbone sequence. A comparison of junction sequences and sequencing read distributions was used to determine the stability of T-DNA insertions over three LBFLFK generations (T3, T4, T5).

Additional method details are provided in the following sections.

B.2.2.2. Preparation of PCR-free shotgun sequencing libraries

Sequencing libraries were made using a TruSeq®[5] DNA PCR-free Library Prep kit to create libraries of randomly broken-up DNA sequences, generated by shearing (“PCR-free shotgun libraries”). Eight PCR-free shotgun libraries were prepared per gDNA sample, and one PCR-free shotgun library was prepared from the LTM593 plasmid DNA sample.

In brief, to prepare a library, gDNA was sheared into fragments at a target size of 550 bp using a focused-ultrasonicator. After confirming successful DNA fragmentation, the ends of DNA fragments were repaired, and small and large DNA fragments were removed from the library. Concentration of the resulting library was assessed by quantitative PCR. Library fragment length distribution was assessed using a 2100 Bioanalyzer®[6].

The eight libraries for each gDNA sample were pooled in equal molarity.

[3] NucleoMag is a registered trademark of MACHEREY-NAGEL & Co.
[4] HiSeq is a registered trademark of Illumina Inc.
[5] TruSeq is a registered trademark of Illumina Inc.
[6] Bioanalyzer is a registered trademark of Agilent Technologies, Inc.

B.2.2.3. Sequencing of short reads

The pooled library samples described above were sequenced on the HiSeq® 2500 system using a high-output run mode (2x125 bp paired end run mode) to produce short sequence reads (approximately 125 bp). Each library pool was sequenced in five flow cell channels, and a sufficient number of sequence reads were obtained for deep coverage of the entire genome (Kovalic et al., 2012).

B.2.2.4. Bioinformatic analysis pipeline

To specifically characterize DNA from LTM593 in LBFLFK, only sequence reads that had sufficient similarity to LTM593 were further analyzed. All sequences that had significant sequence similarity to LTM593 (maximum Expect value (E-value) = 0.0001) were selected from the NGS data set.

B.2.2.4.a. Analysis pipeline steps

Declare variables and merge/format NGS reads per sample

This step was performed in the initial script of the pipeline and was run separately for each sample. All files contributing sequence reads to an individual sample were merged. Following merging, the files were converted into a searchable BLAST database of sequenced reads. This script initiated the remainder of scripts in the bioinformatics pipeline.

Map NGS reads in BLAST database against probe sequences

The next script performed BLAST alignments of sequence reads against eight sequence "probes": LTM593 T-DNA, LTM593 backbone, and six endogenous single-copy genes.

Calculate read coverage for each probe sequence

The third script performed read coverage calculations, which defined the median/mean read depth to compare amongst the probes. During coverage calculation, the bioinformatics pipeline ignored regions of the LTM593 insert containing known endogenous *B. napus* sequences and repeated elements. During analysis, reads aligning to endogenous and repeated sequence regions within the T-DNA also aligned elsewhere in the genome or T-DNA. Therefore, unless ignored, the affected read would be counted multiple times along the insert and genome. This would contribute to increases in apparent read coverage at these positions, altering mean and median values, and impacting the determination of comparative read depth ratios generated when comparing read coverages across probes.

Realign reads to LTM593 T-DNA probe sequences

This step performed a second BLAST alignment of reads that aligned only to the T-DNA probe sequence. Output reads were used to characterize read pairing information and for junction sequence annotation. Top-tier mappings were determined based on BLAST alignment scores and read classifications.

Find structural variants for junction identification

This step identified reads that were aligned (split-read mappings) to a portion of the T-DNA probe sequence and a portion of the genomic flanking region. Split-reads were classified as structural variants to the T-DNA probe and indicated the presence of a junction. Junction positions identified by this function were further used for annotation later in the pipeline.

Perform multiple-sequence alignment on identified junction positions

This step read through the junction files and generated a FASTA file of split-read sequences for each junction position. Independent FASTA files for each junction position clustered the split-read sequences. Once FASTA files were generated, 'multiple-sequence alignments' were performed on each of the files to identify the junction position along the T-DNA probe.

Annotate junction positions

The final script of the bioinformatics pipeline annotated all junctions called based on split-read and read pair alignment information.

B.2.2.4.b. Identification and exclusion of false positive junctions

To identify (and exclude from the T-DNA/genome junction analysis) false-positive junctions, the parental control Kumily was also analyzed using the NGS/bioinformatics pipeline. The junction calls from the Kumily genome, which lacks the presence of the LTM593 vector sequence, signify endogenous split-read mappings that align elsewhere in the genome and share sequence identity with endogenous elements existing in the LTM593 T-DNA. These junctions were found in all samples. They were annotated as false positives and removed from further analysis.

B.2.2.4.c. Quality of NGS and the bioinformatics pipeline

Read depth, read breadth, and uniformity analyses established the quality of the generated NGS data. Read breadth calculations for LBFLFK generations T3, T4, and T5 demonstrated that the distributions of read depth throughout probe lengths were close to a statistical normal distribution and therefore uniform.

The sensitivity level of NGS and related bioinformatics analysis achieved in this study was sufficient to detect 100% of the LTM593 plasmid sequences when spiked at one-tenth molar ratio in conventional control gDNA, confirming the ability of the method to detect any sequences derived from the transformation plasmid.

B.2.3. Analysis of BAC clones (locus specific PCR, Sanger sequencing)

BAC clones RS-2183 and RS-2184, containing Insert1 and Insert2, respectively, plus flanking gDNA, were used as templates for PCR followed by sequencing of the inserts and associated flanking regions in LBFLFK. Genomic DNA from the parental variety Kumily was used to determine the genomic region of Kumily that corresponded to the two insertion sites in LBFLFK. Every nucleotide of the sequence was covered by two independent PCR amplicons. Every PCR fragment was sequenced in both forward and reverse directions (2-fold). Total sequencing coverage from two independent PCR sets was 4-fold.

B.2.3.1. Generation of PCR products

The PCR assays were carried out using a GoTaq®[7] HotStart Green Master Mix. Fifty-five overlapping PCR products that spanned the insert and > 1,000 bp of adjacent 5′ and 3′ flanking DNA sequences were generated from each of the two BAC clones. Moreover, four PCR products were generated, two from each of the two genomic regions of Kumily that correspond to the T-DNA insertion sites in LBFLFK. Each PCR used one primer binding to the 5′ flanking sequences and the other binding to 3′ flanking sequences of LBFLFK Insert1 or LBFLFK Insert2, respectively.

B.2.3.2. Sanger sequencing reactions

All sequences were generated using BigDye®[8] terminator chemistry. Approximately 40 ng of each PCR product was used as template per sequencing reaction.

[7] GoTaq is a registered trademark of Promega Corporation.
[8] BigDye is a registered trademark of Thermo Fisher Scientific.

B.2.3.3. Sequence assembly

Sequences generated from BAC clones RS-2183 and RS-2184 were assembled separately to generate two consensus sequences corresponding to the Locus1 and Locus2 insertion sites, respectively. Consensus sequences were generated by compiling sequences from multiple sequencing reactions from each BAC clone. These consensus sequences were aligned to the sequence of LTM593 to determine the integrity of LBFLFK Insert1 and Insert2 T-DNAs as well as of the respective 5´ and 3´ flanking sequences.

Similarly, sequences generated from Kumily were assembled into separate consensus sequences that corresponded to the genomic Locus1 and Locus2 insertion sites, respectively.

B.2.3.4. Statistical analysis

Quality values (QV scores), similar to *phred* scores, were assigned to all called bases (Ewing and Green, 1998). These QV scores were used during assembly of the single reads and were the basic requirement for calculating the sequence accuracy. In the assembly phase, the QV scores of single reads were used to calculate the consensus confidence.

B.2.3.5. Annotation of insertion site sequences in Kumily

Annotation of insertion site sequences in Kumily was conducted using BLAST against all available canola genome databases, including:

- *B. napus* Darmor-*bzh* v.4.1 (Chalhoub et al., 2014);
- *B. napus* Topaz GBv.1.0 (internal BASF assembly);
- *B. rapa* peptides IVFCAASv (EnsemblPlants, 2017);
- *B. rapa* IVFCAASv1 (Wang et al., 2011; EnsemblPlants, 2017);
- *B. rapa* cDNA IVFCAASv1 (EnsemblPlants, 2017);
- *B. rapa* BRAD v.1.2 (Cheng et al., 2011)
- Unipro/SwissProt (June-21-2016) (UniProt, 2017)

B.2.4. Mendelian inheritance analysis

B.2.4.1. Production of the testing materials

Individual T3 plants that were confirmed homozygous for both LBFLFK Locus1 and LBFLFK Locus2 were crossed to the parental control variety Kumily to produce hemizygous F1 seeds. Confirmed hemizygous F1 plants were self-pollinated to produce segregating F2 seeds. The F2 seeds were pooled, and a randomly picked subsample from the bulk was planted to grow F2 plants. F2 plants that were confirmed hemizygous for both Locus1 and Locus2 were self-pollinated to produce segregating F3 seeds.

B.2.4.2. Zygosity determination

For the determination of the zygosity of both Locus1 and Locus2 in single seeds, a locus-specific duplex real-time TaqMan® PCR was used for each locus. The assay amplified a DNA sequence that spans the junction of the T-DNA and gDNA at the respective integration site. In the same reaction, the assay also amplified the DNA sequence of an endogenous canola gene, which was used to normalize the signal strength of the amplification between samples.

Real-time PCR assays were performed in 96-well microtiter plates. Each microtiter plate contained, as a reference, four reactions with wild-type (Kumily) DNA, four reactions of hemizygous LBFLFK DNA, and four reactions with homozygous LBFLFK DNA. Each plate also contained four reactions of a no template DNA control. Additionally, two standard curves, consisting of four different concentrations of hemizygous LBFLFK DNA or homozygous LBFLFK DNA, were produced as a reference.

Signal strengths were expressed as the cycle at threshold (Ct) value, which is the PCR cycle number at which the fluorescence generated by the assay crosses a specific threshold. The Ct values served as input for statistical analysis to determine zygosity.

B.2.4.3. Statistical analysis

A significance level of $\alpha = 0.05$ (confidence level 95%) was used for all statistical tests.

Zygosity determinations were made based on the real-time PCR analysis raw data. Before the Chi-square analysis, samples with inconclusive zygosity were removed. An inconclusive zygosity determination was made when the sample copy number for the assayed locus was found to be in between the copy numbers set by the controls (wild-type control copy number of 0, heterozygous control copy number of 1.0, homozygous control copy number of 2.0). For example, an inconclusive zygosity call would be any sample with a copy number between 1.3 and 1.7 as it could be either heterozygous or homozygous.

For the F2 generation segregating seed, genotypes were successfully determined for 768 out of 792 randomly picked seeds, with 24 samples removed (or 3.03% of the total sample

population) due to inconclusive zygosity determination. For the F3 generation segregating seed, genotypes were successfully determined for 763 out of 792 randomly picked F3 seeds, with 29 samples removed (or 3.66% of the total sample population) due to inconclusive zygosity determination.

The Ct values that passed the quality checks were assessed using a statistical data analysis script that generated zygosity calls using a linear discriminant analysis (lda) algorithm (Venables and Ripley, 2002). The zygosity calls were conducted with validated R scripts (R Core Team, 2015). Control substances with known zygosity status were used as training sets for the analysis script.

The locus-specific zygosity results derived from the real-time PCR were used as input data for segregation analysis using a Pearson's Chi-square analysis. The Chi-square analysis compares frequencies of observed and frequencies of expected (predicted) observations to calculate a Chi-square value. Depending on the degrees of freedom, this value is used to assign probabilities to accept or reject a hypothesis. The Chi-square formula is:

$$\chi^2 = \sum \frac{(\text{Observed Value} - \text{Expected Value})^2}{\text{Expected Value}}$$

This Chi-square analysis compared the observed segregation ratios of the two LBFLFK inserts to the ratios that are expected if segregation is in accordance with Mendelian inheritance segregation. The analysis was performed using the calculated Chi-square value function of Excel®[9] spreadsheet and data analysis software ("=CHISQ.TEST").

[9] Excel is a registered trademark of Microsoft Corporation.

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Appendix C. Characterization of the Newly Expressed Proteins in EPA+DHA Canola Event LBFLFK – Biochemistry, Materials, Methods, and Results

C.1. Background information on the function and biochemistry of the newly expressed fatty acid elongase and desaturase proteins

C.1.1. Fatty acid synthases and elongases

All organisms are capable of *de novo* synthesis of fatty acids (Nelson and Cox, 2017). This requires acetyl-CoA as a donor of two carbon (C2) units and involves seven different enzymatic activities for elongation by C2 units: acetyl-CoA carboxylase (ACC), acyltransferase (AT), ketoacyl synthase (KS), ketoacyl reductase (KR), hydroxyacyl dehydratase (DH), enoyl reductase (ER), and thioesterase (TE), as well as acyl carrier protein (ACP) to transfer the growing fatty acid between reaction centers. In plants, these reactions are catalyzed by a fatty acid synthase type II complex (FAS type II) that consists of individual soluble mono-functional proteins for each reaction (Ohlrogge and Browse, 1995; Harwood, 1996; Chen et al., 2011; Nelson and Cox, 2017). Plastids of plants, where *de novo* synthesis of fatty acids occurs, contain three different variants of the KS enzyme (beta-ketoacyl-ACP synthase, KAS). KAS enzymes play a key role in determining fatty acid chain length (Cui et al., 2016): elongation of C2 to C4 utilizes KAS III, elongation of C4 to C16 utilizes KAS I, and elongation of C16 to C18 utilizes KAS II (Harwood, 2005).

Fatty acid elongation, extending existing C18 fatty acids by C2 units, requires a four-step reaction cycle that includes KS, KR, DH, and ER activities analogous to the corresponding reaction cycle in *de novo* fatty acid synthesis (Leonard et al., 2004; Jakobsson et al., 2006; Haslam and Kunst, 2013). In contrast to *de novo* fatty acid synthesis, the elongation enzymes are membrane bound, localized in the endoplasmic reticulum, and the acyl chain substrate is bound to CoA instead of ACP (Leonard et al., 2004). The term elongase is context-dependent and can refer to the entire complex, including all four activities, or just the KS component that catalyzes the condensation of a C2 donor to an existing acyl chain acceptor.

Ketoacyl synthases (KS) can be divided into five families (Cantu et al., 2011; Chen et al., 2011). Plants primarily utilize KS components that belong to the KS2-family (FAE-type, Fatty Acid Elongation) for synthesis of waxes, pollen coats, sphingolipids, and suberins but also possess members of KS5-family (ELO-type, Elongation) (Leonard et al., 2004; Haslam and Kunst, 2013). While members of the FAE-type and ELO-type families catalyze similar chemical reactions, their secondary structure is different. ELO-type KS typically have five to seven predicted transmembrane-spanning helices (depending on the algorithm used). FAE-type KS have just one or two predicted transmembrane-spanning helices that serve as a membrane anchor (Leonard et al., 2004; Haslam et al., 2013). Members of the ELO-type KS family have a number of characteristic motifs, such as the highly conserved HXXHH histidine box embedded in the fourth membrane helix (Leonard

et al., 2004) that is critical for enzymatic activity (Denic and Weissman, 2007; Hernandez-Buquer and Blacklock, 2013), as well as KXX(E/D)XXDT, HXXMYXYY, and TXXQXXQ motifs. Experimental evidence suggests that all of these motifs are localized on the cytosolic face of the membrane and are organized in a catalytic ring surrounding the entrance to the substrate binding pocket (Denic and Weissman, 2007). ELO-type KS also differ from other KS proteins in that they do not have an identifiable Cys, His, His/Asn- catalytic triad (Heath and Rock, 2002; Paul et al., 2006; Denic and Weissman, 2007). In fact, the absence of this conserved cysteine renders ELO-type KS enzymes resistant to cerulenin, an antibiotic that targets the cysteine of the catalytic triad of other KS enzymes (Paul et al., 2006; Denic and Weissman, 2007).

Similar to the fatty acid synthase complex (Harwood, 2005; Cui et al., 2016), the KS component of the elongation system was shown to be critical for determining substrate specificity (Denic and Weissman, 2007). The enzymes catalyzing the remaining three steps of the elongation cycle appear to have a broad substrate tolerance, which was demonstrated in both yeast and plants (Millar and Kunst, 1997). Despite belonging to different protein families, the ELO-type and FAE-type KS proteins appear to be interchangeable components of the elongation complex (Paul et al., 2006; Haslam and Kunst, 2013). Therefore, the chain length specificity of an elongation complex can be manipulated by substituting only the ELO or FAE-type components, without the need to substitute the remaining three components of the elongation complex.

All the elongase proteins newly expressed in EPA+DHA canola event LBFLFK display the structural features of an ELO-type KS: they are predicted to have five to seven transmembrane-spanning helices consistent with the currently accepted topology model for ELO-type KS and possess the four motifs (HXXHH, KXX(E/D)XXDT, HXXMYXYY, and TXXQXXQ) conserved among ELO-type KS. The three newly expressed elongases were each demonstrated to catalyze the transfer of C2 units from malonyl-CoA to their respective acyl-CoA substrate. Additional evidence that all the newly expressed elongases are ELO-type KS is the fact that these specific proteins are insensitive to an inhibitor (cerulenin) that inhibits all other KS protein superfamilies (Yilmaz et al., 2017).

C.1.2. Fatty acid desaturases

Fatty acid desaturases catalyze the abstraction of two hydrogen atoms from the hydrocarbon chain of a fatty acid to form a double bond in an unsaturated fatty acid. Desaturases have evolved independently at least twice (Shanklin and Somerville, 1991; Sperling et al., 2003): soluble acyl-ACP desaturases found in the stroma of plastids of plants and integral membrane desaturases found in prokaryotes and eukaryotes (Shanklin and Cahoon, 1998). The latter can be subdivided further into two families that may have evolved independently (Sperling et al., 2003): acyl-CoA desaturases and acyl-lipid desaturases. Members of these two families are localized in the endoplasmic reticulum in animals, plants, fungi, and yeast (Tocher et al., 1998; Sperling et al., 2003), the cytoplasmic membrane of some bacilli (Aguilar et al., 1998), plastids in plants (Ohlrogge and Browse, 1995; Sperling et al., 2003), and the thylakoid membrane in cyanobacteria (Los and Murata, 1998). Plants possess three different families of desaturases: soluble acyl-ACP desaturases in the stroma of plastids, prokaryotic type integral membrane acyl-lipid desaturases in the chloroplast membrane, and eukaryotic-type integral membrane acyl-lipid desaturases in the endoplasmic reticulum. The active site of the soluble ACP-desaturases contains two iron atoms that are essential for the recruitment and activation of molecular oxygen, which is then able to abstract a hydrogen from a methylene group in the acyl-chain (reviewed in Shanklin et al., 2009). The crystal structure of soluble desaturases established how the diiron center is coordinated by characteristic D/EXXH motifs (Lindqvist et al., 1996). Similar to the soluble ACP-desaturases, integral membrane desaturases also use a diiron center for the recruitment and activation of molecular oxygen (Shanklin et al., 1994). The recent crystallization of an integral membrane desaturase showed how three conserved histidine boxes (motifs) (Shanklin et al., 1994), along with an additional conserved histidine, participate in the coordination of the diiron center (Bai et al., 2015; Wang et al., 2015). The conserved histidine is located between the second and third histidine boxes at the C-terminal end of transmembrane helix four on the cytosolic side of the membrane.

Methyl-end desaturases introduce a double bond between an existing double bond and the methyl end of the acyl chain while front-end desaturases introduce a double bond between an existing double bond and the carboxy-end of the acyl chain (reviewed in Sperling et al., 2003). The above-mentioned histidine boxes differ between these two classes of enzymes. Methyl-end desaturases are characterized by the presence of $H(X)_{3-4}H$ (box 1), $H(X)_{2-3}HH$ (box 2), and $H(X)_{2-3}HH$ (box 3), and all eight conserved histidines are essential to the function of these enzymes (Shanklin et al., 1994). For front-end desaturases, box 3 is replaced by $Q(X)_2HH$, and the conserved glutamine is essential for the function of these enzymes (Sayanova et al., 2001). The currently accepted model for the topology of integral membrane desaturases is based on a

combination of hydropathy plots, prediction of transmembrane domains by a variety of algorithms, as well as experiments that established membrane-spanning domains missed by such bioinformatics analysis (Diaz et al., 2002; Sperling et al., 2003; Meesapyodsuk et al., 2007) and was recently confirmed by the crystal structure of such integral membrane desaturases (Bai et al., 2015; Wang et al., 2015). In this model, histidine box 1, located on the cytoplasmic side of the membrane, is preceded by two transmembrane domains, followed by the histidine box 2 on the cytoplasmic face, followed by two further transmembrane-spanning domains that allow for the location of the conserved histidine (right after transmembrane helix 4) and of histidine box 3 on the cytoplasmic face. Notably, often bioinformatic analysis predicts three membrane-spanning helices between histidine box 2 and histidine box 3, which conflicts with experimental analysis (Diaz et al., 2002) and the crystal structure (Bai et al., 2015; Wang et al., 2015). An odd number of transmembrane domains between two histidine boxes sterically precludes both histidine boxes from participation in the coordination of the diiron. Therefore, one of the three predicted hydrophobic domains between histidine box 2 and histidine box 3 is associated with the cytosolic face of the membrane and not spanning the membrane.

All classes of desaturases need a second electron donor besides the substrate fatty acid ester (or thioester) in order to complete the reduction of molecular oxygen to water, releasing one water molecule for each introduced double bond while reactivating the catalytic diiron center for the next catalytic cycle (Buist, 2004). Soluble acyl-ACP desaturases, as well as prokaryotic type acyl-lipid desaturases found in cyanobacteria and plastids of plants, use ferredoxin, ferredoxin reductase, and dihydronicotinamide-adenine dinucleotide phosphate (NADPH). In contrast, eukaryotic type desaturases found in the endoplasmic reticulum use cytochrome b_5, cytochrome b_5 reductase, and primarily nicotinamide adenine dinucleotide (NADH) (Sperling and Heinz, 2001; Meesapyodsuk and Qiu, 2012). Eukaryotic-type desaturases interact with soluble cytochrome b_5 in the case of methyl-end desaturases whereas front-end desaturases possess an N-terminally fused cytochrome b_5 domain recognizable by a characteristic HPGG motif (Sperling and Heinz, 2001).

All the desaturases introduced into EPA+DHA canola event LBFLFK are predicted to have transmembrane-spanning helices consistent with the current accepted topology model for integral membrane desaturases and possess the three histidine boxes conserved among all integral membrane desaturases. All four newly expressed front-end desaturases, D6D(*Ot*), D5D(*Tc*), D4D(*Tc*) and D4D(*Pl*), contain an N-terminal cytochrome b_5 domain as well as the conserved glutamine in histidine box 3 while all three newly expressed methyl-end desaturases, D12D(*Ps*), O3D(*Pir*), and O3D(*Pi*), lack a fused N-terminal cytochrome b_5 domain and have the expected histidine instead of glutamine in histidine box 3. Each of these seven desaturases was shown to abstract two hydrogen atoms from the hydrocarbon chain of their respective fatty acid substrate, forming the expected unsaturated fatty acid product (Yilmaz et al., 2017).

C.1.3. Substrate preferences for the newly expressed elongase and desaturase proteins

The substrate specificity of each of the three elongases and seven desaturases introduced into EPA+DHA canola event LBFLFK has been assessed using yeast strains expressing each of the individual proteins (Yilmaz et al., 2017). In this publication, *in vivo* feeding studies were performed with various fatty acid intermediates in the introduced fatty acid pathway to allow an assessment of specificity of each expressed enzyme. Additionally, using membranes isolated from these yeast expression strains, *in vitro* assays were used by Yilmaz et al. (2017) to further assess backbone specificity (i.e. lipid-linked or Coenzyme A-linked fatty acids) of the proteins.

Results reported by Yilmaz et al. (2017) are consistent with the general characteristics described for fatty acid synthases and elongases (Appendix C.1.1) and fatty acid desaturases (Appendix C.1.2). The reported *in vitro* assays were the basis for the enzymatic assays using PPP preparations to characterize the introduced proteins in LBFLFK.

C.2. Materials used for characterization of the newly expressed proteins

Protein characterization experiments for all newly expressed proteins in EPA+DHA canola event LBFLFK used preparations of proteins from the plants as test substances. Briefly, LBFLFK as well as the parental control canola variety Kumily were grown in the field, and plant-produced proteins (PPP) were isolated from LBFLFK and Kumily immature seeds in a stepwise process. For characterization assessments of AHAS(*At*) [A122TS653N], both PPP and leaf extracts were used.

Generation of reference substances for protein characterization studies was accomplished through expression and purification of proteins in *Escherichia coli*. The cytosolic soluble AHAS(*At*) [A122TS653N] could be produced and purified in sufficient quantities upon expression in *E. coli*. However, large-scale production of the 10 integral membrane desaturases and elongases using heterologous systems failed to yield significant quantities of full-length proteins, except for D6D(*Ot*) and D5D(*Tc*). The challenges in successfully expressing these proteins likely results from the overall hydrophobicity of the proteins that contain multiple membrane-spanning domains (Bill et al., 2011; Bushey et al., 2014). To this end, NC fusion proteins, containing varying lengths of the N-terminus and C-terminus portions of a particular protein to remove internal hydrophobic stretches, were prepared for expression in *E. coli*. All such NC fusion proteins were found to have significantly increased expression levels compared to the full-length proteins. Therefore, NC fusion proteins were used as reference standards in the experiments described herein when full-length proteins were not available in sufficient quantities.

C.3. Methods

C.3.1. Production of seed and preparation of plant-produced proteins (PPP)

Immature seeds of LBFLFK and Kumily were broken by pressing between two glass plates, with the developing embryos then separated from seed coats and placed into 100 mM potassium phosphate, pH 7.2. Isolated embryos were collected from the trap by pouring over an 80-micron mesh-covered strainer. Embryos were continuously ground into buffer EBC (100 mM potassium phosphate, pH 7.2 containing 0.33 M sucrose, 1 mg/ml BSA, 4000 units/ml catalase, 4 mM NADH, and protease inhibitors until a homogenous solution was achieved, which was then filtered through pre-wetted Miracloth. The flow-through was collected in centrifuge tubes. Following centrifugation at 18,000 x *g* for 10 minutes at 4°C, the resulting supernatant (cell-free extract) was again filtered through Miracloth. To produce additional cell-free extract, the resulting insoluble pellet was re-extracted by grinding and centrifuged again. The combined cell-free extracts were subjected to 108,000 x *g* ultracentrifugation for 2 hours at 4°C under vacuum. The supernatant was removed and discarded, and the resulting PPP pellet was rinsed with buffer EBC to remove loose particles without disturbing the packed pellet. PPP pellets were combined, homogenized, aliquoted, and stored at -80°C until needed. In total, about 102 g of Kumily PPP and 136 g of LBFLFK PPP were isolated from about 390,000 (3.1 kg) immature Kumily seeds and 450,000 (3.6 kg) immature LBFLFK seeds, respectively.

C.3.2. Immunopurification of AHAS(*At*) [A122TS653N] protein from plant-produced proteins

To immunopurify AHAS [A122TS653N] from the LBFLFK PPP, 11 ml of PPP (0.38 g PPP/ml) was thawed on ice, and extraction buffer (1X PBS, 1% TritonX-100, and 1X protease inhibitor) was added at a ratio of 10:1 and gently mixed. This preparation was then centrifuged at 21,000 x *g* for 15 minutes at 4°C, and the resulting cleared lysate was used as starting material for immunopurification. The cleared lysate was incubated with an immunopurification resin containing a monoclonal antibody overnight, and then the mixture was loaded onto a gravity flow column. The flow-through was collected, and the column was washed before the AHAS protein was eluted in 1–1.5 ml fractions with an elution buffer (0.1 M glycine, pH 2.5, 0.15 M NaCl). Fractions were concentrated and stored at -80°C.

C.3.3. Preparation of AHAS(*At*) [A122TS653N] protein from leaf

Leaf extracts of LBFLFK and Kumily were prepared by grinding sample leaves in liquid nitrogen. A buffer (containing 100 mM potassium phosphate, pH 7.2, 0.2 M sodium pyruvate, 10 mM $MgCl_2$, ~2 mM TPP, and ~57 µM FAD) was added to make a suspension prior to centrifugation at 10,000 x *g* for 15 min. The supernatant was subjected to ammonium sulfate precipitation followed by centrifugation at 5000 x *g* at 4°C for 15 min. The resulting pellet was resuspended to make the final leaf extract. The total protein concentration of the leaf extract was determined by bicinchoninic acid assay (BCA).

C.3.4. Apparent molecular weight determination and immunoreactivity

PPP samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting to a polyvinylidene fluoride (PVDF) membrane. In brief, samples of Kumily PPP were diluted to 2 mg/ml, and LBFLFK PPP samples were diluted to 1 mg/ml and 2 mg/ml with dilution buffer (1X PBS with protease inhibitors) and mixed with 2X Laemmli buffer with beta-mercaptoethanol (BME). Each reference standard for each newly expressed protein was diluted and mixed with 2X Laemmli buffer. The LBFLFK and Kumily PPP were incubated at 40°C for 30 minutes along with the reference standards for D4D(*Tc*), O3D(*Pir*), D6E(*Tp*), and D5D(*Tc*). The reference standard proteins representing D12D(*Ps*), D6E(*Pp*), D4D(*Pl*), O3D(*Pi*), D5E(*Ot*), D6D(*Ot*), and AHAS(*At*) [A122TS653N] were incubated at 95°C for 10 minutes. Protein samples were separated via electrophoresis and transferred to a PVDF membrane via electrotransfer. Membranes were blocked overnight and then probed with a protein-specific polyclonal or monoclonal antibody. After washes, the membrane was probed with horseradish peroxidase (HRP) conjugated to either anti-goat, anti-rabbit, or anti-mouse IgG at a dilution of 1:10,000, depending on the primary antibody used. After washes, immunoreactive chemiluminescent bands were imaged on the membrane. Molecular weights were determined by comparing the band of interest to the log of the standard curve molecular weights generated vs the R_f value.

C.3.5. Determination of the enzymatic activity of desaturases and elongases

Fatty acid desaturase and elongase enzyme activity was demonstrated by incubating both LBFLFK and Kumily PPP in a solution containing the appropriate substrate and buffer as presented in Table C.1. After incubation, the sample was quenched by the addition of 2 M KOH in a MeOH:H_2O solution (1:4 v:v) and heated at ~90°C for 20 minutes. The sample was then neutralized by the addition of 3 M HCl, and fatty acids were extracted with a MeOH:$CHCl_3$ solution (2:1 v:v) and then with $CHCl_3$. The sample was briefly vortexed, and the chloroform phase (containing fatty acids, bottom layer) was recovered and transferred to a glass vial where it was dried under nitrogen gas. The fatty acids were methylated by re-suspending the dried sample with MeOH containing 2% H_2SO_4 and heating at 90°C for 30 minutes. The fatty acid methyl esters (FAMEs) were extracted through the addition of water and hexane. The sample was vortexed briefly, and the hexane phase (upper layer) was extracted and dried under nitrogen gas then re-suspended in chloroform. In each of the re-suspended samples, radioactivity was assessed using a liquid scintillation counter. The resulting FAMEs from the D12D(*Ps*) assay were separated using a 10% $AgNO_3$ TLC plate with a 1:1 ratio of toluene/heptane solvent as the mobile phase. All other FAMEs were separated with a reverse phase thin-layer chromatography (RP-TLC) plate using 100% acetonitrile solvent as the mobile phase. The TLC plates were visualized on a phosphorimager. The Kumily PPP served as a negative control for all desaturase and elongase enzyme assays. Membrane fractions from yeast (*Saccharomyces cerevisiae*) producing D6D(*Ot*) and D5D(*Tc*) proteins were

used as positive controls for enzyme assays. LBFLFK PPP samples were assayed in duplicate.

Table C.1. Enzyme Activity Substrates and Buffers

Enzyme	Substrate	Vendor	Catalog Number	PPP Amount (µg)
D12D(*Ps*), D12D(*Ps*) [F83L]	[^{14}C]-18:1n-9-CoA	Perkin Elmer	NEC651010UC	20
D6D(*Ot*)	[^{14}C]-18:2n-6-CoA	American Radiolabeled Chemicals Inc./ScanBiRes	ARC 1195	50
D6E(*Tp*) D6E(*Pp*)	C18:3n-6-CoA	Avanti Polar Lipids	870733P	320
	[^{14}C]-malonyl-CoA	Perkin Elmer	NEC612005UC	
D5D(*Tc*)	[^{14}C]-20:3n-6-CoA	American Radiolabeled Chemicals Inc./ScanBiRes	ARC 1596	50
O3D(*Pir*) O3D(*Pi*)	[^{14}C]-20:4n-6-CoA	Moravek Biochemicals	MC 459	100
D5E(*Ot*)	C20:5n-3-CoA	Avanti Polar Lipids	870744P	320
	[^{14}C]-malonyl-CoA	Perkin Elmer	NEC612005UC	
D4D(*Pl*), D4D(*Pl*) [A102S], D4D(*Tc*)	[^{14}C]-22:5n-3-CoA	ScanBiRes	160404-01	40
Desaturase Assay Buffer - 100 mM potassium phosphate at pH 7.2, 330 mM sucrose, 4 mM NADH, 1 mg/ml BSA, and 1X protease inhibitors For D12D(*Ps*), O3D(*Pi*), O3D(*Pir*), D4D(*Pl*), and D4D(*Tc*), include 100 µM 16:0-lysophosphatidylcholine				
Elongase Assay Buffer - 50 mM HEPES at pH 6.8, 1 mM NADPH, 0.1 mM cerulenin, 2 mM $MgCl_2$, and 1X protease inhibitors				

C.3.6. Determination of AHAS(*At*) [A122TS653N] enzymatic activity

C.3.6.1. AHAS enzyme activity in PPP

AHAS enzyme activity in PPP was demonstrated (data not shown) by incubating PPP (both LBFLFK and Kumily preparations) in a solution containing 50 mM potassium phosphate, pH 7.2, 0.1 M sodium pyruvate, 5 mM $MgCl_2$, ~1 mM TPP, and ~29 µM FAD. The AHAS enzyme inhibitor imazamox was dissolved with dimethyl sulfoxide (DMSO) and diluted into enzyme activity buffer to make imazamox concentrations of 0.5, 1.0, 10, 25, 50, and 100 µM prior to adding PPP. After incubation, the samples were quenched by the addition of 5% H_2SO_4 at ~60°C. The samples were then spun down at 17,200 x *g* for 30 minutes. The supernatant of each sample was then loaded into a 96-well plate along with acetoin standards ranging from 0.02–0.5 mM (diluted in enzyme activity buffer). To each well of standard and samples, a 1:1 ratio of 347 mM 1-naphthol (diluted in 4 N NaOH) and 38 mM creatine was added. The plate was heated at ~60°C for 15 minutes to allow for color formation. The plate was read at 530 nm using a plate reader at room temperature.

Kumily PPP was used as a negative control for the AHAS enzyme activity assay inhibition assays. All assays were run in triplicate.

C.3.6.2. AHAS enzyme activity in leaf extracts

Total protein of leaf extract was also used for an AHAS activity assay. AHAS enzyme activity was demonstrated by incubating leaf extract at 37°C in assay buffer containing 50 mM potassium phosphate, pH 7.2, 0.1 M sodium pyruvate, 5 mM $MgCl_2$, ~1 mM TPP, and ~29 µM FAD. Before incubation, negative control samples were pre-quenched by the addition of 5% H_2SO_4. After incubation, the test samples were quenched by the addition of 5% H_2SO_4 and heating at ~60°C for 15 minutes. Acetoin standards were then loaded to the plate ranging from 0.02–0.5 mM. To each well, a mixture of 347 mM 1-naphthol (diluted in 4 N NaOH) and 38 mM creatine was added. The plate was heated at ~60°C for 15 minutes to allow for color formation. The plate was measured at 530 nm using a plate reader at room temperature.

C.3.6.3. AHAS imazamox inhibition and feedback inhibition in leaf extracts

For the analyses of inhibition, leaf extracts were used. For the imazamox inhibition study, imazamox was dissolved with DMSO and then water (1% DMSO final concentration) to create final imazamox concentrations of 1, 2, 5, 10, 20, 50, and 100 µM after adding leaf extract. For the feedback inhibition assay, the AHAS protein feedback regulation inhibitors leucine and valine were dissolved with water to create final Leu/Val concentrations of 5, 10, 20, 50, 100, 200, 500, 1000 µM after adding leaf extract. Leaf extract of LBFLFK and Kumily were analyzed following the same procedure as for the enzymatic assay above. All the samples in enzyme activity and inhibition studies were triplicated, and the final results were averaged.

C.4. Individual protein characterization summaries

C.4.1. Delta-12 desaturase (*Ps*)

C.4.1.1. Structure and function

The delta-12 desaturase (*Ps*) protein D12D(*Ps*), newly expressed in EPA+DHA canola event LBFLFK, is encoded by a coding sequence isolated from the oomycete *Phytophthora sojae*. The deduced D12D(*Ps*) protein consists of 398 amino acids with a calculated molecular mass of 45.6 kDa. The D12D(*Ps*) amino acid sequence, as depicted in Figure C.1, shows the features that are characteristic for a methyl-end integral membrane desaturase. Depicted are the predicted transmembrane helices, three histidine boxes containing eight conserved histidine residues, and the conserved histidine following the C-terminal transmembrane helix.

LBFLFK contains two transfer DNA (T-DNA) inserts that encode the D12D(*Ps*) protein. The two D12D(*Ps*) coding sequences differ by a single nucleotide resulting in a deduced protein that has a [F83L] substitution, referred to as D12D(*Ps*) [F83L], that is predicted to

reside in the first transmembrane-spanning domain of the desaturase (Figure C.1). The D12D(*Ps*) [F83L] protein also consists of 398 amino acids but has a calculated molecular mass of 45.5 kDa. *In vivo* experiments in yeast showed that both proteins, D12D(*Ps*) and D12D(*Ps*) [F83L], catalyze the desaturation reaction required to convert C18:1n-9 into C18:2n-6 (Yilmaz et al., 2017) and have similar conversion efficiencies.

Figure C.1. Deduced Amino Acid Sequence for Delta-12 Desaturase (*Ps*) in EPA+DHA Canola Event LBFLFK

Using the one letter amino acid code for the D12D(*Ps*) protein in event LBFLFK, the position of predicted transmembrane helices and other characteristic protein motifs are indicated. ▒, ▒,= indicates transmembrane helices as predicted by SCAMPI2 (Peters et al., 2016), TMHMM v.2.0 (Krogh et al., 2001), and SOSUI v 1.11 (Hirokawa et al., 1998), respectively. The sequence contains three histidine-box motifs (▢) with eight conserved histidine residues (**H**) typical for desaturases (Shanklin et al., 1994) and an additional conserved histidine (**H**), all of which participate in the coordination of the diiron active center (Bai et al., 2015; Wang et al., 2015). EPA+DHA canola event LBFLFK carries two T-DNA inserts that encode the D12D(*Ps*) protein, one with the amino acid sequence as shown below and one with the amino acid sequence carrying a phenylalanine to leucine substitution at position 83 (D12D(*Ps*) [F83L]), indicated by **F**.

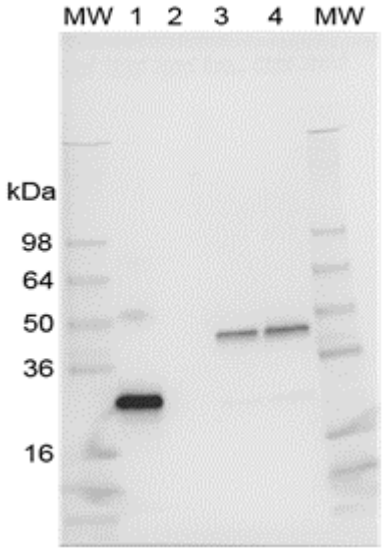
```
1   MAILNPEADS AANLATDSEA KQRQLAEAGY THVEGAPAPL PLELPHFSLR DLRAAIPKHC
61  FERSFVTSTY YMIKNVLTCA AL**F**YAATFID RAGAAAYVLW PVYWFFQGSY LTGVWVIA**H**E
          ========================     ========================
121 C**G**H**Q**AYCSSE VVNNLIGLVL HSALLVPYHS WRIS**H**RK**HH**S NTGSCENDEV FVPVTRSVLA
181 SSWNETLEDS PLYQLYRIVY MLVVGWMPGY LFFNATGPTK YWGKSRSHFN PYSAIYADRE
                ========================
241 RWMIVLSDIF LVAMLAVLAA LVHTFSFNTM VKFYVVPYFI VNAYLVLITY LQ**H**TDTYIPH
       ========================     ========================
301 FREGEWNWLR GALCTVDRSF GPFLDSVVHR IVDT**H**VC**HH**I FSKMPFYHCE EATNAIKPLL
361 GKFYLKDTTP VPVALWRSYT HCKFVEDDGK VVFYKNKL
```

C.4.1.2. Apparent molecular weight and immunoreactivity

Western blot analysis using an anti-D12D(*Ps*) protein antibody was used to show immunoreactivity of the D12D(*Ps*) and the D12D(*Ps*) [F83L] proteins in PPP isolated from LBFLFK (Figure C.2). To demonstrate the specificity of the antibody, a D12D(*Ps*) NC fusion reference protein was included as positive control (Figure C.2, lane 1). An immunoreactive band was observed in the LBFLFK PPP samples at a molecular weight that was in good agreement with the calculated molecular mass of the D12D(*Ps*) and D12D(Ps) [F83L] proteins of 45.6 kDa and 45.5 kDa, respectively (Figure C.2, lanes 3 and 4). No signal was detected in PPP isolated from Kumily, the parental control that does not contain the D12D(*Ps*) coding sequences (Figure C.2, lane 2).

Figure C.2. Western Blot Analysis of Delta-12 Desaturase (*Ps*) in EPA+DHA Canola Event LBFLFK

LBFLFK PPP and Kumily PPP were separated by SDS-PAGE and evaluated by western blot analysis. The membrane was probed with a 1:8,000 dilution of the D12D(*Ps*)-specific antibody and probed with a secondary HRP-conjugated antibody. Approximate molecular weight markers loaded in lanes labeled MW are shown on the left and right side of the blot. The calculated molecular mass of the D12D(*Ps*) and the D12D(*Ps*) [F83L] protein encoded by the coding sequences introduced in EPA+DHA canola event LBFLFK is 45.6 kDa and 45.5 kDa, respectively. The calculated molecular mass of the D12D(*Ps*) NC fusion protein used as a positive control for the D12D(*Ps*)-specific antibody is 21.5 kDa.



	Lane 1	Lane 2	Lane 3	Lane 4
Sample	D12D(*Ps*) NC fusion protein	Kumily PPP	LBFLFK PPP	LBFLFK PPP
Amount Loaded	32 ng	20 µg	10 µg	20 µg

C.4.1.3. Enzymatic activity

The functional activities of the newly expressed D12D(*Ps*) proteins (D12D(*Ps*) and D12D(*Ps*) [F83L]) in PPP isolated from LBFLFK were tested using an *in vitro* assay previously reported for the D12D(*Ps*) enzyme present in membranes isolated from yeast expression strains (Yilmaz et al., 2017). The D12D(*Ps*) was previously shown to desaturate C18:1n-9 to C18:2n-6 when the substrate fatty acid was covalently bound to a lipid, e.g., phosphatidylcholine (PC) (Yilmaz et al., 2017). Therefore, endogenous lysophosphatidylcholine acyl transferase (LPCAT) present in PPP was used to synthesize [^{14}C]-18:1n-9-PC *in situ* by transesterification of [^{14}C]-18:1n-9 from CoA to lysophosphatidylcholine (LPC) (Yilmaz et al., 2017). Specifically, in this assay, PPP (isolated from LBFLFK or Kumily) were incubated with [^{14}C]-18:1n-9-CoA, unlabeled C16:0-lysophosphatidylcholine, and NADH. The resulting fatty acid products were isolated, converted to FAMEs, resolved on a TLC plate, and identified by electronic autoradiography. In the reaction containing LBFLFK PPP (Figure C.3, panel B, lane B and C), a [^{14}C]-FAME was identified that migrates similar to the [^{14}C]-18:2n-6-ME standard (Figure C.3, panel B, lane E), in agreement with the expected product according to the reaction shown in panel A of Figure C.3. This product was not detected when the transfer of [^{14}C]-18:1n-9-CoA to LPC was prevented (Yilmaz et al., 2017). This product was also not detected in the reaction containing Kumily PPP (Figure C.3, panel B, lane A), which does not contain the D12D(*Ps*) coding sequence. Kumily PPP were expected to contain the endogenous *Brassica napus* endoplasmic reticulum D12D (Lee et al., 2013). The absence of [^{14}C]-18:2n-6 product in the reaction containing Kumily PPP indicates a significantly lower endogenous D12D activity relative to the newly expressed D12D(*Ps*).

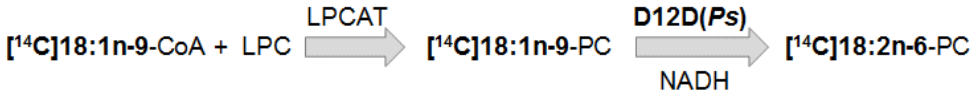
The substrate specificity and activity of the D12D(*Ps*) [F83L] protein, as determined by *in vivo* fatty acid feeding experiments in yeast, was comparable to reported values for the D12D(*Ps*) protein (Yilmaz et al., 2017). Therefore, the results obtained from the presented *in vitro* assays demonstrate the enzymatic activity of the D12D(*Ps*) and/or D12D(*Ps*) [F83L] proteins present in the LBFLFK PPP that is consistent with the previously proposed enzymatic reaction shown in panel A of Figure C.3.

Figure C.3. *In Vitro* Enzymatic Activity of Delta-12 Desaturases (*Ps*) in EPA+DHA Canola Event LBFLFK

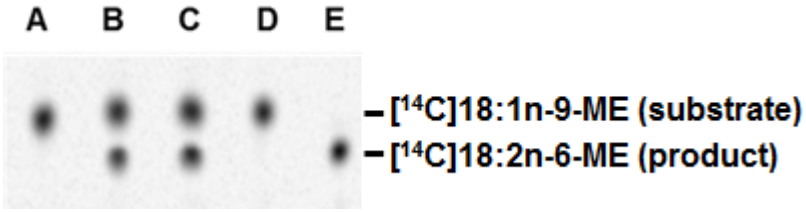
Panel A. D12D(*Ps*) desaturates the fatty acid substrate C18:1n-9 to the fatty acid product C18:2n-6 when the substrate is covalently bound to a lipid, e.g., PC (Yilmaz et al., 2017). In the enzymatic assay, endogenous lysophosphatidylcholine acyl transferase (LPCAT) is used to synthesize [^{14}C]-18:1n-9-PC by transferring [^{14}C]-18:1n-9 from CoA to lysophosphatidylcholine (LPC).

Panel B. PPP (20 µg total protein) were incubated in a buffer solution with [^{14}C]-18:1n-9-CoA substrate, unlabeled C16:0-lysophosphatidylcholine, and NADH. Total lipids were extracted, converted to FAMEs, and separated using a 10% $AgNO_3$ TLC plate with a 1:1 ratio of toluene/heptane solvent as the mobile phase. Shown below is an autoradiographic image of the TLC plate of the resolved [^{14}C]-methyl esters (ME). Kumily PPP were used as a control. Lane A, Kumily PPP (control); lanes B and C, LBFLFK PPP loaded in duplicate; lane D, [^{14}C]-18:1n-9-ME standard (substrate); lane E, [^{14}C]-18:2n-6-ME standard (product).

A.



B.



C.4.2. Delta-6 desaturase (*Ot*)

C.4.2.1. Structure and function

The delta-6 desaturase (*Ot*) protein D6D(*Ot*), newly expressed in EPA+DHA canola event LBFLFK, is encoded by a coding sequence isolated from the marine green microalga *Ostreococcus tauri*. The deduced D6D(*Ot*) protein consists of 456 amino acids with a calculated molecular mass of 51.7 kDa. The D6D(*Ot*) amino acid sequence, as depicted in Figure C.4, shows the features that are characteristic for a front-end integral membrane desaturase. Depicted are the N-terminally fused cytochrome b_5 domain containing the characteristic HPGG motif, the predicted transmembrane helices, three histidine boxes containing seven conserved histidine residues, a conserved glutamine in histidine box 3, and the conserved histidine following the C-terminal transmembrane helix. *In vivo* experiments in yeast showed that this D6D(*Ot*) protein catalyzes the desaturation reaction required to convert C18:2n-6 into C18:3n-6 (Domergue et al., 2005; Yilmaz et al., 2017).

Figure C.4. Deduced Amino Acid Sequence for Delta-6 Desaturase (*Ot*) in EPA+DHA Canola Event LBFLFK

Using the one letter amino acid code for the D6D(*Ot*) protein in event LBFLFK, the position of predicted transmembrane helices and other characteristic protein motifs are indicated. ▒,▒,= indicates transmembrane helices as predicted by SCAMPI2 (Peters et al., 2016), TMHMM v.2.0 (Krogh et al., 2001), and SOSUI v 1.11 (Hirokawa et al., 1998). The sequence contains three histidine-box motifs (▯) with seven conserved histidine residues (H) typical for desaturases (Shanklin et al., 1994) and an additional conserved histidine (H), all of which participate in the coordination of the diiron active center (Bai et al., 2015; Wang et al., 2015). Additionally, the sequence contains a conserved glutamine (Q) in the third histidine box that is essential for activity (Sayanova et al., 2001). Underlined is the cytochrome b_5 domain as annotated by PFAM v31 (Finn et al., 2016), containing the characteristic HPGG motif (Sperling and Heinz, 2001) highlighted in grey.

```
1   MCVETENNDG IPTVEIAFDG ERERAEANVK LSAEKMEPAA LAKTFARRYV VIEGVEYDVT
61  DFKHPGGTVI FYALSNTGAD ATEAFKEFHH RSRKARKALA ALPSRPAKTA KVDDAEMLQD
121 FAKWRKELER DGFFKPSPAH VAYRFAELAA MYALGTYLMY ARYVVSSVLV YACFFGARCG
                                                  ====================
181 WVQHEGGHSS LTGNIWWDKR IQAFTAGFGL AGSGDMWNSM HNKHHATPQK VRHDMDLDTT
    ==
241 PAVAFFNTAV EDNRPRGFSK YWLRLQAWTF IPVTSGLVLL FWMFFLHPSK ALKGGKYEEL
301 VWMLAAHVIR TWTIKAVTGF TAMQSYGLFL ATSWVSGCYL FAHFSTSHTH LDVVPADEHL
361 SWVRYAVDHT IDIDPSQGWV NWLMGYLNCQ VIHHLFPSMP QFRQPEVSRR FVAFAKKWNL
421 NYKVMTYAGA WKATLGNLDN VGKHYYVHGQ HSGKTA
```

C.4.2.2. Apparent molecular weight and immunoreactivity

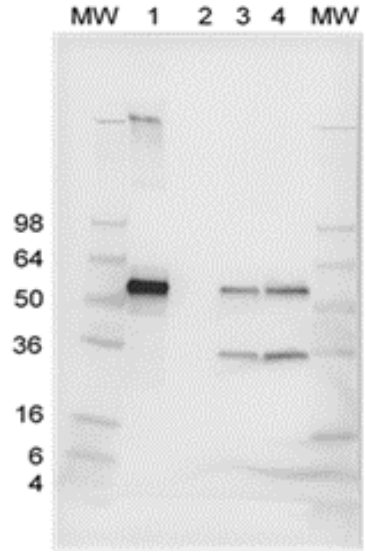
Western blot analysis using an anti-D6D(*Ot*) protein antibody was used to show immunoreactivity of the D6D(*Ot*) protein in PPP isolated from LBFLFK (Figure C.5). To demonstrate the specificity of the antibody, a D6D(*Ot*) full-length reference protein was included as positive control (Figure C.5, lane 1). An immunoreactive band was observed in the LBFLFK PPP samples at a molecular weight that was in good agreement with the calculated molecular mass of the D6D(*Ot*) protein of 51.7 kDa (Figure C.5, lanes 3 and 4). No signal was detected in PPP isolated from Kumily, the parental control that does not contain the D6D(*Ot*) coding sequence (Figure C.5, lane 2). Another immunoreactive band was also observed at ~36 kDa in the LBFLFK PPP (Figure C.5, lanes 3 and 4) that was not observed in Kumily PPP (Figure C.5, lane 2), likely the result of degradation of the D6D(*Ot*) protein in the PPP sample.

C.4.2.3. Enzymatic activity

The functional activity of the newly expressed D6D(*Ot*) enzyme in PPP isolated from LBFLFK was tested using an *in vitro* assay previously reported for the D6D(*Ot*) enzyme present in membranes isolated from yeast expression strains (Yilmaz et al., 2017). *In vivo* and *in vitro* experiments using yeast expression strains showed that the D6D(*Ot*) uses acyl-CoA substrates (e.g., C18:2n-6 bound to coenzyme A) but does not efficiently use phospholipid substrates (Domergue et al., 2005; Yilmaz et al., 2017). Therefore, in the assay, PPP (isolated from LBFLFK or Kumily) were incubated with [^{14}C]18:2n-6-CoA and NADH. The resulting fatty acid products were isolated, converted to FAMEs, resolved on a TLC plate, and identified by electronic autoradiography. In the reaction containing either Kumily or LBFLFK PPP (Figure C.6, panel B, lanes D–F), no [^{14}C]-FAME was identified that migrates similar to the [^{14}C]-18:3n-6-ME standard (Figure C.6, panel B, lane B), indicating the expected product according to the reaction shown in panel A of Figure C.6 was not formed. However, in the reaction containing membranes isolated from yeast expressing the D6D(*Ot*) protein, a [^{14}C]-FAME was identified that migrates similar to the [^{14}C]-18:3n-6-ME standard (Figure C.6, panel B, lanes C and B, respectively), indicating the assay was run appropriately to allow formation of the product by the D6D(*Ot*).

Figure C.5. Western Blot Analysis of Delta-6 Desaturase (*Ot*) in EPA+DHA Canola Event LBFLFK

LBFLFK PPP and Kumily PPP were separated by SDS-PAGE and evaluated by western blot analysis. The membrane was probed with a 1:4000 dilution of the D6D(*Ot*)-specific antibody and probed with a secondary HRP-conjugated antibody. Approximate molecular weight markers loaded in lanes MW are shown on the left and right side of the blot. The calculated molecular mass of the D6D(*Ot*) protein from the coding sequence introduced in EPA+DHA canola event LBFLFK is 51.7 kDa. The calculated molecular mass of the D6D(*Ot*) full-length reference standard protein used as a positive control for the D6D(*Ot*)-specific antibody is 51.7 kDa.



	Lane 1	Lane 2	Lane 3	Lane 4
Sample	D6D(*Ot*) full length protein	Kumily PPP	LBFLFK PPP	LBFLFK PPP
Amount Loaded	40 ng	20 µg	10 µg	20 µg

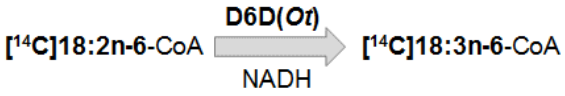
The presence of C18:3n-6 in LBFLFK seeds and the fact that, in LBFLFK, only the newly expressed D6D(*Ot*) enzyme exhibits the D6D activity required to convert C18:2n-6 into C18:3n-6 indicates D6D(*Ot*) is active in seed of LBFLFK. The difficulty in demonstrating *in vitro* activity of the D6D(*Ot*) may result from the central role of acyl-CoAs in lipid metabolism. Many competing enzyme activities rely on acyl-CoA substrates (Waku, 1992; Bates et al., 2009). The high flux of nascent acyl-CoAs into phospholipids via LPCAT was established (Bates et al., 2009) and enables the *in vitro* activity assay of phospholipid dependent desaturases (Yilmaz et al., 2017). In contrast, an assay of acyl-CoA dependent desaturases would be highly affected by the relative activity of enzymes competing for the same acyl-CoA substrate. It is likely that the relative activity of the acyl-CoA dependent D6D(*Ot*) desaturase compared to all other competing enzymes (such as LPCAT and thioesterases) is higher in yeast membranes compared to LBFLFK PPP. However, the D6D(*Ot*) has the intended *in vivo* activity in seeds of LBFLFK.

Figure C.6. *In Vitro* Enzymatic Activity of Delta-6 Desaturase (*Ot*) in EPA+DHA Canola Event LBFLFK

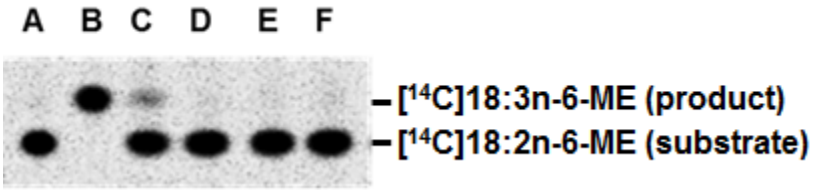
Panel A. D6D(*Ot*) desaturates the fatty acid substrate C18:2n-6 to the fatty acid product C18:3n-6 (Domergue et al., 2005; Yilmaz et al., 2017). *In vivo* and *in vitro* experiments indicate that the D6D(*Ot*) enzyme desaturates fatty acids covalently attached to coenzyme A while *in vitro* experiments further show that this enzyme does not efficiently desaturate fatty acids covalently bound to phosphatidylcholine (PC) (Domergue et al., 2005; Yilmaz et al., 2017).

Panel B. PPP (50 µg total protein) were incubated in a buffer solution with [^{14}C]-18:2n-6-CoA substrate and NADH. Total lipids were extracted, converted to FAMEs, and separated using a reverse phase thin layer chromatography plate with acetonitrile as the mobile phase. Shown below is an autoradiographic image of the TLC plate of the resolved [^{14}C]-methylesters (ME). Kumily PPP were used as a control. Lane A, C18:2n-6-ME substrate; lane B, C18:3n-6-ME product; lane C, yeast membranes containing D6D(*Ot*) (control); lane D, Kumily PPP (control); lanes E and F, LBFLFK PPP in duplicate.

A.



B.



C.4.3. Delta-6 elongase (*Tp*)

C.4.3.1. Structure and function

The delta-6 elongase (*Tp*) protein D6E(*Tp*), newly expressed in EPA+DHA canola event LBFLFK, is encoded by a coding sequence isolated from the marine diatom *Thalassiosira pseudonana*. The deduced D6E(*Tp*) protein consists of 272 amino acids with a calculated molecular mass of 31.8 kDa. The D6E(*Tp*) amino acid sequence, as depicted in Figure C.7, shows the features that are characteristic of ELO-type elongases. Depicted are the predicted transmembrane helices and all four of the signature ELO-motifs. *In vivo* experiments in yeast showed that this D6E(*Tp*) protein catalyzes the elongation reaction required to convert C18:3n-6 into C20:3n-6 (Meyer et al., 2004; Yilmaz et al., 2017).

Figure C.7. Deduced Amino Acid Sequence for Delta-6 Elongase (*Tp*) in EPA+DHA Canola Event LBFLFK

Using the one letter amino acid code for the D6E(*Tp*) protein in event LBFLFK, the position of predicted transmembrane helices and other characteristic protein motifs are indicated. ▒,▒,= indicates transmembrane helices as predicted by SCAMPI2 (Peters et al., 2016), TMHMM v.2.0 (Krogh et al., 2001), and SOSUI v 1.11 (Hirokawa et al., 1998). The sequence contains four motifs (▒) highly conserved among elongases (Leonard et al., 2004; Meyer et al., 2004; Jakobsson et al., 2006), including the characteristic HXXHH histidine box embedded in the fourth membrane helix that is critical for enzymatic activity (Denic and Weissman, 2007; Hernandez-Buquer and Blacklock, 2013). Highly conserved residues are highlighted in black.

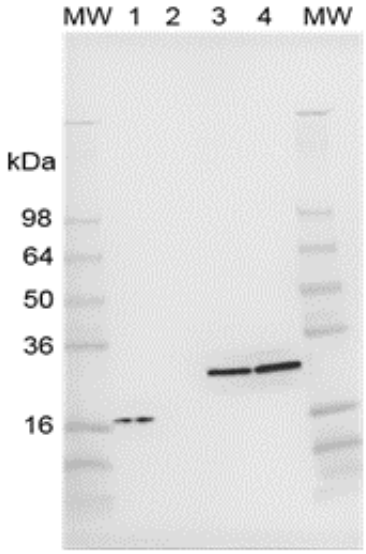
```
1   MDAYNAAMDK IGAAIIDWSD PDGKFRADRE DWWLCDFRSA ITIALIYIAF VILGSAVMQS
                                      ==========================
61  LPAMDPYPIK FLYNVSQIFL CAYMTVEAGF LAYRNGYTVM PCNHFNVNDP PVANLLWLFY
                ==========================           ==============
121 ISKVWDFWDT IFIVLGKKWR QLSFLHVYHH TTIFLFYWLN ANVLYDGDIF LTILLNGFIH
    ==========                                 ====================
181 TVMYTYYFIC MHTKDSKTGK SLPIWWKSSL TAFQLLQFTI MMSQATYLVF HGCDKVSLRI
    ====                       ========================          =
241 TIVYFVYILS LFFLFAQFFV QSYMAPKKKK SA
    ======================
```

C.4.3.2. Apparent molecular weight and immunoreactivity

Western blot analysis using an anti-D6E(*Tp*) protein antibody was used to show immunoreactivity of the D6E(*Tp*) protein in PPP isolated from LBFLFK (Figure C.8). To demonstrate the specificity of the antibody, a D6E(*Tp*) NC fusion reference protein was included as positive control (Figure C.8, lane 1). An immunoreactive band was observed in the LBFLFK PPP samples at a molecular weight that was in good agreement with the calculated molecular mass of the D6E(*Tp*) protein of 31.8 kDa (Figure C.8, lanes 3 and 4). No signal was detected in the PPP isolated from Kumily, the parental control that does not contain the D6E(*Tp*) coding sequence (Figure C.8, lane 2).

Figure C.8. Western Blot Analysis of Delta-6 Elongase (*Tp*) in EPA+DHA Canola Event LBFLFK

LBFLFK PPP and Kumily PPP were separated by SDS-PAGE and evaluated by western blot analysis. The membrane was probed with a 1:1,000 dilution of the D6E(*Tp*)-specific antibody and probed with a secondary HRP-conjugated antibody. Approximate molecular weight markers loaded in lanes MW are shown on the left and right side of the blot. The calculated molecular mass of the D6E(*Tp*) protein from the coding sequence introduced in EPA+DHA canola event LBFLFK is 31.8 kDa. The calculated molecular mass of the D6E(*Tp*) NC fusion protein used as a positive control for the D6E(*Tp*)-specific antibody is 16.6 kDa.



	Lane 1	Lane 2	Lane 3	Lane 4
Sample	D6E(*Tp*) NC fusion protein	Kumily PPP	LBFLFK PPP	LBFLFK PPP
Amount Loaded	30 ng	20 µg	10 µg	20 µg

C.4.3.3. Enzymatic activity

LBFLFK contains newly expressed D6E proteins from two different organisms: D6E(*Tp*) from *Thalassiosira pseudonana* (described in this section), and D6E(*Pp*) from *Physcomitrella patens* (described in section C.4.4). The functional activity assays for the D6Es from both organisms are identical and were demonstrated using membranes isolated from yeast expression strains (Yilmaz et al., 2017). Therefore, in the PPP isolated from LBFLFK, the presence of both D6E was tested using this *in vitro* assay. However, the relative contributions of the individual D6E to the total D6E activity cannot be determined. Specifically, PPP were incubated with the two D6E co-substrates, [^{14}C]-malonyl-CoA and unlabeled C18:3n-6-CoA, in the presence of NADPH. The resulting fatty acid products were isolated, converted to FAMEs, resolved on a TLC plate, and identified by electronic autoradiography. In the reaction containing LBFLFK PPP, a [^{14}C]-FAME was isolated (Figure C.9, panel B, lanes C and D) that migrates similar to the [^{14}C]-20:3n-6-ME standard (Figure C.9, panel B, lane A), in agreement with the expected product according to the reaction shown in panel A of Figure C.9. This product was not detected in the reaction containing Kumily PPP (Figure C.9, panel B, lane B), which does not contain the D6E(*Tp*) or D6E(*Pp*) coding sequences.

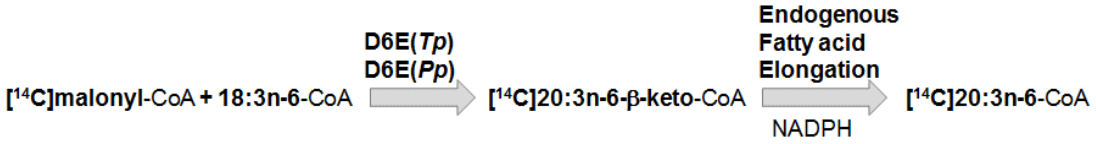
Together, the results obtained from these *in vitro* assays demonstrate D6E activity in the LBFLFK PPP that is not observed in Kumily PPP. The observed D6E activity is consistent with the previously proposed enzymatic reaction (Yilmaz et al., 2017) as shown in panel A of Figure C.9.

Figure C.9. *In Vitro* Enzymatic Activity of Delta-6 Elongase in EPA+DHA Canola Event LBFLFK

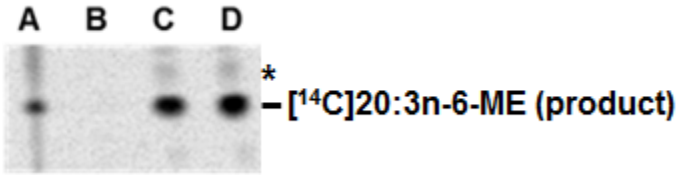
Panel A. D6E(*Tp*) and D6E(*Pp*) both catalyze the transfer of two carbons from [^{14}C]-malonyl-CoA to C18:3n-6-CoA, generating [^{14}C]-20:3n-6-β-keto-CoA, which in the presence of NADPH can be converted to [^{14}C]-20:3n-6-CoA by the endogenous canola elongation complex enzymes.

Panel B. PPP (320 µg total protein) were incubated in a buffer solution with [^{14}C]-malonyl-CoA, unlabeled C18:3n-6-CoA, and NADPH. Depicted is an autoradiographic image of a TLC plate showing separated [^{14}C]-methylesters (MEs) prepared from the total lipids that were extracted from the enzymatic reactions. The resulting FAMEs from the D6E assay were separated using a reverse phase thin layer chromatography plate with acetonitrile as the mobile phase. Lane A, [^{14}C]-20:3n-6-ME standard; lane B, Kumily PPP (control); lanes C and D, LBFLFK PPP in duplicate. The [^{14}C]-labeled malonyl-ME (derived from the substrate [^{14}C]-malonyl-CoA) does not get retained during the extraction process and is therefore not present on the TLC plate. The [^{14}C]-compound observed in lanes C and D that is denoted by an asterisk (*) is derived from the [^{14}C]20:3n-6-β-keto-CoA product during the isolation of the FAMEs (Yilmaz et al., 2017).

A.



B.



C.4.4. Delta-6 elongase (*Pp*)

C.4.4.1. Structure and function

The delta-6 elongase (*Pp*) protein D6E(*Pp*), newly expressed in EPA+DHA canola event LBFLFK, is encoded by a coding sequence isolated from the moss *Physcomitrella patens*. The deduced D6E(*Pp*) protein consists of 290 amino acids with a calculated molecular mass of 33.4 kDa. The D6E(*Pp*) amino acid sequence, as depicted in Figure C.10, shows the features that are characteristic of ELO-type elongases. Depicted are the predicted transmembrane helices and all four of the signature ELO-motifs. *In vivo* experiments in yeast showed that this D6E(*Pp*) protein catalyzes the elongation reaction required to convert C18:3n-6 into C20:3n-6 (Zank et al., 2000; Zank et al., 2002; Domergue et al., 2003; Yilmaz et al., 2017). Additionally, this D6E(*Pp*) was functional upon expression in the seeds of *Nicotiana tabacum*, *Linum usitatissimum*, and *Brassica juncea*, as shown by the successful synthesis of C20:4n-6 and C20:5n-3 in these plants (Abbadi et al., 2004; Wu et al., 2005).

Figure C.10. Deduced Amino Acid Sequence for Delta-6 Elongase (*Pp*) in EPA+DHA Canola Event LBFLFK

Using the one letter amino acid code for the D6E(*Pp*) protein in event LBFLFK, the position of predicted transmembrane helices and other characteristic protein motifs are indicated. ▒, ▒,= indicates transmembrane helices as predicted by SCAMPI2 (Peters et al., 2016), TMHMM v.2.0 (Krogh et al., 2001), and SOSUI v 1.11 (Hirokawa et al., 1998). The sequence contains four motifs (▒) highly conserved among elongases (Leonard et al., 2004; Meyer et al., 2004; Jakobsson et al., 2006), including the characteristic HXXHH histidine box embedded in the fourth membrane helix that is critical for enzymatic activity (Denic and Weissman, 2007; Hernandez-Buquer and Blacklock, 2013). Highly conserved residues are highlighted in black.

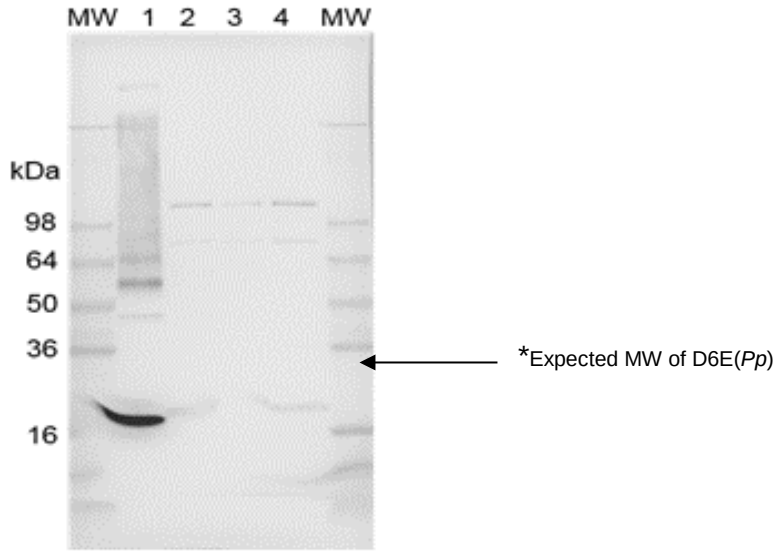
```
1   MEVVERFYGE LDGKVSQGVN ALLGSFGVEL TDTPTTKGLP LVDSPTPIVL GVSVYLTIVI
                                                  ================

61  GGLLWIKARD LKPRASEPFL LQALVLVHNL FCFALSLYMC VGIAYQAITW RYSLWGNAYN
    =======                ========================

121 PKHKEMAILV YLFYMSKYVE FMDTVIMILK RSTRQISFLH VYHHSSISLI WWAIAHHAPG
        ========================     ========================

181 GEAYWSAALN SGVHVLMYAY YFLAACLRSS PKLKNKYLFW GRYLTQFQMF QFMLNLVQAY
          ========================

241 YDMKTNAPYP QWLIKILFYY MISLLFLFGN FYVQKYIKPS DGKQKGAKTE
                         ========================
```

C.4.4.2. Apparent molecular weight and immunoreactivity

Western blot analysis using an anti-D6E(*Pp*) protein antibody was used to show immunoreactivity of the D6E(*Pp*) protein in PPP isolated from LBFLFK (Figure C.11). To demonstrate the specificity of the antibody, a D6E(*Pp*) NC fusion reference protein was included as positive control (Figure C.11, lane 1). An immunoreactive band near the calculated mass of the D6E(*Pp*) protein was not observed in the LBFLFK PPP samples (Figure C.11, lanes 3 and 4). Likewise, no signal was detected in the PPP isolated from Kumily, the parental control that does not contain the *D6E*(*Pp*) coding sequence (Figure C.11, lane 2). Two non-specific bands were seen in both Kumily and LBFLFK PPP at ~68 kDa and 110 kDa.

Figure C.11. Western Blot Analysis of Delta-6 Elongase (*Pp*) in EPA+DHA Canola Event LBFLFK

LBFLFK PPP and Kumily PPP were separated by SDS-PAGE and evaluated by western blot analysis. The membrane was probed with a 1:90 dilution of the D6E(*Pp*)-specific antibody and probed with a secondary HRP-conjugated antibody. Approximate molecular weight markers loaded in lanes MW are shown on the left and right side of the blot. The calculated molecular mass of the D6E(*Pp*) protein from the coding sequence introduced in EPA+DHA canola event LBFLFK is 33.4 kDa. The calculated molecular mass of the D6E(*Pp*) NC fusion protein used as a positive control for the D6E(*Pp*)-specific antibody is 16.8 kDa.



	Lane 1	Lane 2	Lane 3	Lane 4
Sample	D6E(*Pp*) NC fusion protein	Kumily PPP	LBFLFK PPP	LBFLFK PPP
Amount Loaded	50 ng	20 µg	10 µg	20 µg

C.4.4.3. Enzymatic activity

LBFLFK contains newly expressed D6Es from two different organisms: D6E(*Tp*) from *Thalassiosira pseudonana* (previously described in section C.4.3), and D6E(*Pp*) from *Physcomitrella patens* (described in this section). The functional activity assays for the D6Es from both organisms are identical and were demonstrated using membranes isolated from yeast expression strains (Yilmaz et al., 2017). Therefore, in the PPP isolated from LBFLFK, the presence of either D6E protein was tested using this *in vitro* assay (Yilmaz et al., 2017). However, the relative contributions of the individual D6E proteins to the total D6E activity cannot be determined. The results described in section C.4.3.3 demonstrate D6E activity in the LBFLFK PPP. This D6E activity was not observed in Kumily PPP, which does not contain D6E(*Tp*) or D6E(*Pp*) coding sequences. The observed D6E activity is consistent with the previously proposed enzymatic reaction (Yilmaz et al., 2017) shown in panel A of Figure C.9 and could be attributed to either (or both) the D6E(*Tp*) protein or the D6E(*Pp*) protein.

C.4.5. Delta-5 desaturase (*Tc*)

C.4.5.1. Structure and function

The delta-5 desaturase (*Tc*) protein D5D(*Tc*), newly expressed in EPA+DHA canola event LBFLFK, is encoded by a coding sequence isolated from the marine protist *Thraustochytrium* sp. The deduced D5D(*Tc*) protein consists of 439 amino acids with a calculated molecular mass of 49.8 kDa. The D5D(*Tc*) amino acid sequence, as depicted in Figure C.12, shows the features that are characteristic for a front-end integral membrane desaturase. Depicted are the N-terminally fused cytochrome b_5 domain containing the characteristic HPGG motif, the predicted transmembrane helices, three histidine boxes containing seven conserved histidine residues, a conserved glutamine in histidine box 3, and the conserved histidine following the C-terminal transmembrane helix. *In vivo* experiments in yeast showed that this D5D(*Tc*) protein catalyzes the desaturation reaction required to convert C20:3n-6 into C20:4n-6 (Qiu et al., 2001; Yilmaz et al., 2017). Furthermore, this D5D(*Tc*) was functional upon expression in the seeds of *Brassica juncea*, as shown by the successful synthesis of C20:4n-6 and C20:5n-3 (Wu et al., 2005).

C.4.5.2. Apparent molecular weight and immunoreactivity

Western blot analysis using an anti-D5D(*Tc*) protein antibody was used to show immunoreactivity of the D5D(*Tc*) protein in PPP isolated from LBFLFK (Figure C.13). To demonstrate the specificity of the antibody, a D5D(*Tc*) full-length reference protein was included as positive control (Figure C.13, lane 1). An immunoreactive band was observed in the LBFLFK PPP samples at a molecular weight that was in good agreement with the calculated molecular mass of the D5D(*Tc*) protein of 49.8 kDa (Figure C.13, lanes 3 and 4). No signal was detected in PPP isolated from Kumily, the parental control that does not contain the D5D(*Tc*) coding sequence (Figure C.13, lane 2). The immunoreactive proteins observed at approximately 20 kDa in the LBFLFK PPP (Figure C.13, lanes 3 and 4) are not observed in Kumily PPP (Figure C.13, lane 2) and are likely the result of degradation of the D5D(*Tc*) protein.

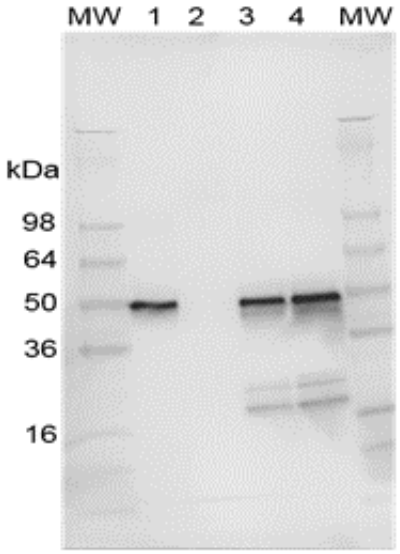
Figure C.12. Deduced Amino Acid Sequence for Delta-5 Desaturase (*Tc*) in EPA+DHA Canola Event LBFLFK

Using the one letter amino acid code for the D5D(*Tc*) protein in event LBFLFK, the position of predicted transmembrane helices and other characteristic protein motifs are indicated. ▒,▒,= indicates transmembrane helices as predicted by SCAMPI2 (Peters et al., 2016), TMHMM v.2.0 (Krogh et al., 2001), and SOSUI v 1.11 (Hirokawa et al., 1998). The sequence contains three histidine-box motifs (▒) with seven conserved histidine residues (H) typical for desaturases (Shanklin et al., 1994) and an additional conserved histidine (H), all of which participate in the coordination of the diiron active center (Bai et al., 2015; Wang et al., 2015). Additionally, the sequence contains a conserved glutamine (Q) in the third histidine box that is essential for activity (Sayanova et al., 2001). Underlined is the cytochrome b_5 domain as annotated by PFAM v31 (Finn et al., 2016) containing the characteristic HPGG motif (Sperling and Heinz, 2001) highlighted in grey.

```
1   MGKGSEGRSA AREMTAEANG DKRKTILIEG VLYDATNFKH PGGSIINFLT EGEAGVDATQ
61  AYREFHQRSG KADKYLKSLP KLDASKVESR FSAKEQARRD AMTRDYAAFR EELVAEGYFD
121 PSIPHMIYRV VEIVALFALS FWLMSKASPT SLVLGVVMNG IAQGRCGWVM HEMGHGSFTG
      ========================  ========================
181 VIWLDDRMCE FFYGVGCGMS GHYWKNQHSK HHAAPNRLEH DVDLNTLPLV AFNERVVRKV
241 KPGSLLALWL RVQAYLFAPV SCLLIGLGWT LYLHPRYMLR TKRHMEFVWI FARYIGWFSL
          ========================          ================
301 MGALGYSPGT SVGMYLCSFG LGCIYIFLQF AVSHTHLPVT NPEDQLHWLE YAADHTVNIS
    =======    ========================
361 TKSWLVTWWM SNLNFQIEHH LFPTAPQFRF KEISPRVEAL FKRHNLPYYD LPYTSAVSTT
421 FANLYSVGHS VGADTKKQD
```

Figure C.13. Western Blot Analysis of Delta-5 Desaturase (*Tc*) in EPA+DHA Canola Event LBFLFK

LBFLFK PPP and Kumily PPP were separated by SDS-PAGE and evaluated by western blot analysis. The membrane was probed with a 1:500 dilution of the D5D(*Tc*)-specific antibody and probed with a secondary HRP-conjugated antibody. Approximate molecular weight markers loaded in lanes MW are shown on the left and right side of the blot. The calculated molecular mass of the D5D(*Tc*) protein encoded by the coding sequence introduced in EPA+DHA canola event LBFLFK is 49.8 kDa. The calculated molecular mass of the D5D(*Tc*) full-length reference standard protein used as a positive control for the D5D(*Tc*)-specific antibody is 49.8 kDa.



	Lane 1	Lane 2	Lane 3	Lane 4
Sample	D5D(*Tc*) full-length protein	Kumily PPP	LBFLFK PPP	LBFLFK PPP
Amount Loaded	8 ng	20 µg	10 µg	20 µg

C.4.5.3. Enzymatic activity

The functional activity of the newly expressed D5D(*Tc*) enzyme in PPP isolated from LBFLFK was tested using an *in vitro* assay previously reported for the D5D(*Tc*) enzyme present in membranes isolated from yeast expression strains (Yilmaz et al., 2017). *In vivo* and *in vitro* experiments using yeast expression strains, as well as bioinformatic analysis, all indicate that the D5D(*Tc*) uses acyl-CoA substrates (e.g., C20:3n-6 bound to coenzyme A) (Li et al., 2016; Senger et al., 2016; Yilmaz et al., 2017). Therefore, in the assay, PPP (isolated from LBFLFK or Kumily) were incubated with [^{14}C]20:3n-6-CoA and NADH. The resulting fatty acid products were isolated, converted to FAMEs, resolved on a TLC plate, and identified by electronic autoradiography. In the reaction containing either Kumily or

LBFLFK PPP (Figure C.14, panel B, lanes D–F), no [^{14}C]-FAME was identified that migrates similar to the [^{14}C]-20:4n-6-ME standard (Figure C.14, panel B, lane B), indicating the expected product according to the reaction shown in panel A of Figure C.14 was not formed. However, in the reaction containing membranes isolated from yeast expressing the D5D(*Tc*) protein, a [^{14}C]-FAME was identified that migrates similar to the [^{14}C]-20:4n-6-ME standard, indicating the assay was run appropriately to allow formation of the product by the D5D(*Tc*) (Figure C.14, panel B, lanes C and B, respectively).

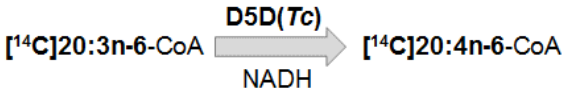
The presence of C20:4n-6 in LBFLFK seeds indicates D5D(*Tc*) is active in seed of LBFLFK. The difficulty in demonstrating *in vitro* activity of the D5D(*Tc*) may result from the central role of acyl-CoAs in lipid metabolism. Many competing enzyme activities rely on acyl-CoA substrates (Waku, 1992; Bates et al., 2009). The high flux of nascent acyl-CoAs into phospholipids via LPCAT was established (Bates et al., 2009) and enables the activity assay of phospholipid dependent desaturases (Yilmaz et al., 2017). In contrast, an assay of acyl-CoA dependent desaturases would be highly affected by the relative activity of enzymes competing for the same acyl-CoA substrate. It is likely the relative activity of the acyl-CoA dependent D6D(*Ot*) desaturase to all other competing enzymes (such as LPCAT and thioesterases) is higher in yeast membranes compared to LBFLFK PPP. However, the D5D(*Tc*) has the intended *in vivo* activity in seeds of LBFLFK.

Figure C.14. *In Vitro* Enzymatic Activity of Delta-5 Desaturase (*Tc*) in EPA+DHA Canola Event LBFLFK

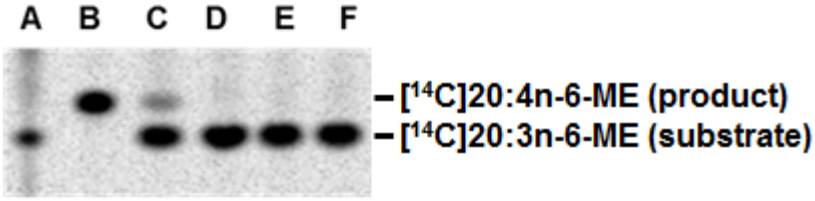
Panel A. D5D(*Tc*) desaturates the fatty acid substrate C20:3n-6 to the fatty acid product C20:4n-6 (Yilmaz et al., 2017). *In vivo* and *in vitro* experiments using yeast expression strains, as well as bioinformatic analysis, all indicate that the D5D(*Tc*) enzyme desaturates fatty acids covalently attached to coenzyme A (Li et al., 2016; Senger et al., 2016; Yilmaz et al., 2017).

Panel B. PPP (50 µg total protein) were incubated in a buffer solution with [^{14}C]-labeled C20:3n-6-CoA substrate and NADH. Total lipids were extracted, converted to FAMEs, and separated using a reverse phase thin layer chromatography plate with acetonitrile as the mobile phase. Shown below is an autoradiographic image of the TLC plate of the resolved [^{14}C]-methyl esters (ME). Lane A, C20:3n-6-ME (substrate standard); lane B, C20:4n-6-ME (product standard); lane C, yeast membranes containing D5D(*Tc*) (control); lane D, Kumily PPP; lanes E and F, LBFLFK PPP in duplicate.

A.



B.



C.4.6. Omega-3 desaturase (*Pir*)

C.4.6.1. Structure and function

The omega-3 desaturase (*Pir*) protein O3D(*Pir*), newly expressed in EPA+DHA canola event LBFLFK, is encoded by a coding sequence from the oomycete *Pythium irregulare*. The deduced O3D(*Pir*) protein consists of 363 amino acids with a calculated molecular mass of 40.4 kDa. The O3D(*Pir*) amino acid sequence, as depicted in Figure C.15, shows the features that are characteristic for a methyl-end integral membrane desaturase. Depicted are the predicted transmembrane helices, three histidine boxes containing eight conserved histidine residues, and the conserved histidine following the C-terminal transmembrane helix. *In vivo* experiments in yeast showed that this O3D(*Pir*) protein catalyzes the reaction required to convert C20:4n-6 into C20:5n-3 (Yilmaz et al., 2017).

Figure C.15. Deduced Amino Acid Sequence for Omega-3 Desaturase (*Pir*) in EPA+DHA Canola Event LBFLFK

Using the one letter amino acid code for the O3D(*Pir*) protein in event LBFLFK, the position of predicted transmembrane helices and other characteristic protein motifs are indicated. ▒,▒,= indicates transmembrane helices as predicted by SCAMPI2 (Peters et al., 2016), TMHMM v.2.0 (Krogh et al., 2001), and SOSUI v 1.11 (Hirokawa et al., 1998). The sequence contains three histidine-box motifs (□) with eight conserved histidine residues (H) typical for desaturases (Shanklin et al., 1994) and an additional conserved histidine (H), all of which participate in the coordination of the diiron active center (Bai et al., 2015; Wang et al., 2015).

```
1   MASTSAAQDA APYEFPSLTE IKRALPSECF EASVPLSLYY TARSLALAGS LAVALSYARA
                                                ▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒
                                                ▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒
                                                ================

61  LPLVQANALL DATLCTGYVL LQGIVFWGFF TVGHDCGHGA FSRSHVLNFS VGTLMHSIIL
                                    ▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒
    ▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒
    ========    ========================

121 TPFESWKLSH RHHHKNTGNI DKDEIFYPQR EADSHPVSRH LVMSLGSAWF AYLFAGFPPR
                                                ▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒
                                                ▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒
                                                ========================

181 TMNHFNPWEA MYVRRVAAVI ISLGVLFAFA GLYSYLTFVL GFTTMAIYYF GPLFIFATML
                                    ▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒
                                    ▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒
                                                ▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒
                                                ▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒▒
    =               ========================    ====================

241 VVTTFLHHND EETPWYADSE WTYVKGNLSS VDRSYGALID NLSHNIGTHQ IHHLFPIIPH
    ▒▒▒▒▒▒▒
    ▒▒▒▒▒▒▒
    ====

301 YKLNDATAAF AKAFPELVRK NAAPIIPTFF RMAAMYAKYG VVDTDAKTFT LKEAKAAAKT
361 KSS
```

C.4.6.2. Apparent molecular weight and immunoreactivity

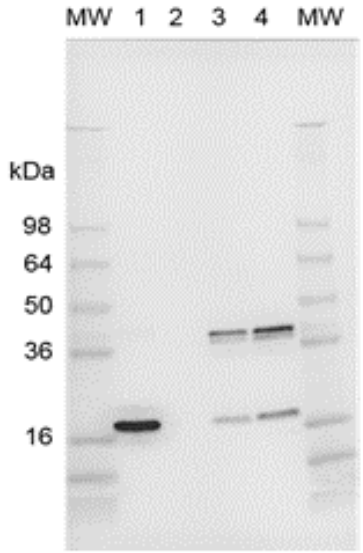
Western blot analysis using an anti-O3D(*Pir*) antibody was used to show immunoreactivity of the O3D(*Pir*) protein in PPP isolated from LBFLFK (Figure C.16). To demonstrate the specificity of the antibody, a O3D(*Pir*) NC fusion reference protein was included as positive control (Figure C.16, lane 1). An immunoreactive band was observed in the LBFLFK PPP samples at a molecular weight that was in good agreement with the calculated molecular mass of the O3D(*Pir*) protein of 40.4 kDa (Figure C.16, lanes 3 and 4). No signal was detected in PPP isolated from Kumily, the parental control that does not contain the *O3D*(*Pir*) coding sequence (Figure C.16, lane 2). The immunoreactive proteins observed at approximately 18 kDa in the LBFLFK PPP (Figure C.16, lanes 3 and 4) were not observed in Kumily PPP (Figure C.16, lane 2) and are likely the result of degradation of the O3D(*Pir*) protein.

C.4.6.3. Enzymatic activity

LBFLFK contains newly expressed omega-3 desaturases (O3Ds) from two different organisms: O3D(*Pir*) from *Pythium irregulare* (described in this section) and O3D(*Pi*) from *Phytophthora infestans* (described in section C.4.7). The functional activity assays for both O3Ds are identical and were demonstrated using membranes isolated from yeast expression strains (Yilmaz et al., 2017). Therefore, in the PPP isolated from LBFLFK, this *in vitro* assay tests for the presence of either O3D protein. Both O3Ds appear to desaturate C20:4n-6 to C20:5n-3 when the substrate fatty acid was covalently bound to a lipid, e.g., phosphatidylcholine (PC) (Yilmaz et al., 2017).

Figure C.16. Western Blot Analysis of Omega-3 Desaturase (*Pir*) in EPA+DHA Canola Event LBFLFK

LBFLFK PPP and Kumily PPP were separated by SDS-PAGE and evaluated by western blot analysis. The membrane was probed with a 1:6,000 dilution of the O3D(*Pir*)-specific antibody and probed with a secondary HRP-conjugated antibody. Approximate molecular weight markers loaded in lanes MW are shown on the left and right side of the blot. The calculated molecular mass of the O3D(*Pir*) protein encoded by the coding sequence introduced in EPA+DHA canola event LBFLFK is 40.4 kDa. The calculated molecular mass of the O3D(*Pir*) NC fusion protein used as a positive control for the O3D(*Pir*)-specific antibody is 18.2 kDa.



	Lane 1	Lane 2	Lane 3	Lane 4
Sample	O3D(*Pir*) NC fusion protein	Kumily PPP	LBFLFK PPP	LBFLFK PPP
Amount Loaded	10 ng	20 µg	10 µg	20 µg

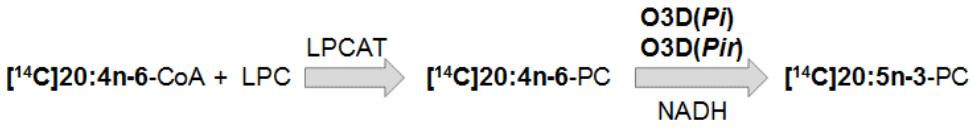
Therefore, endogenous lysophosphatidylcholine acyl transferase (LPCAT) present in PPP was used to synthesize [^{14}C]-20:4n-6-PC *in situ* by transesterification of [^{14}C]-20:4n-6 from CoA to lysophosphatidylcholine (LPC) (Yilmaz et al., 2017). Specifically, in this assay, PPP (isolated from LBFLFK or Kumily) were incubated with [^{14}C]-20:4n-6-CoA, unlabeled C16:0-lysophosphatidylcholine, and NADH. The resulting fatty acid products were isolated, converted to FAMEs, resolved on a TLC plate, and identified by electronic autoradiography. In the reaction containing either Kumily or LBFLFK PPP (Figure C.17, panel B, lanes C-E), no [^{14}C]-FAME was identified that migrates similar to the [^{14}C]-20:5n-3-ME standard (Figure C.17, panel B, lane B), indicating the expected product according to the reaction shown in panel A of Figure C.17 was not formed. The absence of detectable omega-3 desaturase activity may result from the developmental stage of the immature seeds used for the preparation of the LBFLFK PPP as the presence of very long-chain omega-3 fatty acids in the mature seed indicates that O3D activity does occur in LBFLFK plants.

Figure C.17. *In Vitro* Enzymatic Activity of Omega-3 Desaturases in EPA+DHA Canola Event LBFLFK

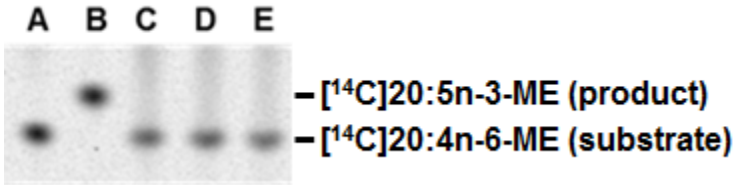
Panel A. O3D(*Pir*) and O3D(*Pi*) both appear to desaturate the fatty acid substrate C20:4n-6 to the fatty acid product C20:5n-3 when the substrate is covalently bound to a lipid, e.g., PC (Yilmaz et al., 2017). In the enzymatic assay, endogenous lysophosphatidylcholine acyl transferase (LPCAT) is used to synthesize C20:4n-6-PC by transferring C20:4n-6 from CoA to lysophosphatidylcholine (LPC).

Panel B. PPP (100 µg total protein) were incubated in a buffer solution with [^{14}C]-20:4n-6-CoA, unlabeled C16:0-lysophosphatidylcholine (LPC), and NADH. Total lipids were extracted, converted to FAMEs, and separated using a 10% $AgNO_3$ thin layer chromatography plate with a 1:1 ratio of toluene/heptane solvent as the mobile phase. Shown below is an autoradiographic image of the TLC plate of the resolved [^{14}C]-methyl esters (ME). Kumily PPP were used as a control. Lane A, C20:4n-6-ME standard; lane B, C20:5n-3-ME standard; lane C, Kumily PPP (control); lanes D and E, LBFLFK PPP in duplicate.

A.



B.



C.4.7. Omega-3 desaturase (*Pi*)

C.4.7.1. Structure and function

The omega-3 desaturase (*Pi*) protein O3D(*Pi*), newly expressed in EPA+DHA canola event LBFLFK, is encoded by a coding sequence from the oomycete *Phytophthora infestans*. The deduced O3D(*Pi*) protein consists of 361 amino acids with a calculated molecular mass of 40.8 kDa. The O3D(*Pi*) amino acid sequence, as depicted in Figure C.18, shows the features that are characteristic for a methyl-end integral membrane desaturase. Depicted are the predicted transmembrane, three histidine boxes containing eight conserved histidine residues, and the conserved histidine following the C-terminal transmembrane helix. *In vivo* experiments in yeast showed that this O3D(*Pi*) protein catalyzes the reaction required to convert C20:4n-6 into C20:5n-3 (Yilmaz et al., 2017).

Figure C.18. Deduced Amino Acid Sequence for Omega-3 Desaturase (*Pi*) in EPA+DHA Canola Event LBFLFK

Using the one letter amino acid code for the O3D(*Pi*) protein in event LBFLFK, the position of predicted transmembrane helices and other characteristic protein motifs are indicated. ▒, ░ ,= indicates transmembrane helices as predicted by SCAMPI2 (Peters et al., 2016), TMHMM v.2.0 (Krogh et al., 2001), and SOSUI v 1.11 (Hirokawa et al., 1998). The sequence contains three histidine-box motifs (☐) with eight conserved histidine residues (H) typical for desaturases (Shanklin et al., 1994) and an additional conserved histidine (H), all of which participate in the coordination of the diiron active center (Bai et al., 2015; Wang et al., 2015).

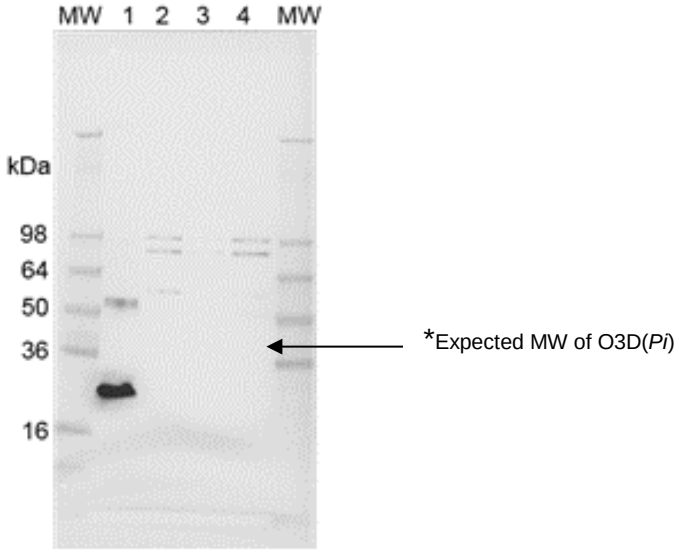
```
1   MATKEAYVFP TLTEIKRSLP KDCFEASVPL SLYYTVRCLV IAVALTFGLN YARALPEVES
                               =========================
61  FWALDAALCT GYILLQGIVF WGFFTVGHDA GHGAFSRYHL LNFVVGTFMH SLILTPFESW
      =========================
121 KLTHRHHHKN TGNIDRDEVF YPQRKADDHP LSRNLILALG AAWLAYLVEG FPPRKVNHFN
                                   ==========================
181 PFEPLFVRQV SAVVISLLAH FFVAGLSIYL SLQLGLKTMA IYYYGPVFVF GSMLVITTFL
               =========================        =====================
241 HHNDEETPWY ADSEWTYVKG NLSSVDRSYG ALIDNLSHNI GTHQIHHLFP IIPHYKLKKA
    ===
301 TAAFHQAFPE LVRKSDEPII KAFFRVGRLY ANYGVVDQEA KLFTLKEAKA ATEAAAKTKS
361 T
```

C.4.7.2. Apparent molecular weight and immunoreactivity

Western blot analysis using an anti-O3D(*Pi*) protein antibody was used to show immunoreactivity of the O3D(*Pi*) protein in PPP isolated from LBFLFK (Figure C.19). To demonstrate the specificity of the antibody, a O3D(*Pi*) NC fusion reference protein was included as positive control (Figure C.19, lane 1). An immunoreactive band near the calculated mass of the O3D(*Pi*) protein of 40.8 kDa was not observed in the LBFLFK PPP samples (Figure C.19, lanes 3 and 4). Likewise, no signal was detected in the PPP isolated from Kumily, the parental control that does not contain the O3D(*Pi*) coding sequence (Figure C.19, lane 2). The immunoreactive proteins observed at approximately 98 kDa and slightly smaller than 98 kDa in LBFLFK PPP and Kumily PPP (Figure C.19, lane 2, lane 4, faint in lane 3) are likely a result from non-specific protein recognition of endogenous canola proteins by the anti-O3D(*Pi*) antibody.

Figure C.19. Western Blot Analysis of Omega-3 Desaturase (*Pi*) in EPA+DHA Canola Event LBFLFK

LBFLFK PPP and Kumily PPP were separated by SDS-PAGE and evaluated by western blot analysis. The membrane was probed with a 1:1,000 dilution of the O3D(*Pi*)-specific antibody and probed with a secondary HRP-conjugated antibody. Approximate molecular weight markers loaded in lanes MW are shown on the left and right side of the blot. The calculated molecular mass of the O3D(*Pi*) protein encoded by the coding sequence introduced in EPA+DHA canola event LBFLFK is 40.8 kDa. The calculated molecular mass of the O3D(*Pi*) NC fusion protein used as a positive control for the O3D(*Pi*)-specific antibody is 22.6 kDa.



	Lane 1	Lane 2	Lane 3	Lane 4
Sample	**O3D(*Pi*) NC fusion protein**	**Kumily PPP**	**LBFLFK PPP**	**LBFLFK PPP**
Amount Loaded	10 ng	20 µg	10 µg	20 µg

C.4.7.3. Enzymatic activity

LBFLFK contains newly expressed O3Ds from two different organisms: O3D(*Pir*) from *Pythium irregulare* (described in section C.4.6) and O3D(*Pi*) from *Phytophthora infestans* (described in this section). The functional activity assays for both O3Ds are identical and were demonstrated using membranes isolated from yeast expression strains (Yilmaz et al., 2017). Therefore, in the PPP isolated from LBFLFK, this *in vitro* assay tests for the presence of either O3D protein. The results described in section C.4.6 show that the PPP fraction isolated from LBFLFK did not contain detectable O3D enzyme activity (Figure C.17). The absence of detectable omega-3 desaturase activity may result from the developmental stage of the immature seeds used for the preparation of the LBFLFK PPP as the presence of very long-chain omega-3 fatty acids in the mature seed indicates that O3D activity does occur in LBFLFK plants.

C.4.8. Delta-5 elongase (*Ot*)

C.4.8.1. Structure and function

The delta-5 elongase (*Ot*) protein D5E(*Ot*), newly expressed in EPA+DHA canola event LBFLFK, is encoded by a coding sequence isolated from the marine green microalga *Ostreococcus tauri*. The deduced D5E(*Ot*) protein consists of 300 amino acids with a calculated molecular mass of 34.2 kDa. The D5E(*Ot*) amino acid sequence, as depicted in Figure C.20, shows the features that are characteristic of ELO-type elongases. Depicted are the predicted transmembrane helices and all four signature ELO-motifs. *In vivo* experiments in yeast showed that this D5E(*Ot*) protein catalyzes the elongation reaction required to convert C20:5n-3 into C22:5n-3 (Meyer et al., 2004; Yilmaz et al., 2017).

Figure C.20. Deduced Amino Acid Sequence for Delta-5 Elongase (*Ot*) in EPA+DHA Canola Event LBFLFK

Using the one letter amino acid code for the D5E(*Ot*) protein in event LBFLFK, the position of predicted transmembrane helices and other characteristic protein motifs are indicated. , ,= indicates transmembrane helices as predicted by SCAMPI2 (Peters et al., 2016), TMHMM v.2.0 (Krogh et al., 2001), and SOSUI v 1.11 (Hirokawa et al., 1998), respectively. The sequence contains four motifs ( ) highly conserved among elongases (Leonard et al., 2004; Meyer et al., 2004; Jakobsson et al., 2006), including the characteristic HXXHH histidine box embedded in the fourth membrane helix that is critical for enzymatic activity (Denic and Weissman, 2007; Hernandez-Buquer and Blacklock, 2013). Highly conserved residues are highlighted in black.

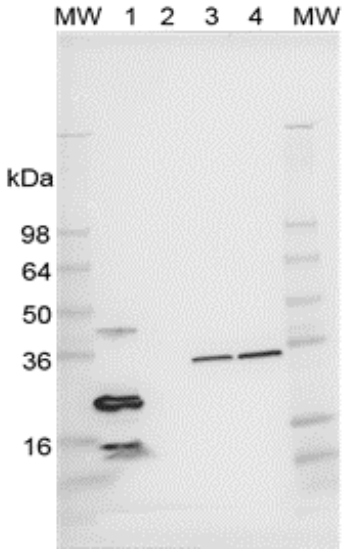
```
1    MSASGALLPA IAFAAYAYAT YAYAFEWSHA NGIDNVDARE WIGALSLRLP AIATTMYLLF
     ==========================
61   CLVGPRLMAK REAFDPKGFM LAYNAYQTAF NVVVLGMFAR EISGLGQPVW GSTMPWSDRK
                       =========================
121  SFKILLGVWL HYNNKYLELL DTVFMVARKK TKQLSFLHVY HHALLIWAWW LVCHLMATND
                                             =========================
181  CIDAYFGAAC NSFIHIVMYS YYLMSALGIR CPWKRYITQA QMLQFVIVFA HAVFVLRQKH
           =========================       =======================
241  CPVTLPWAQM FVMTNMLVLF GNFYLKAYSN KSRGDGASSV KPAETTRAPS VRRTRSRKID
         ========================
```

C.4.8.2. Apparent molecular weight and immunoreactivity

Western blot analysis using an anti-D5E(*Ot*) protein antibody was used to show immunoreactivity of the D5E(*Ot*) protein in PPP isolated from LBFLFK (Figure C.21). The D5E(*Ot*) NC fusion reference protein, included as positive control (Figure C.21, lane 1), displayed an immunoreactive band in good agreement with its calculated molecular mass of 22.2 kDa as well as minor immunoreactive bands correlating to a likely dimer (44 kDa) and degradation products (12 kDa). An immunoreactive band was observed in the LBFLFK PPP samples at a molecular weight that was in good agreement with the calculated molecular mass of the D5E(*Ot*) protein of 34.2 kDa (Figure C.21, lanes 3 and 4). No signal was detected in PPP isolated from Kumily, the parental control that does not contain the D5E(*Ot*) coding sequence (Figure C.21, lane 2).

Figure C.21. Western Blot Analysis of Delta-5 Elongase (*Ot*) in EPA+DHA Canola Event LBFLFK

LBFLFK PPP and Kumily PPP were separated by SDS-PAGE and evaluated by western blot analysis. The membrane was probed with a 1:6000 dilution of the D5E(*Ot*)-specific antibody and probed with a secondary HRP-conjugated antibody. Approximate molecular weight markers loaded in lanes labelled MW are shown on the left and right side of the blot. The calculated molecular mass of the D5E(*Ot*) protein from the coding sequence introduced in EPA+DHA canola event LBFLFK is 34.2 kDa. The calculated molecular mass of the D5E(*Ot*) NC fusion protein used as a positive control for the D5E(*Ot*)-specific antibody is 22.2 kDa.



	Lane 1	Lane 2	Lane 3	Lane 4
Sample	**D5E(*Ot*) NC fusion protein**	**Kumily PPP**	**LBFLFK PPP**	**LBFLFK PPP**
Amount Loaded	30 ng	20 µg	10 µg	20 µg

C.4.8.3. Enzymatic activity

The functional activity of the newly expressed D5E(*Ot*) enzyme in PPP isolated from LBFLFK was tested using an *in vitro* assay previously reported for the D5E(*Ot*) enzyme present in membranes isolated from yeast expression strains (Yilmaz et al., 2017). Specifically, PPP (isolated from LBFLFK or Kumily) were incubated with the two D5E(*Ot*) co-substrates, [^{14}C]-malonyl-CoA and unlabeled C20:5n-3-CoA in the presence of NADPH. The resulting fatty acid products were isolated, converted to FAMEs, resolved on a TLC plate, and identified by electronic autoradiography. In the reaction containing LBFLFK PPP (Figure C.22, panel B, lanes C and D), a [^{14}C]-FAME was identified that migrates similar to the [^{14}C]-22:5n-3-ME standard (Figure C.22, panel B, lane A), in agreement with the expected product according to the reaction shown in panel A of Figure C.22. This product was not detected in the reaction containing parental control Kumily PPP (Figure C.22, panel B, lane B), which does not contain the D5E(*Ot*) coding sequence.

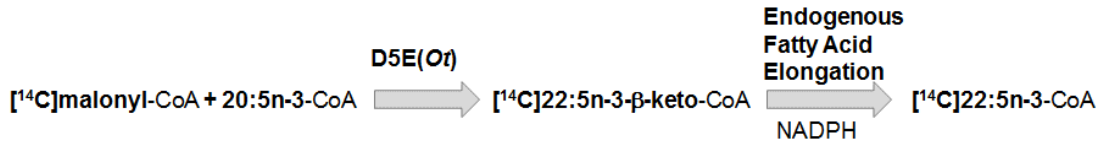
Together, the results obtained from these *in vitro* assays demonstrate that the enzymatic activity of the D5E(*Ot*) protein in the LBFLFK PPP is consistent with the previously (Yilmaz et al., 2017) proposed enzymatic reaction shown in Panel A of Figure C.22.

Figure C.22. *In Vitro* Enzymatic Activity of Delta-5 Elongase (*Ot*) in EPA+DHA Canola Event LBFLFK

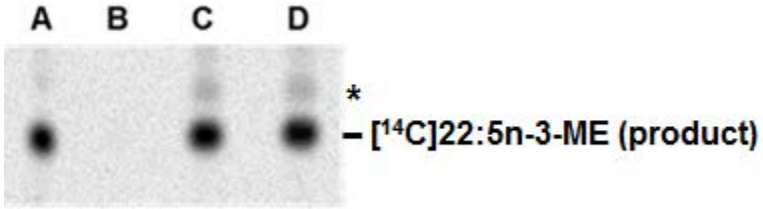
Panel A. D5E(*Ot*) catalyzes the transfer of two carbons from [^{14}C]-malonyl-CoA to C20:5n-3-CoA, generating [^{14}C]-22:5n-3-β-keto-CoA, which in the presence of NADPH can be converted to [^{14}C]-22:5n-3-CoA by the endogenous canola elongation complex enzymes.

Panel B. PPP (320 µg total protein) were incubated in a buffer solution with [^{14}C]-malonyl CoA and unlabeled C20:5n-3-CoA and NADPH. Depicted is an autoradiographic image of a TLC plate showing separated [^{14}C]-methylesters (MEs) prepared from the total lipids that were extracted from the enzymatic reactions. The resulting FAMEs from the D5E(*Ot*) assay were separated using a reverse phase thin layer chromatography plate with acetonitrile as the mobile phase. Lane A, [^{14}C]-22:5n-3-ME standard; lane B, Kumily PPP (control); lanes C and D, LBFLFK PPP in duplicate. The [^{14}C]-labeled malonyl-ME (derived from the substrate [^{14}C]-malonyl-CoA) does not get retained during the extraction process and is therefore not present on the TLC plate. The [^{14}C]-compound observed in lanes C and D that is denoted by an asterisk (*) is derived from the [^{14}C]-22:5n-3-β-keto-CoA product during the isolation of the FAMEs (Yilmaz et al., 2017).

A.



B.



C.4.9. Delta-4 desaturase (*Tc*)

C.4.9.1. Structure and function

The delta-4 desaturase (*Tc*) protein D4D(*Tc*), newly expressed in EPA+DHA canola event LBFLFK, is encoded by a coding sequence isolated from the marine protist *Thraustochytrium* sp. The deduced D4D(*Tc*) protein consists of 519 amino acids with a calculated molecular mass of 59 kDa. The D4D(*Tc*) amino acid sequence, as depicted in Figure C.23, shows the features that are characteristic for a front-end integral membrane desaturase. Depicted are the N-terminally fused cytochrome b_5 domain containing the characteristic HPGG motif, the predicted transmembrane helices, three histidine boxes containing seven conserved histidine residues, a conserved glutamine in histidine box 3, and the conserved histidine following the C-terminal transmembrane helix. *In vivo* experiments in yeast showed that this D4D(*Tc*) protein catalyzes the desaturation reaction required to convert C22:5n-3 into C22:6n-3 (Yilmaz et al., 2017).

Figure C.23. Deduced Amino Acid Sequence for Delta-4 Desaturase (*Tc*) in EPA+DHA Canola Event LBFLFK

Using the one letter amino acid code for the D4D(*Tc*) protein in event LBFLFK, the position of predicted transmembrane helices and other characteristic protein motifs are indicated. ▒, ▒,= indicates transmembrane helices as predicted by SCAMPI2 (Peters et al., 2016), TMHMM v.2.0 (Krogh et al., 2001), and SOSUI v 1.11 (Hirokawa et al., 1998). The sequence contains three histidine-box motifs (▒) with seven conserved histidine residues (H) typical for desaturases (Shanklin et al., 1994) and an additional conserved histidine (H), all of which participate in the coordination of the diiron active center (Bai et al., 2015; Wang et al., 2015). Additionally, the sequence contains a conserved glutamine (Q) in the third histidine box that is essential for activity (Sayanova et al., 2001). Underlined is the cytochrome b_5 domain as annotated by PFAM v31 (Finn et al., 2016), containing the characteristic HPGG motif (Sperling and Heinz, 2001) highlighted in grey.

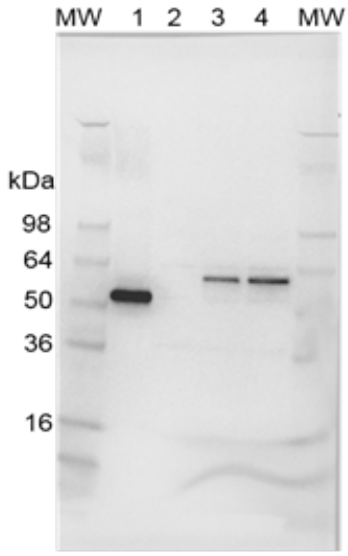
```
1   MTVGYDEEIP FEQVRAHNKP DDAWCAIHGH VYDVTKFASV HPGGDIILLA AGKEATVLYE
61  TYHVRGVSDA VLRKYRIGKL PDGQGGANEK EKRTLSGLSS ASYYTWNSDF YRVMRERVVA
121 RLKERGKARR GGYELWIKAF LLLVGFWSSL YWMCTLDPSF GAILAAMSLG VFAAFVGTCI
181 QHDGNHGAFA QSRWVNKVAG WTLDMIGASG MTWEFQHVLG HHPYTNLIEE ENGLQKVSGK
241 KMDTKLADQE SDPDVFSTYP MMRLHPWHQK RWYHRFQHIY GPFIFGFMTI NKVVTQDVGV
301 VLRKRLFQID AECRYASPMY VARFWIMKAL TVLYMVALPC YMQGPWHGLK LFAIAHFTCG
                          ========================          ========
361 EVLATMFIVN HIIEGVSYAS KDAVKGTMAP PKTMHGVTPM NNTRKEVEAE ASKSGAVVKS
    ================
421 VPLDDWAAVQ CQTSVNWSVG SWFWNHFSGG LNHQIEHHLF PGLSHETYYH IQDVVQSTCA
481 EYGVPYQHEP SLWTAYWKML EHLRQLGNEE THESWQRAA
```

C.4.9.2. Apparent molecular weight and immunoreactivity

Western blot analysis using an anti-D4D(*Tc*) protein antibody was used to show immunoreactivity of the D4D(*Tc*) protein in PPP isolated from LBFLFK (Figure C.24). To demonstrate the specificity of the antibody, a D4D(*Tc*) NC fusion reference protein was included as positive control (Figure C.24, lane 1). An immunoreactive band was observed in the LBFLFK PPP samples at a molecular weight that was in good agreement with the calculated molecular mass of the D4D(*Tc*) protein of 59.0 kDa (Figure C.24, lanes 3 and 4). No signal was detected in PPP isolated from Kumily, the parental control that does not contain the D4D(*Tc*) coding sequence (Figure C.24, lane 2).

Figure C.24. Western Blot Analysis of Delta-4 Desaturase (*Tc*) in EPA+DHA Canola Event LBFLFK

LBFLFK PPP and Kumily PPP were separated by SDS-PAGE and evaluated by western blot analysis. The membrane was probed with a 1:4,000 dilution of the D4D(*Tc*)-specific antibody and probed with a secondary HRP-conjugated antibody. Approximate molecular weight markers loaded in lanes MW are shown on the left and right side of the blot. The calculated molecular mass of the D4D(*Tc*) protein encoded by the coding sequence introduced in EPA+DHA canola event LBFLFK is 59.0 kDa. The calculated molecular mass of the D4D(*Tc*) NC fusion protein used as a positive control for the D4D(*Tc*)-specific antibody is 46.7 kDa (apparent molecular weight slightly higher than 50 kDa).



	Lane 1	Lane 2	Lane 3	Lane 4
Sample	D4D(*Tc*) NC fusion protein	Kumily PPP	LBFLFK PPP	LBFLFK PPP
Amount Loaded	6 ng	20 µg	10 µg	20 µg

C.4.9.3. Enzymatic activity

LBFLFK contains newly expressed D4Ds from two different organisms: D4D(*Pl*) and D4D(*Pl*) [A102S] from *Pavlova lutheri* (described in section C.4.10) and D4D(*Tc*) from *Thraustochytrium* sp. (described in this section). The functional activity assays for the D4Ds from both organisms are identical and were demonstrated using membranes isolated from yeast expression strains (Yilmaz et al., 2017). Therefore, in the PPP isolated from LBFLFK, the presence of all D4Ds was tested using this *in vitro* assay. However, the relative contributions of the individual D4Ds to the total D4D activity cannot be determined. D4D(*Tc*) was previously shown to desaturate C22:5n-3 to C22:6n-3 when the substrate fatty acid was covalently bound to a lipid, e.g., PC, but the D4D(*Pl*) may accept the substrate C22:5n-3-CoA (Yilmaz et al., 2017). Endogenous lysophosphatidylcholine acyl transferase (LPCAT) present in PPP was used to synthesize [^{14}C]-22:5n-3-PC *in situ* by transesterification of [^{14}C]-22:5n-3 from CoA to lysophosphatidylcholine (LPC) (Yilmaz et al., 2017). Specifically, PPP were incubated with [^{14}C]-22:5n-3-CoA, unlabeled C16:0-lysophosphatidylcholine, and NADH. The resulting fatty acid products were isolated, converted to FAMEs, resolved on a TLC plate, and identified by electronic autoradiography. In the reaction containing LBFLFK PPP (Figure C.25, panel B, lanes B and C), a [^{14}C]-FAME was identified that migrates similar to the [^{14}C]-22:6n-3-ME standard (Figure C.25, panel B, lane E), in agreement with the expected product according to the reaction shown in panel A of Figure C.25. This product was also not detected in the reaction containing parental control Kumily PPP (Figure C.25, panel B, lane C), which does not contain the D4D(*Pl*), D4D(*Pl*) [A102S], or D4D(*Tc*) proteins.

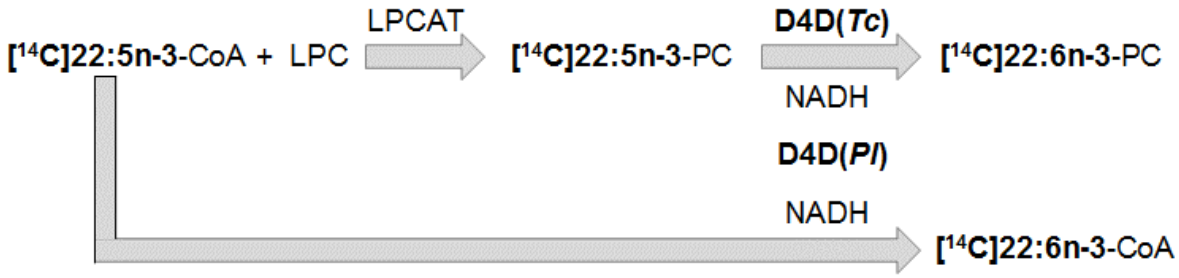
The results obtained from these *in vitro* assays demonstrate D4D activity in the LBFLFK PPP that is not observed in Kumily PPP. The observed D4D activity is consistent with the previously proposed enzymatic reaction (Yilmaz et al., 2017) shown in panel A of Figure C.25 and could be attributed to any or all of the D4D(*Pl*), D4D(*Pl*) [A102S], and D4D(*Tc*) proteins.

Figure C.25. *In Vitro* Enzymatic Activity of Delta-4 Desaturases in EPA+DHA Canola Event LBFLFK

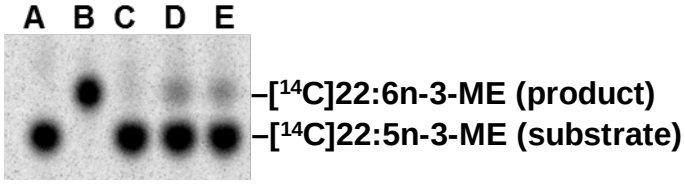
Panel A. D4D(*Tc*) desaturates the fatty acid substrate C22:5n-3 to the fatty acid product C22:6n-3 when the substrate is covalently bound to a lipid (e.g., PC), but the D4D(*Pl*) enzyme may accept the substrate C22:5n-3-CoA (Yilmaz et al., 2017). In the enzymatic assay, endogenous lysophosphatidylcholine acyl transferase (LPCAT) is used to synthesize [^{14}C]-22:5n-3-PC by transferring [^{14}C]-22:5n-3 from CoA to lysophosphatidylcholine (LPC).

Panel B. PPP (40 µg total protein) were incubated in a buffer solution with [^{14}C]-22:5n-3-CoA substrate, unlabeled C16:0-lysophosphatidylcholine, and NADH. Total lipids were extracted, converted to FAMEs, and separated using a 10% $AgNO_3$ thin layer chromatography plate with a 1:1 ratio of toluene/heptane solvent as the mobile phase. Shown below is an autoradiographic image of the TLC plate of the resolved [^{14}C]-methyl esters (ME). Kumily PPP were used as a control. Lane A, C22:5n-3-ME standard (substrate); lane B, C22:6n-3-ME standard (product); lane C, Kumily PPP (control); lane D and E, LBFLFK PPP loaded in duplicate.

A.



B.



C.4.10. Delta-4 desaturase (*Pl*)

C.4.10.1. Structure and function

The delta-4 desaturase (*Pl*) protein D4D(*Pl*), newly expressed in EPA+DHA canola event LBFLFK, is encoded by a coding sequence isolated from the marine microalga *Pavlova lutheri*. The deduced D4D(*Pl*) protein consists of 445 amino acids with a calculated molecular mass of 49.1 kDa. The D4D(*Pl*) amino acid sequence, as depicted in Figure C.26, shows the features that are characteristic for a front-end integral membrane desaturase. Depicted are the N-terminally fused cytochrome b_5 domain containing the characteristic HPGG motif, the predicted transmembrane helices, three histidine boxes containing seven conserved histidine residues, a conserved glutamine in histidine box 3, and the conserved histidine following the C-terminal transmembrane helix.

LBFLFK contains two T-DNA inserts that encode the D4D(*Pl*) protein. The two D4D(*Pl*) coding sequences differ by a single nucleotide resulting in a deduced protein that has a [A102S] substitution, referred to as D4D(*Pl*) [A102S], that is predicted to reside in a linker region between the cytochrome b_5 domain and the first transmembrane-spanning domain of the desaturase. The D4D(*Pl*) [A102S] protein also consists of 445 amino acids with a calculated molecular mass of 49.1 kDa. *In vivo* experiments in yeast showed that both proteins, the D4D(*Pl*) and the D4D(*Pl*) [A102S], catalyze the desaturation reaction required to convert C22:5n-3 into C22:6n-6 (Yilmaz et al., 2017) and have similar conversion efficiencies.

Figure C.26. Deduced Amino Acid Sequence for Delta-4 Desaturase (*Pl*) in EPA+DHA Canola Event LBFLFK

Using the one letter amino acid code for the D4D(*Pl*) protein in event LBFLFK, the position of predicted transmembrane helices and other characteristic protein motifs are indicated. ▒,▒,= indicates transmembrane helices as predicted by SCAMPI2 (Peters et al., 2016), TMHMM v.2.0 (Krogh et al., 2001), and SOSUI v 1.11 (Hirokawa et al., 1998). The sequence contains three histidine-box motifs (▒) with seven conserved histidine residues (H) typical for desaturases (Shanklin et al., 1994) and an additional conserved histidine (H), all of which participate in the coordination of the diiron active center (Bai et al., 2015; Wang et al., 2015). Additionally, the sequence contains a conserved glutamine (Q) in the third histidine box that is essential for activity (Sayanova et al., 2001). Underlined is the cytochrome b_5 domain as annotated by PFAM v31 (Finn et al., 2016), containing the characteristic HPGG motif (Sperling and Heinz, 2001) highlighted in grey. EPA+DHA canola event LBFLFK carries two T-DNA inserts that encode the D4D(*Pl*) protein, one with the amino acid sequence as shown below and one with the amino acid sequence with a substitution of alanine by serine at position 102 (D4D(*Pl*) [A102S]), indicated by **A**.

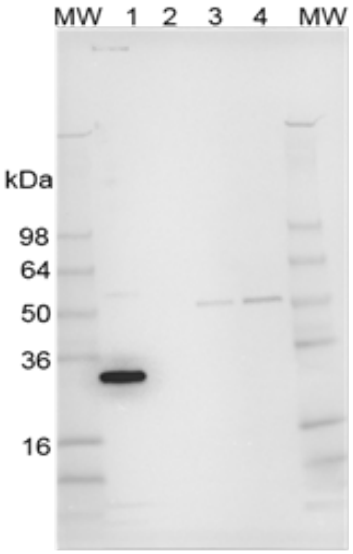
```
1   MPPSAASEGG VAELRAAEVA SYTRKAVDER PDLTIVGDAV YDAKAFRDEH PGGAHFVSLF
61  GGRDATEAFM EYHRRAWPKA RMSKFFVGSL DASEKPTQAD SAYLRLCAEV NALLPKGSGG
121 FAPPSYWLKA AALVVAAVSI EGYMLLRGKT LLLSVFLGLV FAWIGLNIQH DANHGALSRH
                          ========================
181 SVINYCLGYA QDWIGGNMVL WLQEHVVMHH LHTNDVDADP DQKAHGVLRL KPTDGWMPWH
     ========================
241 ALQQLYILPG EAMYAFKLLF LDALELLAWR WEGEKISPLA RALFAPAVAC KLGFWARFVA
       ========================          ======================
301 LPLWLQPTVH TALCICATVC TGSFYLAFFF FISHNFDGVG SVGPKGSLPR SATFVQRQVE
    ==          ========================
361 TSSNVGGYWL GVLNGGLNFQ IEHHLFPRLH HSYYAQIAPV VRTHIEKLGF KYRHFPTVGS
421 NLSSMLQHMG KMGTRPGAEK GGKAE
```

C.4.10.2. Apparent molecular weight and immunoreactivity

Western blot analysis using an anti-D4D(*Pl*) protein antibody was used to show immunoreactivity of the D4D(*Pl*) and D4D(*Pl*) [A102S] proteins in PPP isolated from LBFLFK (Figure C.27). To demonstrate the specificity of the antibody, a D4D(*Pl*) NC fusion reference protein was included as positive control (Figure C.27, lane 1). An immunoreactive band was observed in the LBFLFK PPP samples at a molecular weight that was in good agreement with the calculated molecular mass of the D4D(*Pl*) and D4D(*Pl*) [A102S] proteins of 49.1 kDa (Figure C.27, lanes 3 and 4). No signal was detected in PPP isolated from Kumily, the parental control that does not contain the D4D(*Pl*) coding sequence (Figure C.27, lane 2).

Figure C.27. Western Blot Analysis of Delta-4 Desaturase (*Pl*) in EPA+DHA Canola Event LBFLFK

LBFLFK PPP and Kumily PPP were separated by SDS-PAGE and evaluated by western blot analysis. The membrane was probed with a 1:4,000 dilution of the D4D(*Pl*)-specific antibody and probed with a secondary HRP-conjugated antibody. Approximate molecular weight markers loaded in lanes MW are shown on the left and right side of the blot. The calculated molecular mass of the D4D(*Pl*) and D4D(*Pl*) [A102S] protein encoded by the coding sequences introduced in EPA+DHA canola event LBFLFK is 49.1 kDa. The calculated molecular mass of the D4D(*Pl*) NC fusion protein used as a positive control for the D4D(*Pl*)-specific antibody is 24.4 kDa.



	Lane 1	Lane 2	Lane 3	Lane 4
Sample	D4D(*Pl*) NC fusion protein	Kumily PPP	LBFLFK PPP	LBFLFK PPP
Amount Loaded	7.5 ng	20 µg	10 µg	20 µg

C.4.10.3. Enzymatic activity

LBFLFK contains newly introduced D4Ds from two different organisms: D4D(*Pl*) and D4D(*Pl*) [A102S] from *Pavlova lutheri* (described in this section) and D4D(*Tc*) from *Thraustochytrium* sp. (described in section C.4.9). The functional activity assays for the D4Ds from both organisms are identical and were demonstrated using membranes isolated from yeast expression strains (Yilmaz et al., 2017). Therefore, in the PPP isolated from LBFLFK, the presence of all D4Ds was tested using this *in vitro* assay. However, the relative contributions of the individual D4Ds to the total D4D activity cannot be determined.

The results described in section C.4.9 demonstrate D4D activity in the LBFLFK PPP. This D4D activity was not observed in Kumily PPP, which does not contain D4D(*Pl*), D4D(*Pl*) [A102S], or D4D(*Tc*). The observed D4D activity is consistent with the previously proposed enzymatic reaction (Yilmaz et al., 2017) shown in panel A of Figure C.25 and could be attributed to any or all of the D4D(*Pl*), D4D(*Pl*) [A102S], and D4D(*Tc*) proteins. In yeast *in vivo* feeding experiments, the D4D(*Pl*) [A102S] protein was shown to have substrate specificity and activity comparable to what was reported for the D4D(*Pl*) protein (Yilmaz et al., 2017).

C.4.11. Acetohydroxy acid synthase (*At*) [A122TS653N]

C.4.11.1. Structure and function

The acetoyhydroxy acid synthase protein AHAS(*At*) [A122TS653N], newly expressed in EPA+DHA canola event LBFLFK, is the large subunit of acetohydroxy acid synthase, consisting of 670 amino acids with a calculated molecular mass of 72.6 kDa as depicted in Figure C.28. During transport into the chloroplast, the chloroplast transit peptide is removed to produce the mature AHAS(*At*) [A122TS653N] enzyme, which interacts with the endogenous *Brassica napus* small subunit, enabling typical feedback regulation for AHAS activity. The amino terminus of the mature AHAS(*At*) [A122TS653N] protein was determined by mass spectrometric peptide mapping to be a valine at position 65. The mature AHAS(*At*) [A122TS653N] protein in LBFLFK consists of 606 amino acids with a calculated molecular mass of 66.1 kDa. The sequence introduced into LBFLFK includes nucleotide sequence substitutions such that the translated protein has an alanine residue changed to a threonine at amino acid position 122 (A122T) and a serine residue changed to an asparagine at amino acid position 653 (S653N). These amino acid changes in the plant AHAS protein are known to reduce its binding affinity towards imidazolinone herbicides and thereby result in tolerance to these herbicides while maintaining normal biosynthetic function, including proper feedback regulation (Tan et al., 2005).

Figure C.28. Deduced Amino Acid Sequence for Acetohydroxy Acid Synthase (*At*) [A122TS653N] in EPA+DHA Canola Event LBFLFK

Shown is the one letter amino acid sequence of the AHAS(*At*) [A122TS653N] protein in event LBFLFK. Residues 1–64 (Underlined) represent the chloroplast transit peptide with the mature AHAS(*At*) [A122TS653N] protein starting at valine 65. Amino acid substitutions are A122T and S653N are shown in **bold underlined** text.

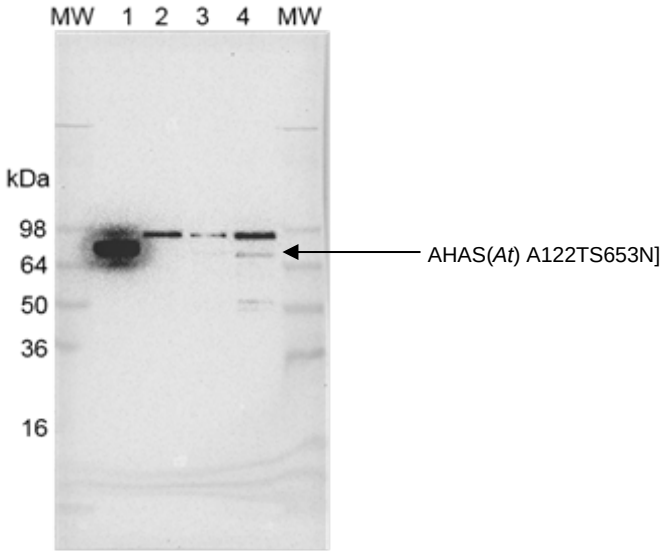
```
1   MAAATTTTTT SSSISFSTKP SPSSSKSPLP ISRFSLPFSL NPNKSSSSSR RRGIKSSSPS
61  SISAVLNTTT NVTTTPSPTK PTKPETFISR FAPDQPRKGA DILVEALERQ GVETVFAYPG
121 GTSMEIHQAL TRSSSIRNVL PRHEQGGVFA AEGYARSSGK PGICIATSGP GATNLVSGLA
181 DALLDSVPLV AITGQVPRRM IGTDAFQETP IVEVTRSITK HNYLVMDVED IPRIIEEAFF
241 LATSGRPGPV LVDVPKDIQQ QLAIPNWEQA MRLPGYMSRM PKPPEDSHLE QIVRLISESK
301 KPVLYVGGGC LNSSDELGRF VELTGIPVAS TLMGLGSYPC DDELSLHMLG MHGTVYANYA
361 VEHSDLLLAF GVRFDDRVTG KLEAFASRAK IVHIDIDSAE IGKNKTPHVS VCGDVKLALQ
421 GMNKVLENRA EELKLDFGVW RNELNVQKQK FPLSFKTFGE AIPPQYAIKV LDELTDGKAI
481 ISTGVGQHQM WAAQFYNYKK PRQWLSSGGL GAMGFGLPAA IGASVANPDA IVVDIDGDGS
541 FIMNVQELAT IRVENLPVKV LLLNNQHLGM VMQWEDRFYK ANRAHTFLGD PAQEDEIFPN
601 MLLFAAACGI PAARVTKKAD LREAIQTMLD TPGPYLLDVI CPHQEHVLPM IPNGGTFNDV
661 ITEGDGRIKY
```

C.4.11.2. Apparent molecular weight and immunoreactivity

Western blot analysis using an anti-AHAS(*At*) [A122TS653N] protein antibody was used to show immunoreactivity of the AHAS(*At*) [A122TS653N] protein in PPP isolated from LBFLFK (Figure C.29). To demonstrate the specificity of the antibody, an AHAS(*At*) [A122TS653N] reference protein was included as positive control (Figure C.29, lane 1). An immunoreactive band was observed in the LBFLFK PPP samples at a molecular weight that is in good agreement with the calculated molecular mass of the AHAS(*At*) [A122TS653N] protein of 66.1 kDa (Figure C.29, lane 3 and 4). There is also a strong non-specific cross-reactivity with an unknown protein at ~98 kDa in both Kumily and LBFLFK PPP (Figure C.29, lanes 2, 3, and 4), which is a significantly higher molecular weight than estimated for endogenous AHAS(*Bn*) protein (~66 kDa). While the cross-reactivity of the utilized antibody to the AHAS(*Bn*) protein could be expected to result in a immunoreactive band in the Kumily PPP samples at a molecular weight that is in agreement with the calculated molecular weight estimated for endogenous AHAS(*Bn*) protein (~66 kDa), the absence of such a band is likely a result of the much lower concentration of AHAS(*Bn*) in Kumily versus total combined concentration of AHAS(*At*) [A122TS653N] and AHAS(*Bn*) in LBFLFK. Molecular weight and immunoreactivity were also confirmed for AHAS(*At*) [A122TS653N] in leaf extracts (data not shown).

Figure C.29. Western Blot Analysis of Acetohydroxy Acid Synthase (*At*) [A122TS653N] in EPA+DHA Canola Event LBFLFK

PPP isolated from EPA+DHA canola event LBFLFK and parental control Kumily were separated by SDS-PAGE and evaluated by western blot analysis. The membrane was probed with a 1:1,000 dilution of the AHAS(*At*) [A122TS653N]-specific antibody and probed with a secondary HRP-conjugated antibody. Approximate molecular weight markers loaded in lanes MW are shown on the left and right side of the blot. The calculated molecular mass of the AHAS(*At*) [A122TS653N] protein encoded by the coding sequence introduced in EPA+DHA canola event LBFLFK is 66.1 kDa. The calculated molecular mass of the reference standard, which also contains a 6x histidine tag and short linker sequence, is 68.1 kDa.



	Lane 1	Lane 2	Lane 3	Lane 4
Sample	AHAS(*At*) [A122TS653N]	Kumily PPP	LBFLFK PPP	LBFLFK PPP
Amount Loaded	5 ng	20 µg	10 µg	20 µg

C.4.11.3. Enzymatic activity

The AHAS enzymatic reaction specifically catalyzes the formation of acetolactate from two pyruvate molecules utilizing the cofactors flavin-adenine dinucleotide (FAD), thiamine pyrophosphate (TPP), and magnesium. Enzymatic activity is assessed spectrophotometrically by the amount of acetolactate formed by the reaction. This activity assay cannot differentiate the production of acetolactate produced by the newly expressed AHAS(*At*) [A122TS653N] or the endogenous AHAS(*Bn*) protein. It is also known that native AHAS activity is highest in young plant tissue (Singh and Shaner, 1995). The enzymatic activity was measured in both leaf tissue and PPP for both LBFLFK and Kumily samples. Only the results of the leaf tissue enzyme activity assays, including inhibition studies, are presented here.

LBFLFK leaf samples showed a specific activity of 0.999 nmol/min/mg while the Kumily leaf samples showed a specific activity of 0.646 nmol/min/mg.

AHAS activities in LBFLFK and Kumily leaf tissues were measured in the presence of increasing concentrations of the herbicide imazamox (1–100 µM) as shown in Figure C.30. LBFLFK leaf samples showed a greater tolerance to increasing imazamox concentrations with 41% activity remaining at 100 µM imazamox compared to the 19% activity remaining in Kumily leaf samples. LBFLFK leaf tissue has both the newly expressed AHAS(*At*) [A122TS653N] and the endogenous genes that produce AHAS(*Bn*) protein with low tolerance to imazamox. Kumily leaf tissue only expresses the endogenous AHAS(*Bn*) protein.

AHAS activities in LBFLFK and Kumily leaf tissues were also measured in the presence of increasing concentrations (5 µM–1 mM) of the AHAS feedback inhibitors leucine and valine (Singh and Shaner, 1995) as shown in Figure C.31. LBFLFK and Kumily leaf tissues showed similar sensitivity to increasing leucine and valine concentrations with 62% and 57% activities remaining at 1 mM, respectively. This result indicates that the newly expressed AHAS(*At*) [A122TS653N] protein maintained the same amount of feedback inhibition from leucine and valine as the endogenous AHAS(*Bn*) proteins.

Figure C.30. Inhibition of Leaf AHAS activity in EPA+DHA Canola event LBFLFK and the Parental Control Variety Kumily by Imidazolinone Herbicide, Imazamox

Leaf extracts from event LBFLFK and the parental variety Kumily were incubated in a buffer containing pyruvate and using thiamine pyrophosphate (TPP) as a co-enzyme with Mg^{2+} and flavin adenine dinucleotide (FAD) as a cofactor. Acetolactate produced by AHAS is converted to acetoin in the presence of acid, and acetoin is detected colorimetrically (A_{530} nm) after interaction with creatine and naphthol. Inhibition was measured with the inclusion of imazamox in the assay buffer at increasing concentrations and reported below as % activity remaining compared to samples without imazamox. Squares (■) represent Kumily leaf extract samples, and circles (●) represent LBFLFK leaf extract samples.

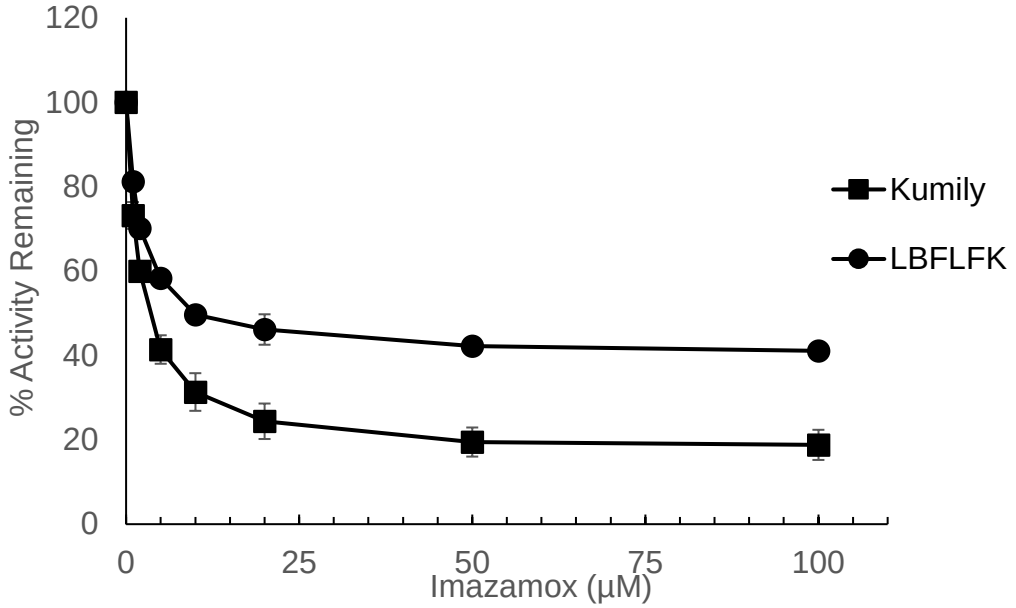
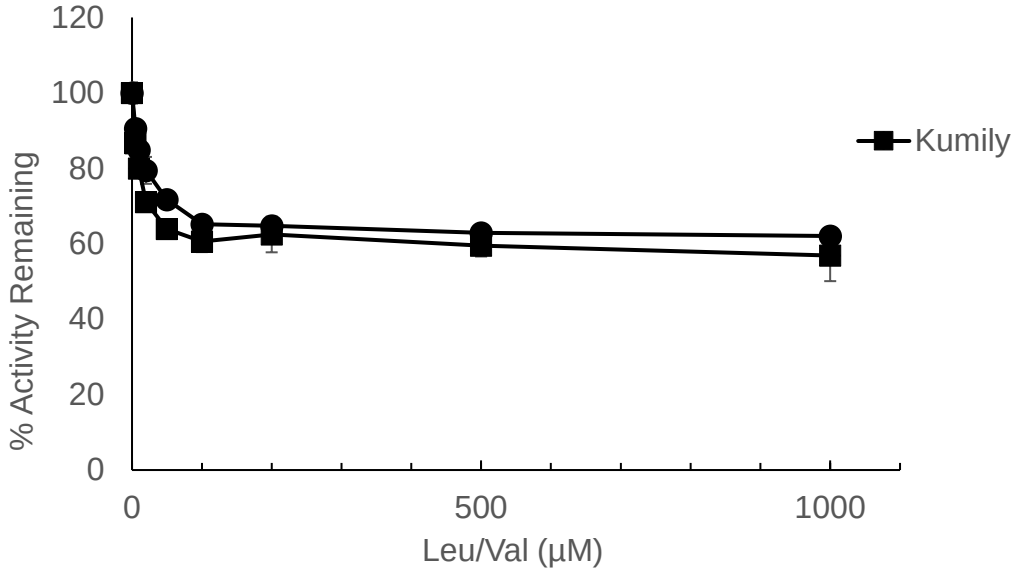


Figure C.31. Feedback Inhibition of Leaf AHAS Activity in EPA+DHA Canola Event LBFLFK and the Parental Control Variety Kumily by Valine and Leucine

Leaf extracts from event LBFLFK and the parental variety Kumily were incubated in a buffer containing pyruvate and using thiamine pyrophosphate (TPP) as a co-enzyme with Mg^{2+} and flavin adenine dinucleotide (FAD) as a cofactor. Acetolactate produced by AHAS is converted to acetoin in the presence of acid, and acetoin is detected colorimetrically (A_{530} nm) after interaction with creatine and naphthol. Inhibition was measured with the inclusion of leucine and valine in the assay buffer at increasing concentrations and reported below as % activity remaining compared to samples without leucine and valine. Squares (■) represent Kumily leaf extract samples, and circles (●) represent LBFLFK leaf extract samples.



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Appendix D. Levels of Newly Expressed Proteins in EPA+DHA Canola Event LBFLFK – Materials, Methods, and Summary of Results

D.1. Materials

D.1.1. Test and control substances

Whole plant at rosette stage, whole plant at flowering stage, immature seed, leaf, root, and mature seed field samples from EPA+DHA canola event LBFLFK were derived from seed lot ID 910019041494. Pollen samples from LBFLFK were derived from seed lot ID 2049146661. The seed lot IDs for Kumily were 910019041495 and 2049146671 for field and pollen samples, respectively.

D.1.2. Protein reference substances

Recombinantly expressed protein standards were prepared in-house for all 11 proteins and served as reference substances.

Large-scale production and purification of the 10 desaturase and elongase proteins using heterologous systems failed to yield suitable quantities of full-length protein for use as reference standards, except for D6D(*Ot*) and D5D(*Tc*). However, removal of hydrophobic stretches between the N-terminus and the C-terminus of the full-length proteins resulted in NC fusion proteins that could be overexpressed and used for the generation of antibodies and as reference substances for the detection of the proteins. The stoichiometry between antibody and target protein was expected to be the same for the NC fusion proteins and the full-length protein.

Each protein reference substance was used to prepare a calibration standard curve in each experiment.

D.1.3. Antibodies

Antibodies used in individual protein characterization experiments are listed in Table D.1.

Table D.1. Antibodies used in Individual Protein Characterization Experiments

Antibody	Target Protein	Antigen	Protein Characterization Experiment	Type	Animal	Grade
AB128	D12D(*Ps*)	N-terminal fragment of full length protein	Apparent Molecular Weight, Glycosylation Analysis, Immunoreactivity, Protein Quantification (ELISA)	polyclonal	goat	Purified IgG
AB129	D12D(*Ps*)	N-terminal fragment of full length protein	Protein Quantification (ELISA)	polyclonal	rabbit	Purified IgG
AB111	D6D(*Ot*)	N-terminal fragment	Apparent Molecular Weight, Glycosylation Analysis, Immunoreactivity	polyclonal	rabbit	Purified IgG
AB404	D6D(*Ot*)	full length protein	Protein Quantification (Wes)	polyclonal	rabbit	Antigen Purified
AB416	D6E(*Tp*)	N-terminal peptide	Protein Quantification (Wes)	polyclonal	goat	Antigen Purified
AB124	D6E(*Tp*)	N-terminal peptide	Apparent Molecular Weight, Glycosylation Analysis, Immunoreactivity	polyclonal	rabbit	Antigen Purified
AB422	D6E(P*p*)	fusion of N-terminal and C-terminal fragment	Apparent Molecular Weight, Immunoreactivity	polyclonal	rabbit	Antigen Purified
AB415	D6E(P*p*)	N-terminal peptide	Protein Quantification	polyclonal	goat	Antigen Purified
AB401	D6E(P*p*)	N-terminal fragment	Protein Quantification	polyclonal	chicken	Antigen Purified
AB353 + AB354	D5D(*Tc*)	N-terminal peptide	Apparent Molecular Weight, Glycosylation Analysis, Immunoreactivity	polyclonal	goat	Antigen Purified
AB417	D5D(*Tc*)	N-terminal peptide	Protein Quantification (ELISA)	polyclonal	goat	Antigen Purified
AB100	D5D(*Tc*)	N-terminal fragment	Protein Quantification (ELISA)	polyclonal	chicken	Purified IgY
AB212	O3D(*Pir*)	N-terminal peptide	Apparent Molecular Weight, Glycosylation Analysis, Immunoreactivity, Protein Quantification (Wes)	polyclonal	rabbit	Antigen Purified
AB403	O3D(*Pi*)	C-terminal peptide	Certificate of Analysis, Apparent Molecular Weight, Immunoreactivity, Protein Quantification (Wes)	monoclonal	mouse	Purified IgG
AB214	D5E(*Ot*)	fusion of N-terminal and C-terminal fragment	Protein Quantification (ELISA)	polyclonal	rabbit	Antigen Purified
AB215	D5E(*Ot*)	fusion of N-terminal and C-terminal fragment	Protein Quantification (ELISA)	polyclonal	chicken	Antigen Purified

Antibody	Target Protein	Antigen	Protein Characterization Experiment	Type	Animal	Grade
AB402	D5E(*Ot*)	N-terminal peptide and C-terminal peptide	Apparent Molecular Weight, Glycosylation Analysis, Immunoreactivity	polyclonal	rabbit	Antigen Purified
AB326	D4D(*Pl*)	fusion of N-terminal and C-terminal fragment	Apparent Molecular Weight, Glycosylation Analysis, Immunoreactivity	polyclonal	rabbit	Antigen Purified
AB363	D4D(*Pl*)	fusion of N-terminal and C-terminal fragment	Protein Quantification (Wes)	polyclonal	rabbit	Antigen Purified
AB217	D4D(*Tc*)	fusion of N-terminal and C-terminal fragment	Protein Quantification (Wes)	polyclonal	rabbit	Antigen Purified
AB366	D4D(*Tc*)	fusion of N-terminal and C-terminal fragment	Apparent Molecular Weight, Glycosylation Analysis, Immunoreactivity	polyclonal	rabbit	Purified IgG
AB414	AHAS(*At*)	N-terminal fragment excluding plastidial targeting signal	Apparent Molecular Weight, Glycosylation Analysis, Immunoreactivity, Protein Quantification (Wes)	polyclonal	rabbit	Antigen Purified
AB461.07-2-5	AHAS(*At*)	fusion of one N-terminal and two C-terminal fragments	Immunopurification	monoclonal	mouse	Purified IgG

D.2. Methods

D.2.1. Field trial design

In the 2015 growing season, four trials were established in the United States at four sites representative of areas where canola production is possible in North America and where the soil type is typical for commercial canola production (Table D.2).

Table D.2. Field Trial Designations, Locations, and Planting Dates

Field Trial Designation	Nearest Town, State	Planting Date
3NRGE	Geneva, MN	20-May-2015
3NRFS	Sun River, MT	25-May-2015
3NRAF	American Falls, ID	28-May-2015
3NREP	Ephrata, WA	19-May-2015

At each location, three entries, including LBFLFK (sprayed), LBFLFK (non-sprayed), and Kumily, each received standard herbicide applications to maintain health of the plots. LBFLFK (sprayed) also received an application of Beyond®[1] herbicide (active ingredient imazamox, an imidazolinone) at a nominal rate of 35–37.7 g a.i./ha. The application of Beyond® herbicide was made at the 3–4 leaf stage when average crop height was 8–13 centimeters.

All other agronomic practices, including use of other standard management practices for weed and pest control, were typical for canola production in the region of each field site and were applied equally to all field plots at a given site.

The study was designed as a single replication of each plot. Plot size ranged from approximately 34 m^2 (366 square feet) to approximately 50 m^2 (538 square feet). Each plot consisted of 14 to 36 rows per plot. Row length ranged from 6 m (20 feet) to 15.2 m (50 feet). Row spacing ranged from 16 cm (6 inches) to approximately 23 cm (9 inches). Plots were spaced at least 15 m (50 feet) apart, within the limits of accuracy of field equipment. Each plot was clearly staked and labeled at planting.

A conventional male sterile variety was used as buffer and border surrounding the trial area at each field site.

[1] Beyond is a registered trademark of BASF Corp.

D.2.2. Tissue sampling and homogenization of field tissue samples

A total of ten test plants (five sprayed and five non-sprayed) and one control plant were collected non-systematically at each sampling point (Table D.3). The leaf, root, and immature seed samples were collected from the same sampled plant. Seeds that were not green in color were not included in the immature seed samples. For collection of mature seed, plants were swathed at approximately BBCH 85 (Meier, 2001), and all seeds from the swathed plants to be sampled were collected once they were visually estimated to have seed moisture below approximately 10 percent (BBCH 99).

All samples were placed in a plastic bag on dry ice within 30 minutes of sample collection and transferred to a -80°C freezer until analysis.

Table D.3. Tissue Samples for Protein Quantification Taken at Each Location

Tissue[1]	Plant Stage (BBCH Scale[2])	# Plants/ Samples	Number Samples/Plot		
			Control	LBFLFK (sprayed)[3]	LBFLFK (non-sprayed)[4]
Whole Plant	Rosette stage (BBCH 16–51)	1	1	5	5
Whole Plant	Flowering stage (BBCH 64–65)	1	1	5	5
Leaf	Early maturity stage (BBCH 75–79)	10	1	5	5
Root	Early maturity stage (BBCH 75–79)	1	1	5	5
Immature Seed[5]	Early maturity stage (BBCH 75–79)	1	1	5	5
Mature Seed[5]	Maturity (BBCH 99)	5	1	5	5
Pollen	Flowering stage (BBCH 61–63)	411/353[6]	1	N/A	2

[1] Tissue collection (except pollen) was replicated at four field sites.
[2] BBCH scale as determined for canola (Meier, 2001).
[3] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[4] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[5] Seeds were removed from pods. Only seeds at the appropriate developmental stage were collected.
[6] Pollen of 411 plants was used for two pollen samples of Kumily and 353 plants for two pollen samples of LBFLFK.

Tissues were homogenized into powdered samples to a consistency appropriate for quantitative analysis, weighed into primary storage containers, aliquoted into separate containers for expression analysis, and stored again at approximately -80°C in a freezer.

D.2.3. Production and sampling of pollen in the greenhouse

LBFLFK and Kumily were grown in parallel in separate bays of a greenhouse. Two cycles of production were used. Pollen collection was initiated when plants reached at least BBCH 61 (first flowers opened). To maximize the amount of pollen that could be collected from each set of plants, pollen collection was performed over a period of 14–15 days. Pollen was collected from open flowers and sieved, weighed, placed on dry ice, and transferred to a -80°C freezer once the sampling for the day was completed. Prior to shipping samples for analysis, all samples of the same genotype and cycle were combined to generate bulk samples (two LBFLFK and two Kumily samples).

D.2.4. Moisture analysis and conversion of protein level measurements from fresh to dry weight

To estimate the moisture content of each analyzed tissue sample, aliquots of a pre-determined sample weight were dried, re-weighed, and percentage moisture was calculated.

For tissue samples collected from the field sites, three samples per tissue type were analyzed for moisture from each field site: a subsample of Kumily, a pool of subsamples from LBFLFK (non-sprayed), and a pool of subsamples from LBFLFK (sprayed). For the pollen samples collected from the greenhouse, three subsamples from each bulk sample were analyzed.

The average percent moisture for each sample or sample pool was used to determine a dry weight conversion factor (DWCF). The DWCF was used to convert protein levels assessed on a fresh weight basis into levels reported on a dry weight basis. For details of the calculations used to determine values for average percent moisture and DWCF, see section D.3 below.

D.2.5. Protein quantification

Each frozen tissue sample was weighed into a polypropylene tube along with a stainless-steel bead and an appropriate volume of a method-specific extraction buffer for extraction of each of the newly expressed proteins. The extracted samples were diluted to at least the validated minimum required dilution (MRD) and analyzed using either enzyme-linked immunosorbent assay (ELISA) or quantitative western blot.

Procedural quality control (QC) samples were included in each protein quantification experiment to ensure each run was within acceptance criteria determined during method validation (50–120% recovery of the nominal fortified protein reference standard concentration). QC samples were prepared by fortifying Kumily tissue of the sample type being analyzed with the corresponding protein reference standard. The fortified extraction

buffers were prepared at two concentration levels, 1–2X the limit of quantitation (LOQ) and at the approximate midpoint of the calibration range. Exceptions for meeting the preset acceptance criteria were made when results for a sample type were low across field sites or if repeat analysis yielded similar recoveries. Newly expressed protein quantification results were not normalized to procedural QC recovery values.

Each of the homogenized sample lysates was analyzed for quantity of the newly expressed proteins using validated quantitative immunoassays.

Each of the eleven immunoassays was developed utilizing antibodies specific to a newly expressed protein and corresponding reference substance. Extraction and dilution buffers and extraction conditions were optimized for each protein and each tissue type.

Validation of the quantitative immunoassays included determination of the following method performance quality characteristics: calibration range, MRD, and quantification range, including limits of quantitation and detection.

The method calibration range was established using a protein reference standard with concentrations evenly distributed across the range and fit with an appropriate regression. The MRD was defined as the dilution of each tissue sample lysate at which interference from matrix effects was negligible. The method LOQ was defined by the lowest amount of reference substance that could reliably be recovered when spiked into a given canola tissue. The method limit of detection (LOD) was defined as the lowest reference substance concentration in the calibration range. The LOQ and LOD are reported on both a fresh weight and dry weight basis for each method and are included in Table D.15 to Table D.24.

D.2.5.1. Quantification via ELISA

Validated ELISAs were utilized to determine the amounts of D12D(*Ps*), D6E(*Pp*), D5D(*Tc*), and D5E(*Ot*) present in the tissue samples.

A capture antibody, specific for the target protein, was coated on an immunoassay plate. Protein reference standards and sample extracts were added to the plate, binding the targeted protein to the coating antibody. Unbound substances were washed away between each reagent incubation step. A detection antibody, specific for the target protein, was then added to the plate, binding to the target protein and creating a ‘sandwich’ between the two antibodies. A peroxidase-labeled secondary antibody, specific to the species of the detection antibody, was added, followed by a tetramethylbenzidine substrate solution that caused chromogenic color development correlating to the amount of newly expressed protein present in the samples. Color development was stopped by addition of 1N HCl, and the intensity of the color was measured spectrophotometrically at 450 nanometers (nm). Sample concentrations were interpolated from a calibration curve

fit to the reference standards using a 4- or 5-parameter logistic regression. Each homogenized tissue sample lysate was analyzed once in technical triplicate except for pollen samples, which were analyzed three times in technical triplicate. The corresponding optical densities (OD) were used to determine the newly expressed protein content.

D.2.5.1.a. D12D(Ps)

The validated D12D(*Ps*) immunoassay is an ELISA method that uses a polyclonal capture antibody (AB128) in combination with a polyclonal detection antibody (AB129). Both antibodies were generated with an N-terminal D12D(*Ps*) protein fragment antigen. The method specifically detects the delta-12 desaturase (*Ps*) NC fusion protein (Std D12D-0114) reference substance and the newly expressed D12D(*Ps*) protein.

Table D.4. D12D(*Ps*) Validated Method Parameters

Standardized D12D(*Ps*) Method Parameters		
Capture Antibody	AB128, goat (19.5 mg/ml diluted to 6.50 µg/ml	
Detection Antibody	AB129, rabbit (2.90 mg/ml) diluted to 0.97 µg/ml	
Secondary Antibody	Donkey anti-rabbit IgG (H+L) HRP (horseradish peroxidase)	
Calibration Range	0.117–15.0 nanograms (ng)/ml	
Extraction Buffer	1X TBS (50 mM Tris, 150 mM NaCl, pH 7.6), 1% Triton™[2] X-100, 1X EDTA-free protease inhibitor	
Tissue-Specific D12D(*Ps*) Method Parameters		
Tissue Type	**Extraction Ratio (tissue mass:buffer volume)**	**Minimum Required Dilution**
Whole Plant at Rosette Stage	1:15	32X
Whole Plant at Flowering Stage	1:15	32X
Immature Seed	1:15	64X
Leaf	1:30	128X
Root	1:15	8X
Mature Seed	1:30	16X
Pollen	1:60	4X

[2] Triton is a trademark of The Dow Chemical Company.

D.2.5.1.b. D6E(*Pp*)

The validated D6E(*Pp*) immunoassay is an ELISA method that uses a polyclonal capture antibody (AB415) in combination with a polyclonal detection antibody (AB401). AB415 was generated with an N-terminal D6E(*Pp*) peptide antigen while AB401 was generated with an N-terminal D6E(*Pp*) protein fragment antigen. The method specifically detects the delta-6 elongase (*Pp*) NC fusion protein (Std D6EPp-0114) reference substance and the newly expressed D6E(*Pp*) protein.

Table D.5. D6E(*Pp*) Validated Method Parameters

Standardized D6E(*Pp*) Method Parameters	
Capture Antibody	AB415, goat (1.01 mg/ml) diluted to 2.00 µg/ml
Detection Antibody	AB401, chicken (0.45 mg/ml) diluted to 0.30 µg/ml
Secondary Antibody	Donkey anti-chicken IgY (H+L) HRP
Calibration Range	0.50–64.00 ng/ml
Extraction Buffer	1X TBST (25 mM Tris pH 7.4, 3 mM KCl, 140 mM NaCl, 0.05% TWEEN®-20[3]), 1% Triton™ X-100, 1X EDTA-free protease inhibitor

Tissue-Specific D6E(*Pp*) Method Parameters		
Tissue Type	**Extraction Ratio (tissue mass:buffer volume)**	**Minimum Required Dilution**
Whole Plant at Rosette Stage	1:30	16X
Whole Plant at Flowering Stage	1:30	16X
Immature Seed	1:30	32X
Leaf	1:30	32X
Root	1:30	16X
Mature Seed	1:15	64X
Pollen	1:60	16X

[3] TWEEN is a registered trademark of Croda Americas LLC.

D.2.5.1.c. D5D(*Tc*)

The validated D5D(*Tc*) immunoassay is an ELISA method that uses a polyclonal capture antibody (AB417) in combination with a polyclonal detection antibody (AB100). AB417 was generated with an N-terminal D5D(*Tc*) peptide antigen while AB100 was generated with an N-terminal D5D(*Tc*) protein fragment antigen. The method specifically detects the delta-5 desaturase (*Tc*) full length protein (Std D5D-0113) reference substance and the newly expressed D5D(*Tc*) protein.

Table D.6. D5D(*Tc*) Validated Method Parameters

Standardized D5D(*Tc*) Method Parameters	
Capture Antibody	AB417, goat (1.00 mg/ml) diluted to 0.45 µg/ml
Detection Antibody	AB100, chicken (11.5 mg/ml) diluted to 0.30 µg/ml
Secondary Antibody	Donkey anti-chicken IgY (H+L) HRP
Calibration Range	2.00–256.00 ng/ml
Extraction Buffer # 1	1X TBST (25 mM Tris pH 7.4, 3 mM KCl, 140 mM NaCl, 0.05% Tween®-20), 1% Triton™ X-100, 1X EDTA-free protease inhibitor, 1% LysoFos Choline-12
Extraction Buffer # 2	1X TBST (25 mM Tris pH 7.4, 3 mM KCl, 140 mM NaCl, 0.05% Tween®-20), 1% Triton™ X-100, 1X EDTA-free protease inhibitor, 1% LysoFos Choline-12, 2.5% 2-mercaptoethanol
Dilution Buffer	1X TBST (25 mM Tris pH 7.4, 3 mM KCl, 140 mM NaCl, 0.05% Tween®-20), 1% Triton™ X-100, 1X EDTA-free protease inhibitor

Tissue-Specific D5D(*Tc*) Method Parameters			
Tissue Type	**Extraction Ratio (tissue mass:buffer volume)**	**Minimum Required Dilution**	**Extraction Buffer**
Whole Plant at Rosette Stage	1:60	32X	Extraction Buffer #1
Whole Plant at Flowering Stage	1:60	32X	Extraction Buffer #2
Immature Seed	1:30	64X	Extraction Buffer #1
Leaf	1:60	64X	Extraction Buffer #1
Root	1:30	32X	Extraction Buffer #1
Mature Seed	1:15	16X	Extraction Buffer #1
Pollen	1:60	16X	Dilution Buffer

D.2.5.1.d. D5E(*Ot*)

The validated D5E(*Ot*) immunoassay is an ELISA method that uses a polyclonal capture antibody (AB214) in combination with a polyclonal detection antibody (AB215). Both antibodies were generated with a D5E(*Ot*) NC fusion protein antigen. The method specifically detects the delta-5 elongase (*Ot*) NC fusion protein (Std D5E-0114) reference substance and the newly expressed D5E(*Ot*) protein.

Table D.7. D5E(*Ot*) Validated Method Parameters

Standardized D5E(*Ot*) Method Parameters	
Capture Antibody	AB214, rabbit (0.679 mg/ml) diluted to 0.45 µg/ml
Detection Antibody	AB215, chicken (0.60 mg/ml) diluted to 0.40 µg/ml
Secondary Antibody	Donkey anti-chicken IgY (H+L) HRP
Calibration Range	0.33–20.00 ng/ml
Extraction Buffer # 1	1X TBST (25 mM Tris pH 7.4, 3 mM KCl, 140 mM NaCl, 0.05% Tween®-20), 1% Triton™ X-100, 1X EDTA-free protease inhibitor, 2.5% 2-mercaptoethanol
Extraction Buffer # 2	1X TBST (25 mM Tris pH 7.4, 3 mM KCl, 140 mM NaCl, 0.05% Tween®-20), 1% Triton™ X-100, 1X EDTA-free protease inhibitor, 2.5% 2-mercaptoethanol, 1% BSA (bovine serum albumin)
Extraction Buffer # 3	1X TBST (25 mM Tris pH 7.4, 3 mM KCl, 140 mM NaCl, 0.05% Tween®-20), 1% Triton™ X-100, 1X EDTA-free protease inhibitor, 1% 2-mercaptoethanol, 1% BSA, 1% SDS (sodium dodecyl sulfate)
Dilution Buffer # 1	1X TBST (25 mM Tris pH 7.4, 3 mM KCl, 140 mM NaCl, 0.05% Tween®-20), 1% Triton™ X-100, 1X EDTA-free protease inhibitor
Dilution Buffer # 2	1X TBST (25 mM Tris pH 7.4, 3 mM KCl, 140 mM NaCl, 0.05% Tween®-20), 1% Triton™ X-100, 1X EDTA-free protease inhibitor, 1% BSA

Tissue-Specific D5E(*Ot*) Method Parameters				
Tissue Type	**Extraction Ratio (tissue mass:buffer volume)**	**Minimum Required Dilution**	**Extraction Buffer**	**Dilution Buffer**
Whole Plant at Rosette Stage	1:30	32X	Extraction Buffer #2	Dilution Buffer #2
Whole Plant at Flowering Stage	1:30	32X	Extraction Buffer #2	Dilution Buffer #2
Immature Seed	1:30	64X	Extraction Buffer #1	Dilution Buffer #1
Leaf	1:60	32X	Extraction Buffer #2	Dilution Buffer #2
Root	1:50	16X	Extraction Buffer #2	Dilution Buffer #2
Mature Seed	1:30	64X	Extraction Buffer #1	Dilution Buffer #1
Pollen	1:60	64X	Extraction Buffer #3	Dilution Buffer #1

D.2.5.2. Quantification via western blot analysis

Validated quantitative western blot methods were used to determine the amounts of D6D(*Ot*), D6E(*Tp*), O3D(*Pi*), O3D(*Pir*), D4D(*Pl*), D4D(*Tc*), and AHAS(*At*) [A122TS653N] present in the tissue samples.

Samples and reference standards were separated by molecular weight within a capillary, immobilized to the capillary, and then detected with a protein-specific primary antibody. The detection antibody was then probed with a peroxidase-linked secondary antibody specific to the species of the detection antibody. Finally, the addition of a luminol-peroxide substrate generated chemiluminescent signals. These signals were recorded with a camera, the resulting digital images were processed into electropherograms, and the chemiluminescent peak areas were quantified using the computer software Compass (v 2.7.1, ProteinSimple, San Jose, CA). Sample concentrations were interpolated from a calibration curve fit to the reference standards using linear regression. Each homogenized tissue sample lysate was analyzed once as a single technical replicate except for pollen samples, which were analyzed three times as a single technical replicate. The corresponding peak areas were used to determine the newly expressed protein content.

D.2.5.2.a. D6D(*Ot*)

The validated D6D(*Ot*) immunoassay is a quantitative western blot method that uses a polyclonal primary antibody (AB404) generated with full-length D6D(*Ot*) protein antigen. The method specifically detects the delta-6 desaturase (*Ot*) full length protein (Std D6D-0114) reference substance and the newly expressed D6D(*Ot*) protein.

Table D.8. D6D(*Ot*) Validated Method Parameters

Standardized D6D(*Ot*) Method Parameters		
Primary Antibody	AB404, rabbit (0.1 mg/ml) diluted to 5 µg/ml	
Secondary Antibody	Anti-rabbit HRP, undiluted	
Calibration Range	7.50–120.00 ng/ml	
Extraction Buffer	1X TBS (25 mM Tris pH 7.4, 3 mM KCl, 140 mM NaCl), 1% Fos-Choline-12, 1X EDTA-free protease inhibitor	
Tissue-Specific D6D(*Ot*) Method Parameters		
Tissue Type	**Extraction Ratio (tissue mass:buffer volume)**	**Minimum Required Dilution**
Whole Plant at Rosette Stage	1:30	16X
Whole Plant at Flowering Stage	1:30	16X
Immature Seed	1:15	16X
Leaf	1:30	16X
Root	1:30	16X
Mature Seed	1:30	16X
Pollen	1:30	8X

D.2.5.2.b. D6E(*Tp*)

The validated D6E(*Tp*) immunoassay is a quantitative western blot method that uses a polyclonal primary antibody (AB416) generated with an N-terminal D6E(*Tp*) peptide antigen. The method specifically detects the delta-6 elongase (*Tp*) NC fusion protein reference substance (Std D6ETp-0217) and the newly expressed D6E(*Tp*) protein.

Table D.9. D6E(*Tp*) Validated Method Parameters

Standardized D6E(*Tp*) Method Parameters	
Primary Antibody	AB416, goat (1.0 mg/ml) diluted to 66.67 µg/ml
Secondary Antibody	Biotin-SP-donkey anti-goat IgG diluted to 1.3 µg/ml in combination with Streptavidin poly-HRP diluted to 0.167 µg/mL
Calibration Range	51.80–250.00 ng/ml
Extraction Buffer	25 mM Tris pH8, 5 mM KCl, 800 mM NaCl, 0.5% DDM (n-dodecyl-D-maltoside), 0.8% Tween®-20, 3 mM TCEP (tris(2-carboxyethyl)phosphine), 1X EDTA-free protease inhibitor

Tissue-Specific D6E(*Tp*) Method Parameters		
Tissue Type	**Extraction Ratio (tissue mass:buffer volume)**	**Minimum Required Dilution**
Whole Plant at Rosette Stage	1:10	16X
Whole Plant at Flowering Stage	1:10	16X
Immature Seed	1:20	40X
Leaf	1:10	16X
Root	1:5	16X
Mature Seed	1:40	56X
Pollen	1:20	24X

D.2.5.2.c. O3D(*Pi*)

The validated O3D(*Pi*) immunoassay is a quantitative western blot method that uses a monoclonal primary antibody (AB403) generated with C-terminal O3D(*Pi*) peptide antigen. The method specifically detects the omega-3 desaturase (*Pi*) NC fusion protein (Std O3DPi-0115) reference substance and the newly expressed O3D(*Pi*) protein.

Table D.10. O3D(*Pi*) Validated Method Parameters

Standardized O3D(*Pi*) Method Parameters		
Primary Antibody	AB403, mouse (1.18 mg/ml) diluted to 118 µg/ml	
Secondary Antibody	Goat anti-mouse poly-HRP, diluted to 33.3 µg/ml	
Calibration Range	5.72–60.00 ng/ml	
Extraction Buffer	1X PBS, 1% Triton™ X-100, 2mM TCEP, 5 mM EDTA, 10 mM Sodium Metabisulfite, 1:50 Protease Inhibitor Cocktail	
Tissue-Specific O3D(*Pi*) Method Parameters		
Tissue Type	**Extraction Ratio (tissue mass:buffer volume)**	**Minimum Required Dilution**
Whole Plant at Rosette Stage	1:30	32X
Whole Plant at Flowering Stage	1:30	64X
Immature Seed	1:15	16X
Leaf	1:50	64X
Root	1:30	32X
Mature Seed	1:30	80X
Pollen	1:20	32X

D.2.5.2.d. O3D(*Pir*)

The validated O3D(*Pir*) immunoassay is a quantitative western blot method that uses a polyclonal primary antibody (AB212) generated with N-terminal O3D(*Pir*) peptide antigen. The method specifically detects the omega-3 desaturase (*Pir*) NC fusion protein (Std O3Dpir-0114) reference substance and the newly expressed O3D(*Pir*) protein.

Table D.11. O3D(*Pir*) Validated Method Parameters

Standardized O3D(*Pir*) Method Parameters		
Primary Antibody	AB212, rabbit (2.18 mg/ml) diluted to 21.80 µg/ml	
Secondary Antibody	Anti-rabbit HRP, undiluted	
Calibration Range	5.72–60.00 ng/ml	
Extraction Buffer	1X TBS, 0.1% Tween®-20, 1% Triton™ X-100, 1X EDTA-free protease inhibitor	
Tissue-Specific O3D(*Pir*) Method Parameters		
Tissue Type	**Extraction Ratio (tissue mass:buffer volume)**	**Minimum Required Dilution**
Whole Plant at Rosette Stage	1:30	30X
Whole Plant at Flowering Stage	1:30	30X
Immature Seed	1:30	30X
Leaf	1:30	40X
Root	1:50	70X
Mature Seed	1:30	90X
Pollen	1:25	20X

D.2.5.2.e. D4D(*Pl*)

The validated D4D(*Pl*) immunoassay is a quantitative western blot method that uses a polyclonal primary antibody (AB363) generated with a D4D(*Pl*) NC fusion protein antigen. The method specifically detects the delta-4 desaturase (*Pl*) NC fusion protein (Std D4DPl-0114) reference substance and the newly expressed protein D4D(*Pl*) protein.

Table D.12. D4D(*Pl*) Validated Method Parameters

Standardized D4D(*Pl*) Method Parameters		
Primary Antibody	AB363, rabbit (0.8 mg/ml) diluted to 16 µg/ml	
Secondary Antibody	Anti-rabbit HRP, undiluted	
Calibration Range	3.81–40 ng/ml	
Extraction Buffer	50 mM Tris pH 9.5, 150 mM NaCl, 2 mM EDTA, 2 mM TCEP, 1% Triton™ X-100, 1X EDTA-free protease inhibitor	
Tissue-Specific D4D(*Pl*) Method Parameters		
Tissue Type	**Extraction Ratio (tissue mass:buffer volume)**	**Minimum Required Dilution**
Whole Plant at Rosette Stage	1:15	16X
Whole Plant at Flowering Stage	1:15	24X
Immature Seed	1:15	8X
Leaf	1:15	16X
Root	1:15	24X
Mature Seed	1:15	16X
Pollen	1:15	16X

D.2.5.2.f. D4D(*Tc*)

The validated D4D(*Tc*) immunoassay is a quantitative western blot method that uses a polyclonal primary antibody (AB217) generated with a D4D(*Tc*) NC fusion protein antigen. The method specifically detects the delta-4 desaturase (*Tc*) NC fusion protein (Std D4DTc-0114) reference substance and the newly expressed D4D(*Tc*) protein.

Table D.13. D4D(*Tc*) Validated Method Parameters

Standardized D4D(*Tc*) Method Parameters		
Primary Antibody	AB217, rabbit (3.74 mg/ml) diluted to 12.5 µg/ml	
Secondary Antibody	Anti-rabbit HRP, undiluted	
Calibration Range	3.29–25.00 ng/ml	
Extraction Buffer	50 mM Tris pH 9.5, 150 mM NaCl, 2 mM EDTA, 2 mM TCEP, 1% Triton™ X-100, 1X EDTA-free protease inhibitor	
Tissue-Specific D4D(*Tc*) Method Parameters		
Tissue Type	**Extraction Ratio (tissue mass:buffer volume)**	**Minimum Required Dilution**
Whole Plant at Rosette Stage	1:10	16X
Whole Plant at Flowering Stage	1:20	32X
Immature Seed	1:20	20X
Leaf	1:20	32X
Root	1:10	32X
Mature Seed	1:30	28X
Pollen	1:10	16X

D.2.5.2.g. AHAS(*At*) [A122TS653N]

The validated AHAS(At) [A122TS653N] immunoassay is a quantitative western blot method that uses a polyclonal antibody (AB414) generated with AHAS(*At*) N-terminal fragment excluding the plastidial targeting signal as antigen. The method specifically detects the acetohydroxy acid synthase (AtAHAS) [A122TS653N] (Std AHAS-0114) reference substance, the newly expressed acetohydroxy acid synthase (AHAS(*At*) [A122TS653N]), and the native *Brassica napus* acetohydroxy acid synthase (AHAS(*Bn*)) proteins. During optimization of the immunoassay, the MRD was adjusted such that AHAS(*Bn*) expressed in Kumily was below the LOD of the method while good recovery of reference standard with procedural QC samples was maintained. Therefore, detection of AHAS(*Bn*) was not expected in the plant tissue samples, and the AHAS concentrations reported by the method therefore represent just the newly expressed AHAS(*At*) [A122TS653N].

Table D.14. AHAS(*At*) [A122TS653N] Validated Method Parameters

Standardized AHAS(*At*) [A122TS653N] Method Parameters			
Primary Antibody	AB414, rabbit (0.2 mg/ml) diluted to 13.3 µg/ml		
Secondary Antibody	Anti-rabbit HRP, undiluted		
Calibration Range	0.63–20.00 ng/ml		
Extraction Buffer 1	1X PBS, 1% Triton™ X-100, 2 mM TCEP, 1:50 Protease Inhibitor Cocktail		
Extraction Buffer 2	120 mM Tris pH 8.0, 20% glycerol, 2% lithium dodecyl sulfate, 50 mM TCEP, final pH 12.0		
Pollen Dilution Buffer	120 mM Tris pH 8.0, 20% glycerol, 2% lithium dodecyl sulfate, 50 mM TCEP, final pH 8.0		
Tissue-Specific AHAS(*At*) [A122TS653N] Method Parameters			
Tissue Type	**Extraction Ratio (tissue mass:buffer volume)**	**Minimum Required Dilution**	**Extraction Buffer**
Whole Plant at Rosette Stage	1:15	20X	Extraction Buffer 1
Whole Plant at Flowering Stage	1:15	20X	Extraction Buffer 1
Immature Seed	1:30	24X	Extraction Buffer 1
Leaf	1:20	12X	Extraction Buffer 1
Root	1:10	8X	Extraction Buffer 1
Mature Seed	1:40	24X	Extraction Buffer 1
Pollen	1:100	48X	Extraction Buffer 2

D.3. Data analysis and statistics

Calculated concentrations of newly expressed proteins in each sample were interpolated from protein reference standard calibration curves fit to a 4- or 5-parameter logistic regression for ELISA or linear regression for western blot, respectively.

The quantified concentration for each of the newly expressed proteins in lysates prepared from each homogenized tissue sample was adjusted to account for the tissue to extraction buffer ratio and any subsequent dilution into buffer. This value was then corrected for the molecular weight difference between the reference standard and the corresponding newly expressed protein using the molecular weight correction factor (MWCF). The resultant value was represented as the concentration of newly expressed protein on a fresh weight basis. To report the quantified value on a dry weight basis, the fresh weight expression level was multiplied by the DWCF described below. In cases where the measured protein level exceeded the upper limit of quantitation (ULOQ) of the method, another aliquot of the sample was extracted, diluted at a higher dilution, and re-analyzed.

The following formulas were used to calculate final results based on the sample dilution, extraction ratio, MWCF, and DWCF:

Dilution Adjusted Concentration Determination:

$$(X_1) \times (\text{Dil}) = X_2$$

Where:

X_1 = calculated concentration
Dil = dilution factor
X_2 = dilution adjusted concentration

Extraction Adjusted Concentration Determination:

$$(X_2) \times [\text{ER}] = X_3$$

Where:

X_2 = dilution adjusted concentration
ER = extraction ratio
X_3 = extraction adjusted concentration

Molecular Weight Correction Factor (MWCF):

$$\frac{A}{B} = MWCF$$

Where:

A = molecular weight of newly expressed protein
B = molecular weight of reference substance

Molecular Weight Corrected Protein Concentration Determined from Reference Substance (also described as concentration expressed as fresh weight of sample):

$$(X_3) \times MWCF = y$$

Where:

y = final protein concentration
X_3 = extraction adjusted concentration of newly expressed protein
MWCF = molecular weight correction factor

Percent Moisture (PM) Calculations:

$$\left[\frac{m_1 - m_2}{m_1}\right] \times 100 = PM$$

Where:

m_1 = weight of fresh weight tissue
m_2 = weight of lyophilized tissue
PM = percent moisture

Dry Weight Conversion Factor (DWCF):

$$\left[\frac{100}{100 - PM}\right] = DWCF$$

Where:

PM = percent moisture
DWCF = dry weight conversion factor

Concentration expressed on a dry weight basis:

$(X_1) \times (DWCF) = (X_2)$

Where:

X_1 = concentration expressed as fresh weight (FW) of sample
DWCF = dry weight conversion factor
X_2 = concentration expressed as dry weight (DW) of sample

Protein levels that are below the LOD are reported as < LOD while protein levels that are greater than the LOD but below the LOQ are reported as < LOQ. For any sample datasets that contained at least two quantifiable tissue samples (out of twenty), the mean and range were determined across all individual samples. Fresh weight samples that were < LOQ or < LOD were substituted with the fresh weight LOQ or LOD value. Dry weight samples that were < LOQ or < LOD were substituted with the dry weight LOQ or LOD value. Ranges are reported for each newly expressed protein across all individual samples for each treatment and sample type.

D.4. Tissue-specific protein levels in EPA+DHA canola event LBFLFK

Table D.15. D12D(*Ps*) Protein Levels in EPA+DHA Canola Event LBFLFK Tissues

Tissue[3]	LBFLFK (non–sprayed)[1]		LBFLFK (sprayed)[2]		Method Quantification and Detection Limits	
	Mean (SD)[4] min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	Mean (SD) min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	LOQ LOD (µg/g FW)	LOQ LOD (µg/g DW)
Whole Plant (Rosette Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	0.51 0.12	5.22 1.22
Whole Plant (Flowering Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	0.51 0.12	4.76 1.11
Leaf	NA < LOD	NA < LOD	NA <LOD	NA <LOD	4.07 0.95	38.18 8.93
Root	NA < LOD	NA < LOD	NA <LOD	NA <LOD	0.13 0.03	0.64 0.15
Immature Seed	1.25 (0.32) < LOQ–1.93	3.98 (1.30) < LOQ–6.56	1.15 (0.44) < LOD–1.97	3.72 (1.69) < LOD–6.99	1.02 0.24	2.97 0.70
Mature Seed	0.79 (0.19) 0.51–1.10	0.93 (0.23) 0.57–1.24	0.71 (0.19) < LOQ–1.12	0.83 (0.23) < LOQ–1.29	0.51 0.12	0.55 0.13
Pollen	NA < LOD	NA < LOD	ND ND	ND ND	0.25 0.06	0.28 0.07

< LOQ = below the limit of quantitation; < LOD = below the limit of detection; ND = not determined; NA = not applicable; mean and standard deviation were not calculated for those datasets that did not contain two or more values ≥ LOQ.

[1] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[3] n = 20 for whole plant at rosette stage, whole plant at flowering stage, immature seed, leaf, root, and mature seed non-sprayed and sprayed (with Beyond® herbicide) datasets; n = 2 for pollen dataset, non-sprayed only.

[4] Mean, standard deviation (SD), and range (Minimum (min)–Maximum (max)) of protein concentrations are reported on a µg/g fresh weight (FW) and dry weight (DW) basis.

Table D.16. D6D(*Ot*) Protein Levels in EPA+DHA Canola Event LBFLFK Tissues

Tissue[3]	LBFLFK (non–sprayed)[1]		LBFLFK (sprayed)[2]		Method Quantification and Detection Limits	
	Mean (SD)[4] min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	Mean (SD) min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	LOQ LOD (µg/g FW)	LOQ LOD (µg/g DW)
Whole Plant (Rosette Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	10.80 3.60	110.81 36.94
Whole Plant (Flowering Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	10.80 3.60	101.09 33.70
Leaf	NA < LOD	NA < LOD	NA < LOD	NA < LOD	10.80 3.60	101.30 33.77
Root	NA < LOD	NA < LOD	NA < LOD	NA < LOD	10.80 3.60	54.54 18.18
Immature Seed	8.97 (4.86) < LOQ–20.00	29.90 (17.71) < LOQ–68.00	9.16 (4.80) < LOD–18.18	30.89 (18.45) < LOD–64.54	5.40 1.80	15.77 5.26
Mature Seed	34.65 (15.46) 18.16–65.96	40.22 (16.87) 21.43–74.53	35.76 (14.27) 19.17–66.05	41.80 (16.11) 23.00–75.96	10.80 3.60	11.77 3.92
Pollen	NA < LOD	NA < LOD	ND ND	ND ND	3.60 1.80	3.96 1.98

< LOQ = below the limit of quantitation; < LOD = below the limit of detection; ND = not determined; NA = not applicable; mean and standard deviation were not calculated for those datasets that did not contain two or more values ≥ LOQ.

[1] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[3] n = 20 for whole plant at rosette stage, whole plant at flowering stage, immature seed, leaf, root, and mature seed non-sprayed and sprayed (with Beyond® herbicide) datasets; n = 2 for pollen dataset, non-sprayed only.

[4] Mean, standard deviation (SD), and range (Minimum (min)–Maximum (max)) of protein concentrations are reported on a µg/g fresh weight (FW) and dry weight (DW) basis.

Table D.17. D6E(*Tp*) Protein Levels in EPA+DHA Canola Event LBFLFK Tissues

Tissue[3]	LBFLFK (non–sprayed)[1]		LBFLFK (sprayed)[2]		Method Quantification and Detection Limits	
	Mean (SD)[4] min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	Mean (SD) min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	LOQ LOD (µg/g FW)	LOQ LOD (µg/g DW)
Whole Plant (Rosette Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	24.58 15.91	252.15 163.27
Whole Plant (Flowering Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	24.58 15.91	230.03 148.95
Leaf	NA < LOD	NA < LOD	NA < LOD	NA < LOD	24.58 15.91	230.52 149.26
Root	NA < LOD	NA < LOD	NA < LOD	NA < LOD	12.29 7.96	62.05 40.18
Immature Seed	180.40 (42.33) < LOQ–301.84	600.86 (181.74) < LOQ–1038.33	185.25 (48.05) < LOD–271.99	626.21 (194.98) < LOD–900.29	153.60 79.56	448.51 232.33
Mature Seed	793.02 (232.25) 399.59–1181.34	936.43 (309.07) 471.52–1488.49	779.21 (229.40) 372.33–1229.15	915.86 (276.98) 446.80–1376.65	344.06 222.78	375.03 242.83
Pollen	NA < LOD–< LOQ	NA < LOD–< LOQ	ND ND	ND ND	55.30 47.74	60.83 52.51

< LOQ = below the limit of quantitation; < LOD = below the limit of detection; ND = not determined; NA = not applicable; mean and standard deviation were not calculated for those datasets that did not contain two or more values ≥ LOQ.

[1] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[3] n = 20 for whole plant at rosette stage, whole plant at flowering stage, immature seed, leaf, root, and mature seed non-sprayed and sprayed (with Beyond® herbicide) datasets; n = 2 for pollen dataset, non-sprayed only.

[4] Mean, standard deviation (SD), and range (Minimum (min)–Maximum (max)) of protein concentrations are reported on a µg/g fresh weight (FW) and dry weight (DW) basis.

Table D.18. D6E(*Pp*) Protein Levels in EPA+DHA Canola Event LBFLFK Tissues

Tissue[3]	LBFLFK (non–sprayed)[1]		LBFLFK (sprayed)[2]		Method Quantification and Detection Limits	
	Mean (SD)[4] min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	Mean (SD) min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	LOQ LOD (µg/g FW)	LOQ LOD (µg/g DW)
Whole Plant (Rosette Stage)	NA < LOD	NA < LOD	NA < LOD–< LOQ	NA < LOD–< LOQ	2.85 0.48	29.25 4.88
Whole Plant (Flowering Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	2.85 0.48	26.69 4.45
Leaf	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–5.72	NA < LOD–< LOQ[1]	5.70 0.95	53.49 8.91
Root	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD	NA < LOD	2.85 0.48	14.40 2.40
Immature Seed	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	5.70 0.95	16.65 2.78
Mature Seed	NA < LOD	NA < LOD	NA < LOD	NA < LOD	5.70 0.95	6.22 1.04
Pollen	NA < LOD	NA < LOD	ND ND	ND ND	5.70 0.95	6.27 1.05

< LOQ = below the limit of quantitation; < LOD = below the limit of detection; ND = not determined; NA = not applicable; mean and standard deviation were not calculated for those datasets that did not contain two or more values ≥ LOQ.

[1] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[3] n = 20 for whole plant at rosette stage, whole plant at flowering stage, immature seed, leaf, root, and mature seed non-sprayed and sprayed (with Beyond® herbicide) datasets; n = 2 for pollen dataset, non-sprayed only.

[4] Mean, standard deviation (SD), and range (Minimum (min)–Maximum (max)) of protein concentrations are reported on a µg/g fresh weight (FW) and dry weight (DW) basis.

Table D.19. D5D(*Tc*) Protein Levels in EPA+DHA Canola Event LBFLFK Tissues

Tissue[3]	LBFLFK (non–sprayed)[1]		LBFLFK (sprayed)[2]		Method Quantification and Detection Limits	
	Mean (SD)[4] min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	Mean (SD) min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	LOQ LOD (µg/g FW)	LOQ LOD (µg/g DW)
Whole Plant (Rosette Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	9.60 3.84	98.50 39.40
Whole Plant (Flowering Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	9.60 3.84	89.86 35.94
Leaf	NA < LOD	NA < LOD	NA < LOD	NA < LOD	19.20 7.68	180.10 72.04
Root	NA < LOD	NA < LOD	NA < LOD	NA < LOD	4.80 1.92	24.24 9.70
Immature Seed	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	9.60 3.84	28.03 11.21
Mature Seed	1.19 (0.35) < LOD–1.66	1.33 (0.40) < LOD–1.88	1.38 (0.46) < LOD–2.56	1.53 (0.55) < LOD–2.94	1.20 0.48	1.31 0.52
Pollen	NA < LOD	NA < LOD	ND ND	ND ND	4.80 1.92	5.28 2.11

< LOQ = below the limit of quantitation; < LOD = below the limit of detection; ND = not determined; NA = not applicable; mean and standard deviation were not calculated for those datasets that did not contain two or more values ≥ LOQ.

[1] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[3] n = 20 for whole plant at rosette stage, whole plant at flowering stage, immature seed, leaf, root, and mature seed non-sprayed and sprayed (with Beyond® herbicide) datasets; n = 2 for pollen dataset, non-sprayed only.

[4] Mean, standard deviation (SD), and range (Minimum (min)–Maximum (max)) of protein concentrations are reported on a µg/g fresh weight (FW) and dry weight (DW) basis.

Table D.20. O3D(*Pir*) Protein Levels in EPA+DHA Canola Event LBFLFK Tissues

Tissue[3]	LBFLFK (non–sprayed)[1]		LBFLFK (sprayed)[2]		Method Quantification and Detection Limits	
	Mean (SD)[4] min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	Mean (SD) min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	LOQ LOD (µg/g FW)	LOQ LOD (µg/g DW)
Whole Plant (Rosette Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	39.96 11.43	409.99 117.26
Whole Plant (Flowering Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	39.96 11.43	374.03 106.97
Leaf	NA < LOD	NA < LOD	NA < LOD	NA < LOD	53.28 15.24	499.77 142.93
Root	NA < LOD	NA < LOD	NA < LOD	NA < LOD	155.40 44.44	784.77 224.44
Immature Seed	47.66 (18.21) < LOQ–77.41	162.36 (70.06) < LOQ–266.29	48.06 (24.43) <LOD–109.14	169.26 (86.39) <LOD–361.25	29.97 11.43	87.51 33.37
Mature Seed	428.26 (140.79) 168.54–623.80	504.38 (169.24) 188.76–704.89	474.27 (157.65) 188.70–693.86	561.61 (196.20) 211.34–832.63	107.89 34.29	117.60 37.37
Pollen	NA < LOD	NA < LOD	ND ND	ND ND	16.65 6.35	18.32 6.98

< LOQ = below the limit of quantitation; < LOD = below the limit of detection; ND = not determined; NA = not applicable; mean and standard deviation were not calculated for those datasets that did not contain two or more values ≥ LOQ.

[1] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[3] n = 20 for whole plant at rosette stage, whole plant at flowering stage, immature seed, leaf, root, and mature seed non-sprayed and sprayed (with Beyond® herbicide) datasets; n = 2 for pollen dataset, non-sprayed only.

[4] Mean, standard deviation (SD), and range (Minimum (min)–Maximum (max)) of protein concentrations are reported on a µg/g fresh weight (FW) and dry weight (DW) basis.

Table D.21. O3D(*Pi*) Protein Levels in EPA+DHA Canola Event LBFLFK Tissues

Tissue[3]	LBFLFK (non–sprayed)[1]		LBFLFK (sprayed)[2]		Method Quantification and Detection Limits	
	Mean (SD)[4] min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	Mean (SD) min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	LOQ LOD (µg/g FW)	LOQ LOD (µg/g DW)
Whole Plant (Rosette Stage)	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	29.54 9.94	303.07 101.97
Whole Plant (Flowering Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	59.08 19.88	552.97 186.06
Leaf	NA < LOD	NA < LOD	NA < LOD	NA < LOD	98.46 33.13	923.59 310.76
Root	NA < LOD	NA < LOD	NA < LOD	NA < LOD	29.54 9.94	149.17 50.19
Immature Seed	NA < LOD	NA < LOD	NA < LOD	NA < LOD	6.52 2.48	19.03 7.26
Mature Seed	NA < LOD	NA < LOD	NA < LOD	NA < LOD	65.16 24.85	71.02 27.08
Pollen	NA < LOD	NA < LOD	ND ND	ND ND	19.69 6.63	21.66 7.29

< LOQ = below the limit of quantitation; < LOD = below the limit of detection; ND = not determined; NA = not applicable; mean and standard deviation were not calculated for those datasets that did not contain two or more values ≥ LOQ.

[1] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[3] n = 20 for whole plant at rosette stage, whole plant at flowering stage, immature seed, leaf, root, and mature seed non-sprayed and sprayed (with Beyond® herbicide) datasets; n = 2 for pollen dataset, non-sprayed only.

[4] Mean, standard deviation (SD), and range (Minimum (min)–Maximum (max)) of protein concentrations are reported on a µg/g fresh weight (FW) and dry weight (DW) basis.

Table D.22. D5E(*Ot*) Protein Levels in EPA+DHA Canola Event LBFLFK Tissues

Tissue[3]	LBFLFK (non–sprayed)[1]		LBFLFK (sprayed)[2]		Method Quantification and Detection Limits	
	Mean (SD)[4] min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	Mean (SD) min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	LOQ LOD (µg/g FW)	LOQ LOD (µg/g DW)
Whole Plant (Rosette Stage)	NA < LOD	NA < LOD	NA < LOD–< LOQ	NA < LOD–< LOQ	2.23 0.49	22.90 4.99
Whole Plant (Flowering Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	2.23 0.49	20.89 4.55
Leaf	NA < LOD	NA < LOD	NA < LOD	NA < LOD	4.46 0.97	41.87 9.13
Root	NA < LOD	NA < LOD	NA < LOD	NA < LOD	1.86 0.41	9.39 2.05
Immature Seed	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	NA < LOD–< LOQ	4.46 0.97	13.03 2.84
Mature Seed	13.32 (3.75) 7.63–18.27	15.48 (4.00) 9.00–20.65	13.14 (3.35) 6.99–18.27	15.36 (3.63) 8.39–21.01	4.46 0.97	4.87 1.06
Pollen	NA < LOD	NA < LOD	ND ND	ND ND	8.93 1.95	9.82 2.14

< LOQ = below the limit of quantitation; < LOD = below the limit of detection; ND = not determined; NA = not applicable; mean and standard deviation were not calculated for those datasets that did not contain two or more values ≥ LOQ.

[1] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[3] n = 20 for whole plant at rosette stage, whole plant at flowering stage, immature seed, leaf, root, and mature seed non-sprayed and sprayed (with Beyond® herbicide) datasets; n = 2 for pollen dataset, non-sprayed only.

[4] Mean, standard deviation (SD), and range (Minimum (min)–Maximum (max)) of protein concentrations are reported on a µg/g fresh weight (FW) and dry weight (DW) basis.

Table D.23. D4D(*Pl*) Protein Levels in EPA+DHA Canola Event LBFLFK Tissues

Tissue[3]	LBFLFK (non–sprayed)[1]		LBFLFK (sprayed)[2]		Method Quantification and Detection Limits	
	Mean (SD)[4] min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	Mean (SD) min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	LOQ LOD (µg/g FW)	LOQ LOD (µg/g DW)
Whole Plant (Rosette Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	2.89 1.84	29.70 18.86
Whole Plant (Flowering Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	4.34 2.76	40.64 25.80
Leaf	NA < LOD	NA < LOD	NA < LOD	NA < LOD	2.89 1.84	27.15 17.24
Root	NA < LOD	NA < LOD	NA < LOD	NA < LOD	4.34 2.76	21.93 13.92
Immature Seed	4.19 (1.72) < LOD–7.04	16.16 (6.18) < LOD–27.80	3.68 (1.78) < LOD–7.06	13.28 (6.67) < LOD–25.06	1.93 0.92	5.63 2.68
Mature Seed	3.74 (1.91) < LOD–7.24	4.16 (2.18) < LOD–8.18	3.60 (1.96) < LOD–7.28	4.03 (2.29) < LOD–8.37	3.86 1.83	4.21 2.00
Pollen	NA < LOD	NA < LOD	ND ND	ND ND	2.41 1.84	2.65 2.02

< LOQ = below the limit of quantitation; < LOD = below the limit of detection; ND = not determined; NA = not applicable; mean and standard deviation were not calculated for those datasets that did not contain two or more values ≥ LOQ.

[1] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[3] n = 20 for whole plant at rosette stage, whole plant at flowering stage, immature seed, leaf, root, and mature seed non-sprayed and sprayed (with Beyond® herbicide) datasets; n = 2 for pollen dataset, non-sprayed only.

[4] Mean, standard deviation (SD), and range (Minimum (min)–Maximum (max)) of protein concentrations are reported on a µg/g fresh weight (FW) and dry weight (DW) basis.

Table D.24. D4D(*Tc*) Protein Levels in EPA+DHA Canola Event LBFLFK Tissues

Tissue[3]	LBFLFK (non–sprayed)[1]		LBFLFK (sprayed)[2]		Method Quantification and Detection Limits	
	Mean (SD)[4] min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	Mean (SD) min–max (µg/g FW)	Mean (SD) min–max (µg/g DW)	LOQ LOD (µg/g FW)	LOQ LOD (µg/g DW)
Whole Plant (Rosette Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	1.61 0.66	16.55 6.81
Whole Plant (Flowering Stage)	NA < LOD	NA < LOD	NA < LOD	NA < LOD	6.45 2.65	60.38 24.83
Leaf	NA < LOD	NA < LOD	NA < LOD	NA < LOD	6.45 2.65	60.51 24.89
Root	NA < LOD	NA < LOD	NA < LOD	NA < LOD	3.23 1.33	16.29 6.70
Immature Seed	7.88 (7.63) < LOD–30.05	29.02 (26.96) < LOD–102.17	6.29 (5.03) < LOD–18.96	22.53 (18.72) < LOD–62.76	3.02 1.66	8.83 4.84
Mature Seed	10.55 (4.75) < LOD–20.36	11.81 (5.43) < LOD–23.01	9.66 (3.12) < LOD–15.89	10.88 (3.71) < LOD–18.27	8.47 3.49	9.23 3.80
Pollen	NA < LOD	NA < LOD	ND ND	ND ND	1.41 0.66	1.55 0.73

< LOQ = below the limit of quantitation; < LOD = below the limit of detection; ND = not determined; NA = not applicable; mean and standard deviation were not calculated for those datasets that did not contain two or more values ≥ LOQ.

[1] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[3] n = 20 for whole plant at rosette stage, whole plant at flowering stage, immature seed, leaf, root, and mature seed non-sprayed and sprayed (with Beyond® herbicide) datasets; n = 2 for pollen dataset, non-sprayed only.

[4] Mean, standard deviation (SD), and range (Minimum (min)–Maximum (max)) of protein concentrations are reported on a µg/g fresh weight (FW) and dry weight (DW) basis.

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Appendix E. Agronomic and Phenotypic Field Trial Analysis of EPA+DHA Canola Event LBFLFK – Study Design, Statistical Model, By-Site Analysis

EPA+DHA canola event LBFLFK and the parental control Kumily served as test and control entries, respectively, for field trial studies of the agronomic and phenotypic characteristics. Six commercial canola varieties were also included in the trials as references to provide a comparative range. Test, control, and reference lines studied in the 2014/15 winter season and the 2015 spring season are listed in Table E.1. The identity of the test substance was confirmed using event-specific assays that tested for the integrity and copy number of the DNA insertions. In these trials, the LBFLFK entry tested included two treatments, LBFLFK (sprayed) that received an application of Beyond®[1] herbicide at the 3–4 leaf stage, and LBFLFK (non-sprayed) that was not sprayed with Beyond® herbicide. Plots were otherwise treated the same at each individual location.

Table E.1. Test, Control, and Reference Substances

Line/Variety	Purpose	BASF Seed Lot ID	Growing Season
LBFLFK	Test Substance	2047830031	Winter 2014/15
Kumily	Parental Control Substance	2047837301	Winter 2014/15
Q2	Reference Substance	910018225702	Winter 2014/15
46A65	Reference Substance	910018225703	Winter 2014/15
IMC105	Reference Substance	910018225707	Winter 2014/15
IMC302	Reference Substance	910018225705	Winter 2014/15
Wizzard	Reference Substance	910018225704	Winter 2014/15
Orinoco	Reference Substance	910018225706	Winter 2014/15
LBFLFK	Test Substance	910019041494	Spring 2015
Kumily	Parental Control Substance	910019041495	Spring 2015
Q2	Reference Substance	910018225702	Spring 2015
46A65	Reference Substance	910018225703	Spring 2015
IMC105	Reference Substance	910018225707	Spring 2015
IMC302	Reference Substance	910018225705	Spring 2015
Wizzard	Reference Substance	910018225704	Spring 2015
Orinoco	Reference Substance	910018225706	Spring 2015

This appendix provides general background information on the design of the field trial studies, the statistical approach that was used to compare the collected field data on LBFLFK to Kumily, and provides the results of the by-site statistical comparisons that were performed for data sets with a significant entry-by-site interaction or variance heterogeneity.

[1] Beyond is a registered trademark of BASF Corp.

E.1. Field sites and trial design

Winter trials in 2014/15 were conducted at six locations in Georgia, Louisiana, and Texas (Table E.2). Each field trial was designed as a randomized complete block with four replications. Plot size was approximately 24 m^2 (258 square feet). Each plot consisted of 18 rows of approximately 5 m (16 ft) long. Row spacing was approximately 20 cm (8 in). Plots were spaced 8.2 m (27 ft) apart, within the limits of accuracy of field equipment. Exceptional weather was noted at five of the six sites (Table E.3). Daily maximum and minimum temperatures and daily precipitation were recorded on-site or at a nearby weather station.

Spring trials in 2015 were conducted at eight locations in Iowa, Minnesota, North Dakota, Montana, Washington, and South Dakota (Table E.2). Each field trial was designed as a randomized complete block with four replications. Plot size was approximately 28.8 m^2 (310 ft^2). Each plot consisted of 18 rows of approximately 6 m (19.5 ft) long. Row spacing was approximately 20 cm (8 in). Plots were spaced 9.2 m (30 ft) apart, within the limits of accuracy of field equipment. Exceptional weather was noted at one of the eight sites (Table E.3). Daily maximum and minimum temperatures and daily precipitation were recorded on-site or at a nearby government weather station.

Table E.2. Field Trial Designations, Locations, and Planting Dates for 2014/15 and 2015 Seasons

Field Trial Designation	Nearest Town, State	Planting Date	Trial Season
3SRBLY1	Beasley, TX	20-November-2014	Winter 2014/15
3SRJV	Jeffersonville, GA	03-December-2014	Winter 2014/15
3SRKT	Kendleton, TX	03-December-2014	Winter 2014/15
3SROM	Odem, TX	18-November-2014	Winter 2014/15
3SRRH	Rio Hondo, TX	19-November-2014	Winter 2014/15
3SRWN1	Washington, LA	14-November-2014	Winter 2014/15
3NRLS	Lime Springs, IA	13-May-2015	Spring 2015
3NRGE	Geneva, MN	20-May-2015	Spring 2015
3NRSC	Sartell, MN	21-May-2015	Spring 2015
3NRCB	Campbell, MN	22-May-2015	Spring 2015
3NRNW-1	Northwood, ND	22-May-2015	Spring 2015
3NRMA-2	Malta, MT	23-May-2015	Spring 2015
3NREP	Ephrata, WA	19-May-2015	Spring 2015
3NRBRK	Brookings, SD	02-June-2015	Spring 2015

Table E.3. Exceptional Weather Conditions

Trial ID	Type	Date	Duration (days)
3SRBLY1	Higher than normal precipitation	March – May 2015	~90 days
3SRJV	Colder than normal temperatures	January 2015	~30 days
3SRKT	Higher than normal precipitation	March – May 2015	~90 days
3SROM	None	NA	NA
3SRRH	Higher than normal precipitation	March – April 2015	~60 days
3SRWN1	Extremely cold temperatures	February – March 2015	~60 days
3NRLS	None	NA	NA
3NRGE	None	NA	NA
3NRSC	None	NA	NA
3NRCB	None	NA	NA
3NRNW-1	None	NA	NA
3NRMA-2	None	NA	NA
3NREP	Heat exceeded 10-year norm	May – August	120
3NRBRK	None	NA	NA

Trial IDs above the line are winter 2014/15 field site IDs, and below the line are spring 2015 field site IDs.
NA = not applicable

For both seasons, to minimize cross-pollination between plots, plots of a conventional male sterile canola variety were used as a pollen buffer. The male-sterile canola variety also surrounded the entire trial area at each field location. In addition, a fallow area of approximately 1.6 m (~5.5 ft) width surrounded each plot.

At each location, planting simulated commercial cultivation using appropriate equipment for the size of the plots. The seeding rate was 17–25 seeds/m. All cultural practices such as tillage, seed bed preparation, fertilization, pest control, etc., were applied uniformly, and reflective of commercial production, to the entire trial area (including borders) at each location.

During the 2014/15 winter trials, insecticide application was made at two sites (3SRKT and 3SRRH). At 3SRKT, during evaluation at BBCH 60 (start of flowering), most plots had an insect damage rating of 1 (mild symptoms of minor insect feeding). However, there were three reference plots that had an aphid damage rating of 2 (intermediate damage that required mitigation), so an insecticide was applied. At the 3SRRH site, because conditions were favorable for diamondback moths, multiple proactive pesticide applications were made. During the 2015 spring trials, insecticide and fungicides were applied at three locations (3NRWN-1, 3NRMA-2, and 3NREP). At both the 3NRNW-1 and 3NREP sites, fungicide was used proactively (before any infestation was seen) to prevent potential downy mildew infestation. At the 3NRMA-2 and 3NREP sites, pesticides were used proactively to control flea beetle and aphids, respectively.

At all sites, plots were swathed when approximately 40–60% of the seed had changed color from green. Plots were threshed using stationary threshing machines between 6 and 29 days after swathing.

E.2. Agronomic and phenotypic characteristics assessed

Agronomic and phenotypic data were collected in the field for the following characteristics: field emergence, early plant stand, seedling vigor, days to start of flowering, days to end of flowering, days to maturity, plant height, lodging, pod shattering, final plant stand, yield, and plant response to biotic and abiotic stressors. Pod count was additionally determined during the spring 2015 season. Seedling vigor was determined at BBCH 12–13 (Meier, 2001) using a rating scale between 1 and 5, where 1 represented optimal seedling vigor. Plant lodging and pod shattering were assessed at BBCH 85–86 using a rating scale between 1 and 10, where 1 represented a low amount of shatter or lodging and 10 represented a high degree of pod shattering or plant lodging. Biotic and abiotic stressors were evaluated at BBCH 12–16, BBCH 60–62, BBCH 69, and BBCH 85–86 using the categorical scale shown in Table E.4.

Table E.4. Evaluation Scale for Biotic and Abiotic Stressors

Scale	Severity of Plant Damage
0	No damage or symptoms observed
1	Mild damage; < 10% of the plants in a plot affected; symptoms not damaging to plant development (e.g., minor feeding, minor lesions, nutrient deficiency, chlorosis); mitigation likely not required
2	Moderate damage; < 30% of the plants in a plot affected; likely requires mitigation
3	Severe damage; > 30% of the plants in a plot affected; symptoms damaging to plant development (e.g., stunting or death); mitigation unlikely to be effective

E.3. Statistical analysis method

Data meeting at least one of the following three criteria were considered not suitable for analysis of variance (ANOVA).

- The characteristic had 6 or less distinct values.
- The mode of the characteristic had more than 40% frequency.
- More than 40% of the site-entry combinations had null variance.

For agronomic and phenotypic measurements not suitable for statistical analysis (including those that were ordinal), results were summarized using across-site means and ranges. These summary statistics are reported separately from the data analyzed by ANOVA.

The following linear model (also referred to as “overall model”) was fitted to each variable that was suitable for ANOVA:

$$y_{ijk}=\mu+l_i+b_{j(i)}+T_k+lT_{ik}+\varepsilon_{ijk} \quad (1)$$

In this model, y_{ijk} is the response variable measured on block *j* within site *i* for test material *k*, μ is the overall mean effect, l_i is the random effect of site *i*, $b_{j(i)}$ is the random effect of block *j* within site *i*, T_k is the fixed effect of entry *k*, lT_{ik} is the random entry-by-site interaction term associated with test material *k* within site *i*, and ε_{ijk} is a random experimental error term associated with test material *k* from block *j* within site *i*. This is a linear mixed model because it contains both random and fixed terms. Random experimental errors are assumed to be independently and normally distributed with zero mean and common variance σ^2_ε.

E.3.1. Normality assessment and outlier detection

The linear mixed model specified above was fitted to each original response variable. To account for possible deviations from normality, alternative transformations included:

- $y'=\text{arcsine}(\sqrt{y/100})$ only for characteristics measured as % within the range 0 to 100% (transformation referred to as “asin”).
- $y'=\ln(y+1)$ and $y'=\sqrt{y+1}$ for all other characteristics (transformations referred to as “log” and “sqrt”).

This procedure allowed estimation of Studentized residuals associated with each observation. Normality of the residuals estimated for all fitted models (i.e. all original and transformed variables) was assessed using Lilliefors Normality tests at a 0.01 significance level (Kolmogorov-Smirnov-like statistics) (Conover, 1999). Outliers were defined by any data point that had a Studentized residual whose absolute value was greater than or equal to 6.

Quantile-quantile plots (Q-Q) plots and histograms built using Studentized residuals from fitted models were used to select a transformation for response variables for which ANOVA assumptions did not hold.

Observations having an absolute value of the associated Studentized residual greater than or equal to 6 were considered extreme data points (outliers) and were excluded when performing statistical analyses.

E.3.2. Comparison of means

ANOVA methods (McIntosh, 1983; Kuznetsova et al., 2015) were applied on the overall model to compare the estimated characteristic means of Kumily against the estimated characteristic means of both LBFLFK (sprayed) and LBFLFK (non-sprayed). All measurements from reference varieties were excluded from ANOVA-based pairwise comparisons of interest but were used for assessing normality of estimated residuals, outlier identification, and for estimating the range of references. The statistical significance for the ANOVA F-tests was predetermined to be at the 5% level ($\alpha = 0.05$).

The following model was used to carry out individual site analyses whenever a significant entry-by-site interaction occurred:

$$y_{jk}=\mu+b_j+T_k+\varepsilon_{jk} \quad (2)$$

where y_{jk} is the observed response for entry *k* at block *j*, μ is the site mean, b_j is the random effect of block *j*, T_k is the fixed effect of entry *k*, and ε_{jk} is the random residual error term associated with entry *k* at block *j*. Comparisons were then made between the estimated mean of Kumily and both the mean of LBFLFK (sprayed) and the mean of LBFLFK (non-sprayed). All significance tests were performed at the 95% confidence level.

Characteristic means were also graphically compared against the reference varieties using the smallest and largest entry-by-site means of the six reference canola varieties.

All analyses were carried out using R statistical software (R Core Team, 2016).

E.4. By-site statistical analysis tables

Table E.5 to Table E.9 below provide the results of the by-site analysis for characteristic data sets that had significant entry-by-site interactions from the winter 2014/15 field trials. The by-site analysis for characteristic data sets that had significant entry-by-site interactions during the spring 2015 trials are presented in Table E.10 to Table E.12.

Table E.5. Field Emergence (%) – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)	ANOVA p-value
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	Entry effect[4]
3SRBLY1	63.75 (3.01) 58–67	53 (3.01) 50–58	10.75 (4.12) (0.04)*	59.25–75.25	55.25 (3.01) 46–67	8.5 (4.12) (0.085)	0.0862
3SRJV	61 (3.13) 50–68	33.5 (3.13) 28–42	27.5 (4.42) (<0.001)*	36.25–56.5	43.75 (3.13) 40–48	17.25 (4.42) (0.004)*	0.0005*
3SRKT	68 (3.61) 63–75	59.5 (3.61) 46–71	8.5 (5.03) (0.142)	63.75–69.75	54 (3.61) 50–58	14 (5.03) (0.032)*	0.0809
3SROM	64.75 (3.77) 58–75	59.5 (3.77) 46–67	5.25 (3.82) (0.218)	58.25–69	59.5 (3.77) 54–63	5.25 (3.82) (0.218)	0.3495
3SRRH	52 (2.09) 46–58	40 (2.09) 38–42	12 (2.17) (0.002)*	42–57.25	39.75 (2.09) 33–42	12.25 (2.17) (0.001)*	0.0020*
3SRWN1	68.25 (3.28) 62–77	31.5 (3.28) 23–36	36.75 (4.64) (<0.001)*	56–80.25	38.5 (3.28) 30–46	29.75 (4.64) (<0.001)*	0.0001*

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.
[4] * indicates p≤ 0.05 for entry effect.

Table E.6. Early Plant Stand (Count) – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)	ANOVA p-value
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	Entry effect[4]
3SRBLY1	152.75 (7.58) 136–164	127.5 (7.58) 117–139	25.25 (10.03) (0.045*)	142.75–178.75	130.5 (7.58) 106–157	22.25 (10.03) (0.068)	0.0864
3SRJV	146.5 (7.29) 121–162	81 (7.29) 68–101	65.5 (10.31) (<0.001*)	87.25–135.5	104.75 (7.29) 96–115	41.75 (10.31) (0.003*)	0.0004*
3SRKT	163 (8.58) 149–181	143.25 (8.58) 113–169	19.75 (11.95) (0.15)	154.25–170.75	128 (8.58) 117–141	35 (11.95) (0.026*)	0.0691
3SROM	153 (8.56) 135–180	142.5 (8.56) 113–159	10.5 (9.29) (0.302)	139–164	142 (8.56) 129–148	11 (9.29) (0.281)	0.4571
3SRRH	97.25 (17.25) 12–137	93.75 (17.25) 85–103	3.5 (24.39) (0.889)	99–135.5	95 (17.25) 80–102	2.25 (24.39) (0.928)	0.9895
3SRWN1	163.5 (7.99) 149–185	75.5 (7.99) 55–87	88 (11.3) (< 0.001*)	134.25–192.25	91.75 (7.99) 71–110	71.75 (11.3) (< 0.001*)	0.0001*

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.
[4] * indicates p≤ 0.05 for entry effect.

Table E.7. Days to End of Flowering – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)	ANOVA p-value
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	Entry effect[4]
3SRBLY1	132.25 (0.55) 131–133	135 (0.55) 133–137	-2.75 (0.61) (0.004*)	131.25–137	133 (0.55) 133–133	-0.75 (0.61) (0.267)	0.0103*
3SRJV	141 (1.5) 138–148	146.5 (1.5) 145–148	-5.5 (1.81) (0.023*)	139.75–152.5	140 (1.5) 139–142	1 (1.81) (0.601)	0.0235*
3SRKT	122 (0) 122–122	122 (0) 122–122	NA	122–124	122 (0) 122–122	NA	NA
3SROM	139 (1.15) 139–139	141 (1.15) 139–147	-2 (1.63) (0.257)	129.75–146.5	139 (1.15) 139–139	0 (1.63) (1)	0.4122
3SRRH	129 (0.61) 129–129	129.75 (0.61) 129–132	-0.75 (0.87) (0.409)	119–137.75	129.75 (0.61) 129–132	-0.75 (0.87) (0.409)	0.6224
3SRWN1	152 (0) 152–152	152 (0) 152–152	NA	146–152	152 (0) 152–152	NA	NA

NA = not applicable

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[3] Difference test p-value: * indicates p≤ 0.05.

[4] * indicates p≤ 0.05 for entry effect.

Table E.8. Days to Maturity – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)	ANOVA p-value
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	Entry effect[4]
3SRBLY1	160.25 (0.46) 159–162	162 (0.46) 162–162	-1.75 (0.61) (0.029*)	159–160.5	161.25 (0.46) 161–162	-1 (0.61) (0.154)	0.0751
3SRJV	162.5 (0.71) 161–163	166 (0.71) 163–167	-3.5 (0.91) (0.009*)	161–166	162.5 (0.71) 161–163	0 (0.91) (1)	0.0129*
3SRKT	149.75 (0.83) 149–152	150.5 (0.83) 149–152	-0.75 (1.17) (0.542)	147.5–152	150.5 (0.83) 149–152	-0.75 (1.17) (0.542)	0.7684
3SROM	158 (0) 158–158	158 (0) 158–158	NA	150–159	158 (0) 158–158	NA	NA
3SRRH	151 (0) 151–151	151 (0) 151–151	NA	138–151	151 (0) 151–151	NA	NA
3SRWN1	171 (0) 171–171	171 (0) 171–171	NA	171–171	171 (0) 171–171	NA	NA

NA = not applicable
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.
[4] * indicates p≤ 0.05 for entry effect.

Table E.9. Final Plant Stand (Count) – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)	ANOVA p-value
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	Entry effect[4]
3SRBLY1	157.5 (10.87) 130–183	118 (10.87) 100–152	39.5 (13.36) (0.025*)	128–169.75	118.75 (10.87) 104–133	38.75 (13.36) (0.027*)	0.0408*
3SRJV	141.25 (8.03) 120–164	84.5 (8.03) 62–95	56.75 (7.19) (< 0.001*)	91.25–134.5	108.25 (8.03) 98–127	33 (7.19) (0.004*)	0.0007*
3SRKT	93.25 (8.95) 81–106	86.25 (8.95) 55–111	7 (12.66) (0.594)	99.25–111.75	96.25 (8.95) 77–113	3 (12.66) (0.818)	0.7282
3SROM	148.25 (6.78) 138–157	138.5 (6.78) 113–158	9.75 (9.59) (0.336)	130.25–166.25	144 (6.78) 131–154	4.25 (9.59) (0.668)	0.6116
3SRRH	98.25 (4.69) 80–107	80.25 (4.69) 69–86	18 (2.91) (0.001*)	77.5–139.25	82 (4.69) 73–91	16.25 (2.91) (0.001*)	0.0015*
3SRWN1	161.25 (7.81) 144–179	74.25 (7.81) 60–84	87 (11.05) (< 0.001*)	132.75–189	90.75 (7.81) 70–113	70.5 (11.05) (< 0.001*)	0.0001*

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.
[4] * indicates p≤ 0.05 for entry effect.

Table E.10. Plant Height (cm) – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)	ANOVA p-value
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) p-value[3]	min–max	Mean (SE) min–max	Difference (SE) p-value[3]	Entry effect[4]
3NRBRK	141.55 (1.54) 135.80–146.80	138.20 (1.54) 136.80–140.00	3.35 (2.16) 0.1717	147.25–159.05	139.35 (1.54) 136.80–141.40	2.20 (2.16) 0.3474	0.3533
3NRCB	52.70 (0.76) 51.20–53.20	51.40 (0.76) 49.20–54.00	1.30 (0.74) 0.1298	52.50–60.00	51.80 (0.76) 50.40–53.20	0.90 (0.74) 0.2701	0.2746
3NREP	114.45 (2.82) 108.20–119.80	111.75 (2.82) 105.60–119.20	2.70 (2.18) 0.2623	104.00–137.00	111.15 (2.82) 104.20–114.80	3.30 (2.18) 0.1813	0.3403
3NRGE	133.60 (1.86) 131.20–137.20	137.20 (1.86) 135.60–138.80	-3.60 (2.63) 0.2198	139.60–159.80	141.40 (1.86) 133.60–146.40	-7.80 (2.63) 0.0250*	0.0462*
3NRLS	151.15 (6.78) 139.20–166.80	144.80 (6.78) 126.20–163.40	6.35 (4.15) 0.1770	145.90–170.35	149.20 (6.78) 132.00–160.40	1.95 (4.15) 0.6552	0.3574
3NRMA-2	82.30 (2.10) 74.80–90.00	87.45 (2.10) 83.60–91.40	-5.15 (2.71) 0.1061	68.95–93.00	82.15 (2.10) 79.60–84.40	0.15 (2.71) 0.9577	0.1641
3NRNW-1	125.05 (6.74) 106.60–140.80	128.55 (6.74) 117.60–153.20	-3.50 (8.54) 0.6963	123.80–141.65	121.55 (6.74) 116.60–127.20	3.50 (8.54) 0.6963	0.7275
3NRSC	130.00 (3.57) 124.00–143.00	124.50 (3.57) 122.00–128.00	5.50 (3.98) 0.2162	136.00–151.25	130.50 (3.57) 122.00–142.00	-0.50 (3.98) 0.9041	0.3170

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.
[4] * indicates p≤ 0.05 for entry effect.

Table E.11. Pod Count – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)	ANOVA p-value
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) p-value[3]	min–max	Mean (SE) min–max	Difference (SE) p-value[3]	Entry effect[4]
3NRBRK	159.76 (0.07) 148.40–164.60	211.02 (0.07) 193.00–226.20	-51.26 0.0154*	120.70–181.80	162.14 (0.07) 127.60–199.20	-2.38 0.8646	0.0260*
3NRCB	155.43 (0.07) 146.20–181.00	168.77 (0.07) 135.20–211.60	-13.34 0.2774	132.25–183.80	164.81 (0.07) 147.80–195.20	-9.38 0.4278	0.5096
3NREP	98.28 (0.10) 82.00–125.20	94.35 (0.10) 84.00–116.60	3.92 0.6556	85.55–114.70	73.51 (0.10) 53.80–88.20	24.77 0.0156*	0.0311*
3NRGE	78.27 (0.09) 56.40–101.60	90.60 (0.09) 76.00–101.60	-12.33 0.2789	72.05–96.25	93.91 (0.09) 78.60–105.60	-15.65 0.1886	0.3361
3NRLS	114.57 (0.12) 102.40–139.80	108.09 (0.12) 64.40–149.40	6.48 0.6686	130.85–195.45	146.72 (0.12) 130.00–170.40	-32.15 0.1044	0.1163
3NRMA-2	184.13 (0.13) 142.80–228.00	129.05 (0.13) 78.60–177.00	55.08 0.0799	104.30–170.70	132.01 (0.13) 101.20–163.80	52.12 0.0962	0.1399
3NRNW-1	120.73 (0.13) 104.00–162.80	133.02 (0.13) 110.40–170.20	-12.3 0.6218	138.75–219.65	114.48 (0.13) 85.20–198.00	6.24 0.7857	0.7295
3NRSC	190.57 (0.06) 174.60–206.20	171.38 (0.06) 152.00–206.40	19.19 0.2180	209.95–278.95	165.47 (0.06) 136.60–189.20	25.1 0.1169	0.2416

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.
[4] * indicates p≤ 0.05 for entry effect.

Table E.12. Seed Moisture (%) – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)	ANOVA p-value
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) p-value[3]	min–max	Mean (SE) min–max	Difference (SE) p-value[3]	Entry effect[4]
3NRBRK	7.20 (0.23) 7.00–7.50	7.67 (0.23) 7.10–8.20	-0.47 (0.32) 0.1915	6.60–7.08	8.43 (0.23) 8.10–9.10	-1.23 (0.32) 0.0090*	0.0129
3NRCB	6.88 (0.06) 6.70–7.10	7.78 (0.06) 7.70–7.80	-0.90 (0.08) 0.0000*	6.42–7.28	7.85 (0.06) 7.80–8.00	-0.97 (0.08) 0.0000*	0.0000
3NREP	5.72 (0.06) 5.70–5.80	6.80 (0.06) 6.70–6.90	-1.07 (0.07) 0.0000*	5.40–6.05	6.83 (0.06) 6.60–7.00	-1.10 (0.07) 0.0000*	0.0000
3NRGE	8.38 (0.20) 8.00–9.00	9.62 (0.20) 8.90–10.10	-1.25 (0.27) 0.0035*	7.70–8.47	9.10 (0.20) 8.90–9.30	-0.72 (0.27) 0.0358*	0.0101
3NRLS	7.15 (0.23) 7.10–7.30	8.55 (0.23) 7.80–9.20	-1.40 (0.24) 0.0010*	6.38–7.47	7.88 (0.23) 7.60–8.20	-0.72 (0.24) 0.0217*	0.0030
3NRMA-2	7.33 (0.30) 7.00–7.70	9.00 (0.30) 7.70–9.50	-1.68 (0.28) 0.0009*	5.78–6.68	8.40 (0.30) 7.80–8.70	-1.07 (0.28) 0.0081*	0.0026
3NRNW-1	5.42 (0.11) 5.10–5.80	6.38 (0.11) 6.20–6.60	-0.95 (0.11) 0.0001*	5.35–6.25	6.47 (0.11) 6.40–6.60	-1.05 (0.11) 0.0001*	0.0001
3NRSC	5.97 (0.21) 5.70–6.20	6.67 (0.21) 5.70–7.10	-0.70 (0.21) 0.0143*	5.65–6.38	6.80 (0.21) 6.60–7.00	-0.82 (0.21) 0.0069*	0.0142

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates $p \leq 0.05$.
[4] * indicates $p \leq 0.05$ for entry effect.

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Appendix F. Seed Germination and Dormancy Analysis of EPA+DHA Canola Event LBFLFK – Study Design, Statistical Model, By-Site Analysis

Canola seed samples harvested from agronomic and phenotypic analysis field trial plots (spring 2015), including six locations in Iowa, Minnesota, North Dakota, South Dakota, and Washington (Table F.1), were subjected to several seed germination assessments. EPA+DHA canola event LBFLFK and the parental control Kumily served as the test and control entries, respectively, and six commercial canola varieties were included as references to provide a representative data range (Table F.2). The LBFLFK samples tested included two treatments, LBFLFK (sprayed) that received an application of Beyond®[1] herbicide during the field trial, and LBFLFK (non-sprayed) that was not sprayed with Beyond® herbicide.

Table F.1. Trial Designations and Locations from which Seed was Harvested

Field Trial ID	Nearest Town
3NRLS	Lime Springs, IA
3NRNW-1	Northwood, ND
3NRCB	Campbell, MN
3NRGE	Geneva, MN
3NRBRK	Brookings, SD
3NREP	Ephrata, WA

Table F.2. Test, Control, and Reference Substances for Seed Germination and Dormancy Analysis

Line/Variety	Purpose	BASF Seed Lot ID
LBFLFK	Test Substance	910019041494
Kumily	Parental Control Substance	910019041495
Q2	Reference Substance	910018225702
46A65	Reference Substance	910018225703
IMC105	Reference Substance	910018225707
IMC302	Reference Substance	910018225705
Wizzard	Reference Substance	910018225704
Orinoco	Reference Substance	910018225706

[1] Beyond is a registered trademark of BASF Corp.

This appendix is meant to supplement the information provided in the main text of the petition, where it was established that the EPA+DHA trait may impact the germination potential of LBFLFK seed. Provided herein are additional details of the seed germination assessments that were performed, including information on the study design, the statistical model, and the results of the by-site analysis for the cold germination and secondary dormancy assays that showed a statistically significant entry-by-site interaction.

F.1. Trial design and testing facility

Germination and dormancy analyses were performed by SGS North America (Brookings, SD). A randomized complete block design, the same as for the field trial, was used.

F.2. Germination and dormancy analysis

Four types of germination tests were conducted as described below.

F.2.1. AOSA “standard germination”

Each replicate (100 canola seeds from each harvested plot) was placed on top of a moistened paper towel. Samples were placed in a germination chamber for 3 days under the following conditions: 16 hours dark at 15 ± 2°C and 8 hours daylight conditions at 25 ± 2°C. After 3 days, the samples were evaluated for a first count of normal seedlings (see “Evaluation” below) and then returned to the germination chamber for an additional 4 days before a final evaluation of seed germination was made. The total percent germination was calculated as the sum of the total germinated seedlings at 3 and 7 days.

After the final evaluation for germination, any firm non-germinated seeds were further tested for viability (dormancy) using a tetrazolium (TZ) test. The seed coats were removed and the seeds soaked in a 1% weight per unit volume (w/v) solution of 2,3,5-triphenyl tetrazolium chloride for ~4 hours at 30–35°C. Seeds with adequate red staining on the essential embryonic structure (radical tip stained slightly darker, cotyledons intact) were counted as viable (AOSA, 2010).

F.2.2. Warm germination

Each replicate was placed on top of a flat paper towel (76#) on a tray moistened with approximately 125 ml of tap water and placed in a germination chamber (5125 lumens daylight conditions for 8 hours followed by 16 hours in the dark) set to a constant 25 ± 2°C for 3 days, after which the samples were evaluated for a first count of normal seedlings (see “Evaluation” below). Paper towels were remoistened as needed. The samples were then returned to the germination chamber for an additional 4 days, after which time a final evaluation of seed germination was made. At the final evaluation, normal seedlings, abnormal seedlings, non-germinated seeds (seeds that are firm to the touch and have not germinated), and dead seeds (seeds that have not germinated and compress when gently pressed) were scored. After the final germination evaluation, any non-germinated seed that were firm were further tested for viability (dormancy) using a TZ test.

F.2.3. Cold germination

Each replicate of 100 seeds was placed on moistened creped cellulose paper, covered evenly with ½ to ¾ inch of sand, and placed at 10 ± 2°C for 10 days in a germination chamber (24 hours dark). After the 10 days, the samples were evaluated for a first count of normal seedlings (see “Evaluation” below) and then returned to a germination chamber set at a constant 25 ± 2°C (8 hours light followed by 16 hours dark). After 3 days, emerged seedlings were counted.

F.2.4. Secondary/dark dormancy germination

A canola seed dormancy induction method (Schatzki et al., 2013) was employed to assess for any differences in secondary/dark dormancy potential. Each replicate of 100 seeds was placed on filter paper moistened with polyethylene glycol solution in a Petri dish. The planted seed was placed at 18 ± 2°C in a germination chamber without lights (24 hours dark) for 14 days. After dormancy induction, the seed was rinsed with distilled water and returned to the 18 ± 2°C germination chamber (24 hours dark). Seedling emergence counts were performed at 2 and 4 days. Any remaining non-germinated seed after the 4-day (18-day total) count was rinsed with distilled water and incubated for an additional 10 days (28-day total) at 18 ± 2°C (24 hours dark) before another evaluation of emergence. After this, any remaining non-germinated seed was re-moistened and placed in a chamber with alternating dark-light (12 hours dark at 5 ± 2°C, 12 hours light at 25 ± 2°C) and counted at 31 and 35 days total (3 and 7 days after moving to the new chamber).

Any non-germinated seeds that were firm were further tested for viability using a TZ test. The seed coats were removed and the seeds soaked in a 1% w/v solution of 2,3,5-triphenyl tetrazolium chloride for ~4 hours at 30–35°C. Seeds with adequate red staining on the essential embryonic structure (radical tip stained slightly darker, cotyledons intact) were counted as viable (AOSA, 2010).

F.2.5. Evaluation

For all of the germination assessments described above, an evaluation based on the Association of Official Seed Analysts (AOSA) Rules for Testing Seeds (AOSA, 2014) was followed. Any fungal growth or other observable quality problem with the samples was also recorded.

F.3. Statistical analysis method

Across-site analyses were carried out for all germination and dormancy characteristics that were considered suitable for parametric statistical analysis (ANOVA). Data, transformed where necessary (based on Kolmogorov-Smirnov normality tests at a 0.05 significance level, Q-Q plots, and histograms), were subjected to mixed model ANOVA based on the model in equation (1) using the R package lmerTest (Kuznetsova et al., 2015).

$$Y_{ijk} = U + T_i + S_j + B(S)_{jk} + ST_{jk} + e_{ijk} \quad (1)$$

In this model, Y_{ijk} is the observed response for entry i at block k within site j, U is the overall mean, T_i is the fixed effect of entry i, S_j is the random effect of site j, $B(S)_{jk}$ is the random effect of block k within site j, ST_{jk} is the entry-by-site random interaction effect associated with site j and entry i, and e_{ijk} is the residual error. Data from standard residual plots were used to assess site variance homogeneity assumption, and appropriate statistical procedures were applied when performing mean comparisons (Kuznetsova et al., 2015; Pinheiro et al., 2017).

Significance of the marginal entry effect was evaluated using F-tests. Comparison of the across-sites mean for each LBFLFK treatment (sprayed and non-sprayed) against the mean of the parental control Kumily was conducted using t-tests.

Across-sites means for LBFLFK (sprayed and non-sprayed) and Kumily were also graphically compared against reference varieties using the smallest and largest entry-by-site means of the six reference canola varieties.

Individual site analyses were performed if a significant entry-by-site interaction occurred. The analysis was implemented using the mixed model in equation (2):

$$Y_{ik} = U + T_i + B_k + e_{ik} \quad (2)$$

where Y_{ik} is the observed response for entry i at block k, U is the site mean, T_i is the fixed effect of entry i, B_k is the random effect of block k, and e_{ik} is the residual error. Comparison of characteristic means from each LBFLFK treatment against the parental control Kumily were performed.

A significance level of $\alpha = 0.05$ (confidence level 95%) was used for all statistical tests. All analyses were carried out using R statistical software (R Core Team, 2016).

F.4. By-site analysis results

Results of the by-site analysis performed for the cold germination assay and the secondary dormancy assessments that showed a significant entry-by-site interactionare presented in Table F.3 to Table F.5 below.

Table F.3. By-Site Statistical Analysis of Seed Germination after Cold Conditions – 10 days

Site[1]	Control (Kumily)	LBFLFK (sprayed)[2]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[3]	Control minus LBFLFK (non-sprayed)
	Mean min–max	Mean min–max	Difference (p-value)[4]	min–max	Mean min–max	Difference (p-value)[4]
3NRBRK	58.41 49–72	4.59 3–7	53.82 (< 0.001)*	34.25–59.5	3.66 1–10	54.75 (< 0.001*)
3NRCB	64.36 42–74	2.97 1–5	61.38 (< 0.001)*	26.75–63.25	3.02 0–6	61.34 (< 0.001*)
3NREP	70.58 61–82	6.11 2–13	64.47 (< 0.001)*	40–58.75	1.46 1–2	69.12 (< 0.001*)
3NRGE	47.21 36–59	3.34 1–8	43.87 (< 0.001)*	30.75–45.75	2.04 0–5	45.17 (< 0.001*)
3NRLS	39.42 24–56	3.54 1–7	35.88 (0.001)*	22.25–41.75	1.77 0–8	37.65 (< 0.001*)
3NRNW-1	82.95 75–90	7.9 4–13	75.05 (< 0.001)*	34.5–68.5	11.73 5–27	71.22 (< 0.001*)

[1] Data were arc sine transformed. Means were back transformed, and differences are differences of back transformed means.
[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[3] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[4] Difference test p-value: * indicates p≤ 0.05.

Table F.4. By-Site Statistical Analysis of Seed Germination after Cold Conditions – 13 days

Site[1]	Control (Kumily)	LBFLFK (sprayed)[2]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[3]	Control minus LBFLFK (non-sprayed)
	Mean min–max	Mean min–max	Difference (p-value)[4]	min–max	Mean min–max	Difference (p-value)[4]
3NRBRK	83.73 78–89	62.11 57–72	21.62 (0.002)*	85.5–92.67	61.37 52–71	22.36 (0.001*)
3NRCB	84.65 74–89	68.09 60–73	16.56 (0.006)*	88–94.5	61.09 56–70	23.56 (0.001*)
3NREP	95.67 92–99	70.58 48–91	25.1 (0.015)*	80.75–94.5	36.01 28–54	59.67 (< 0.001*)
3NRGE	80.15 76–86	60.19 48–71	19.96 (0.002)*	72.5–84.5	65.42 57–75	14.72 (0.009*)
3NRLS	66.99 57–78	45.73 40–56	21.27 (0.005)*	58.5–74.75	41.42 34–51	25.57 (0.002*)
3NRNW-1	96.87 90–100	78.3 75–81	18.57 (0.001)*	93–94.75	76.58 73–81	20.3 (< 0.001*)

[1] Data were arc sine transformed. Means were back transformed, and differences are differences of back transformed means.
[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[3] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[4] Difference test p-value: * indicates p≤ 0.05.

Table F.5. By-Site Statistical Analysis of Percent Total Seed Germinated - Secondary Dormancy Assay

Site[1]	Control (Kumily)	LBFLFK (sprayed)[2]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[3]	Control minus LBFLFK (non-sprayed)
	Mean min–max	Mean min–max	Difference (p-value)[4]	min–max	Mean min–max	Difference (p-value)[4]
3NRBRK	92.22 89.13–95	89.69 83–91.92	2.53 (0.248)	87.6–97.44	88.66 85.15–90.29	3.56 (0.129)
3NRCB	96.58 93.07–98.94	80.26 76–83	16.32 (< 0.001)*	90.63–100	78.32 72.92–86.73	18.26 (< 0.001*)
3NREP	99.53 97.03–100	89.51 75.76–96.94	10.02 (0.007)*	96.41–100	78.09 74–83	21.44 (< 0.001*)
3NRGE	90.72 78.22–95.96	74.09 67–81	16.62 (0.008)*	64.28–96	74.81 66.67–82	15.91 (0.01*)
3NRLS	79.8 68–88.78	59.07 46.46–74.47	20.73 (0.009)*	68.78–85.25	52.51 45.83–59.18	27.29 (0.003*)
3NRNW-1	95.87 95–98	89.8 85–95.1	6.07 (0.022)*	90.22–99.25	84.38 82.35–87	11.49 (0.002*)

[1] Data were arc sine transformed. Means were back transformed, and differences are differences of back transformed means.
[2] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[3] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[4] Difference test p-value: * indicates p≤ 0.05.

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Appendix G. Pollen Morphology and Viability Analysis of EPA+DHA Canola Event LBFLFK – Methods

As described in the main petition document, EPA+DHA canola event LBFLFK and the parental control Kumily served as test and control entries for a comparative evaluation of canola pollen morphology and viability. Three commercial canola varieties were also included as reference entries to provide a range of values. The test, control, and reference substances are listed in Table G.1.

Table G.1. Test, Control, and Reference Substances for Pollen Viability and Morphology Analysis

Line/Variety	Purpose	BASF Seed Lot ID
LBFLFK	Test Substance	910017879621
Kumily	Parental Control Substance	910020076790
46A65	Reference Substance	910020076787
IMC302	Reference Substance	910020076789
Wizzard	Reference Substance	910020076793

Described are the methods that were used for this analysis including details of the statistical model.

G.1. Trial design

Plants were grown in the greenhouse under conditions that promote canola flowering. Plants were arranged in a randomized complete block design with 16 replicates for each entry. Pollen was prepared from flowers sampled when the stigma was at approximately half the height of the anthers.

G.2. Pollen morphology and viability analysis

Individual plant pollen samples from a total of 16 plants per entry were imaged for viability and morphology analysis. At least 170 pollen grains from each sample were evaluated for viability, and 15 pollen grains from each sample were evaluated for morphology. The characteristics quantified were percent viable pollen, pollen grain length, and pollen grain width.

For the viability analysis, each pollen grain sample was incubated in a 15% sucrose solution containing fluorescein diacetate (0.2 mg/ml, weight/volume). Aliquots of each sample were placed on microscope slides and imaged with a fluorescence microscope. Images were evaluated for the number of viable (fluorescing) pollen grains and the number of non-viable (non-fluorescing) pollen grains to quantify the percent viable pollen for each sample. These images were also used to quantify pollen grain length and width using image analysis software.

G.3. Pollen germination analysis

Individual plant pollen samples from a total of 12 plants per test, control, and reference entry were sampled for pollen germination assays. At least 175 pollen grains from each sample were imaged and evaluated. Each pollen grain sample was incubated in pollen germination medium (17% sucrose, 2 mM $CaCl_2$, 1.65 mM H_3BO_3, pH 7) on a microscope slide. After 6–8 hours of incubation, multiple images per sample were acquired using a microscope. Images were evaluated for the total number of visible pollen and the total number of germinated pollen to quantify the pollen germination rate for each sample.

G.4. Statistical analysis method

Linear mixed model analysis of variance (ANOVA) methods (Kuznetsova et al., 2015) were used for performing mean comparisons (Tukey HSD test) between EPA+DHA canola event LBFLFK and the parental control Kumily for percent pollen germination, percent viable pollen, pollen grain length, and pollen grain width using R statistical software (R Core Team, 2016). The statistical significance for the ANOVA F-test was predetermined to be at the 5% level ($\alpha = 0.05$). Conventional reference varieties were used to establish a reference range for each response variable.

The following linear model was fitted to each response variable:

$$y_{ij}=\mu+b_i+T_j+\varepsilon_{ij}$$

Where y_{ij} is the response variable measured within block *i* for test material *j*, μ is the overall mean effect, b_i is the random effect of block *i*, T_j is the fixed effect of test material *j*, and ε_{ij} is a random experimental error term associated with test material *j* within block *i*.

G.4.1. Data transformation and outlier detection

For y response variables measured as a percentage (i.e. germinated pollen and viable pollen), the following expression was used to get y' transformed values:

$$y'=\arcsin(\sqrt{y/100})$$

The linear mixed model above was fitted to each original response variable as well as to both arcsine transformed variables and to natural logarithmic and square root transformed values. The natural logarithm and square root transformation were applied only to continuous characteristics (i.e. pollen length and pollen width). This procedure allowed estimation of Studentized residuals associated with each observation. Outliers were defined by any data point that had a Studentized residual with an absolute value greater than 6. Normality of the residuals estimated for all fitted models (i.e. all original and

transformed variables) was assessed using a Kolmogorov-Smirnov normality test at a 0.01 significance level.

G.4.2. Comparison of means

ANOVA F-tests were performed to test whether there was an effect of test material (entry) on the mean of each measured characteristic. Specific comparisons were made between the estimated means of Kumily and LBFLFK. The Tukey HSD adjustment was applied when performing these specific mean comparisons. All significance tests were performed at the 95% confidence level.

All analyses were carried out using R statistical software (R Core Team, 2016).

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Kuznetsova A, Brockhoff P and Christensen R, 2015, online. lmerTest: Tests in Linear Mixed Effects Models. R package version 2.0-29. Accessed on January 18, 2016. available at http://CRAN.R-project.org/package=lmerTest

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Appendix H. Environmental Interaction Analysis of EPA+DHA Canola Event LBFLFK

This appendix provides further details on the ecological interactions comparative survey study using field-grown EPA+DHA canola event LBFLFK that is described in the main text of the petition. Details on the trial design and methods, including the statistical models, applied for the assessment of environmental interactions and more detailed results of the study are provided.

For this study, LBFLFK and the parental canola variety Kumily served as test and control entries. Three commercial canola varieties were also included to provide a reference range. Test, control, and reference entries are listed in Table H.1. LBFLFK received an application of Beyond®[1] herbicide at the 3–4 leaf stage.

Table H.1. Test, Control, and Reference Substances for Environmental Interaction Analysis

Line/Variety	Purpose	BASF Seed Lot ID
LBFLFK	Test Substance	910019112248
Kumily	Parental Control Substance	910019112249
Q2	Reference Substance	910018225702
46A65	Reference Substance	910018225703
Wizzard	Reference Substance	910018225704

H.1. Field sites and trial design

Data was collect at three sites that included the states of Idaho, South Dakota, and Minnesota (Table H.2). Each trial was designed as a randomized complete block with four replications of five entries. Each plot consisted of ~48 rows, and each row was ~10 m (32.8 feet) long with a seeding rate consistent with commercial practice. A fallow area with a minimum of 1.6 m (5.3 feet) in width surrounded each side of each plot.

Table H.2. Trial Designations and Locations for Environmental Interaction Analysis

Field Trial ID	Location
3NRAF	American Falls, ID
3NRBRK	Brookings, SD
3NRCB	Campbell, MN

[1] Beyond is a registered trademark of BASF Corp.

H.2. Agricultural practice

Activities related to preparation for planting, including tillage, seed bed preparation, fertilization, and weed control, were applied uniformly at each location to the entire field site area. Maintenance herbicide applications were used according to label directions. No fungicides or insecticides were used.

H.3. Environmental interactions analyses

Sampling methods for insects and other arthropods consisted of visual observations, sticky traps, and pitfall traps. For these assessments, sampling intervals at different dates and the following developmental stages (Meier, 2001) of the plants were used: 2–6 leaf (BBCH 12–16), at first flower (BBCH 60–61), at end of flowering (BBCH 69), and at maturity (BBCH 83–87). The sticky traps and pitfall traps were deployed in each plot for 3 days at each of the four different growth stages assessed. An assessment of arthropod community composition was performed within each plot according to the abundance of each organism for each sampling method at each sampling time point. The number of arthropods and organism growth stage (such as larvae, nymphs, and adults) were recorded. The arthropods identified were classified at the family taxonomic level for the more common taxa or a higher taxa level for the less common taxa (Triplehorn and Johnson, 2005).

An evaluation of earthworm density was also conducted in the sampling area at two sampling time points (BBCH 60–61 and BBCH 83–87). Two square frames measuring approximately 20 cm x 20 cm were placed in each plot at the front and back of the sampling area. Within each frame, approximately 10 liters of soil was removed to a depth of ~25 cm and inspected to collect all the earthworms. The worms collected were counted and weighed.

H.4. Statistical analysis method

The R statistical software (R Core Team, 2016) was used for statistical analyses, using the “lme4” package of the R software.

H.4.1. Shannon-Weaver index

The Shannon-Weaver (S-W) index (Shannon, 1948) was determined in order to assess the biodiversity in each combination of plant growth stage and sampling method. To this end, the treatment effect on diversity was analyzed for each sampling method and stage of crop development. The S-W index was calculated using the following formula:

$$H = -\sum_{i=1}^{S} p_i . \ln(p_i)$$

Where H is the S-W index value, p_i is the proportion of individuals that belong to taxa i, and S is the number of different taxa. The S-W index was calculated for the data obtained for each sampling method. At each observation time point, all the organisms observed were included in the S-W index calculation. The S-W index evaluates the number of taxa (species richness) present in the test, control, and reference plots and the relative amount (abundance) of each species. Index values may vary from "0" (low diversity) to ∞ (high diversity). Similar approaches to assess the influence on invertebrate diversity of a biotechnology-derived crop species have previously been used (Bai et al., 2012).

Individual site analyses were carried out for S-W index data for each method at each stage. Data were subjected to mixed model analysis of variance (ANOVA) based on the model in the following equation using R package lmerTest (Kuznetsova et al., 2015):

$$Y_{ij} = U + T_i + B_j + e_{ij}$$

In this model, Y_{ij} is the observed response for entry i at site j, U is the overall mean, T_i is the entry effect, B_j is the effect of block, and e_{ij} is the residual error. Entry effect was considered as fixed while the effect of block was considered as random. Reference varieties were not included in the ANOVA to avoid inflation of the residual error, but the maximum and minimum values provided by the references were used for comparisons. Paired contrasts for treatment effect were conducted using t-tests. Significant differences were declared at the 95% confidence level ($\alpha = 0.05$).

H.4.2. Invertebrate count data

The average number of each invertebrate taxon in each sampling method and growth stage of the plant was analyzed. Data on any assessed taxon represented by less than four counts or with the frequency of the most commonly observed value greater than or equal to 40% were not considered suitable for ANOVA, and those data were excluded from analysis.

Individual site analyses were performed for each method at each stage. Data were subjected to generalized linear mixed model ANOVA (Stroup, 2012) with Poisson distribution based on the equation model below using “glmer” function in R package lme4 (Bates et al., 2015):

$$ln(Y_{ij}) = U + T_i + B_j + e_{ij}$$

In this model, Y_{ij} is the organism counts for entry i at site j with Poisson distribution, U is the overall mean, T_i is the entry effect, B_j is the effect of block, and e_{ij} is the residual error. Entry effect was considered as fixed while the effect of block was considered as random. Reference varieties were not included in the ANOVA to avoid inflation of the residual error.

Paired contrasts for treatment effect of counting assessments were conducted using t-tests. Significant differences were declared at the 95% confidence level (α = 0.05). The back-transformed results were also provided for transformed data. For data not suitable for ANOVA, only the descriptive statistics and range were reported.

H.5. Results of the ecological interactions assessment

The main arthropods found in canola fields included ground beetles (Carabidae), springtails (Collembola), leaf beetles (Chrysomelidae), flies (Diptera), lady beetles (Coccinellidae), and diamondback moths (Plutellidae). Table H.3 to Table H.5 provide a summary of the arthropod organism counts made across all sites for visual observations, sticky trap captures, and pitfall fall trap captures, respectively. The details of these results are discussed in sections H.5.1 to H.5.3. The results of the survey of earthworms are provided in section H.5.4.

Table H.3. Visual Observations of Plant-Dwelling and Flying Arthropods Across All Varieties and Locations

Taxa Enumerated Order: Family	Common Names	Primary Roles	Total	% Total
Coleoptera:				
Chrysomelidae	Leaf beetles	Herbivore	2424	32
Coccinellidae	Lady beetles	Predator	2091	28
Meloidae	Blister beetles	Predator	6	0.08
Lepidoptera:				
Plutellidae	Diamondback moths	Herbivore	2482	33
Noctuidae	Cabbage looper	Herbivore	2	0.03
Crambidae	Beet webworms	Herbivore	9	0.12
Other Lepidoptera	NA	NA	8	0.11
Diptera:				
Syrphidae	Hoverflies	Pollinator	1	0.01
Other Diptera	NA	NA	9	0.12
Hemiptera:				
Pentatomidae	Stink bugs	Herbivore	360	4.8
Miridae	Tarnished plant bugs	Herbivore	5	0.07
Aphididae	Aphids	Herbivore	15	0.20
Hymenoptera:				
Apoidea	Bees	Pollinator	3	0.04
Dermaptera:				
Forficulidae	Earwigs	Omnivore	1	0.01
Araneae	Spiders	Predator	80	1.07
Total			**7496**	**100**

NA = Not applicable

Table H.4. Sticky Trap Captures of Flying and Foliage-Dwelling Taxa Across All Varieties and Locations

Taxa Enumerated Order: Family	Common Names	Primary Roles	Total	% Total
Coleoptera:				
Chrysomelidae	Flea beetles	Herbivore	7793	18
Coccinellidae	Lady beetles	Predator	1124	3
Meloidae	Blister beetles	Predator	3	0.01
Lepidoptera:				
Plutellidae	Diamondback moths	Herbivore	1012	2.3
Pieridae	Cabbage worms	Herbivore	42	0.1
Pieridae	Imported cabbage worms	Herbivore	9	0.02
Other Lepidoptera	NA	NA	4	0.01
Diptera:				
Syrphidae	Hoverflies	Pollinator	132	0.30
Other Diptera	NA	NA	31427	72.6
Hemiptera:				
Pentatomidae	Stink bugs	Herbivore	2	0.005
Miridae	Tarnished plant bugs	Herbivore	386	0.89
Cicadellidae	Leafhoppers	Herbivore	22	0.05
Hymenoptera:				
Apoidea	Bees	Pollinators	572	1.3
NI	Wasps	Predators/pollinators	2	0.005
NI	Parasitic wasps	Parasitoids	19	0.04
Neuroptera	Lacewings	Predators	443	1
Ephemeroptera	Mayfly	Predator	21	0.05
Dermaptera:				
Forficulidae	Earwigs	Omnivore	2	0.005
Odonata	Dragonfly	Predator	1	0.002
Araneae	Spiders	Predator	271	0.63
Total			**43287**	**100**

NI = Family not identified; NA = Not applicable

Table H.5. Pitfall Trap Captures of Ground Surface-Dwelling Invertebrate Taxa Across All Varieties and Locations

Taxa Enumerated Order: Family	Common Names	Primary Roles	Total[1]	% Total
Coleoptera:				
Carabidae	Ground beetles	Predators	3989	29
Chrysomelidae	Leaf beetles	Herbivore	612	4
Coccinellidae	Lady beetles	Predator	305	2
Meloidae	Blister beetles	Predator	2	0.01
Other Coleoptera	NA	NA	2	0.01
Lepidoptera:				
Plutellidae	Diamondback moths	Herbivore	56	0.4
Pieridae	Cabbage worms	Herbivore	4	0.03
Noctuidae	Cabbage looper	Herbivore	19	0.14
Other Lepidoptera	NA	NA	15	0.11
Diptera:				
Syrphidae	Hoverflies	Pollinator	47	0.3
Tipulidae	Crane flies		1	0.01
Other Diptera	NA	NA	470	3.4
Hemiptera:				
Miridae	Tarnished plant bugs	Herbivore	72	0.5
Aphididae	Aphids	Herbivore	36	0.3
Hymenoptera:				
Apoidea	Bees	Pollinators	15	0.1
Formicidae	Ants	Detritivore	4	0.03
NI	Wasps	Predators/pollinators	4	0.03
NI	Parasitic wasps	Parasitoids	30	0.2
Collembola	Springtails	Indirect decomposers	4865	35.7
Orthoptera	Grasshoppers	Herbivore	28	0.2
Dermaptera:				
Forficulidae	Earwigs	Omnivore	1	0.01
Thysanoptera	Thrips	Herbivore	1431	10.5
Araneae	Spiders	Predator	1622	12
Total			**13630**	**100**

NI = Family not identified; NA = Not applicable
[1] Total includes both immature and adult arthropods

H.5.1. Plant-dwelling and flying arthropods: visual observations

H.5.1.1. Diversity and abundance

Whole plant visual observations were used to detect groups of plant-dwelling and flying arthropods on the plants at the time of evaluation. Visual counts in the field trials included representatives of canola pests, beneficial invertebrate predators, and other functional groups such as pollinators. The S-W index and the statistical comparisons between LBFLFK and Kumily plots by site, taxa, and growth stage are presented in Table H.6 and Table H.7, respectively. With visual observations, the S-W index increased with plant development, generally with the maximum at BBCH 69 (flowering stage) in both LBFLFK and Kumily plots. At BBCH 69, the S-W index was slightly higher (not significant) in Kumily plots compared to the LBFLFK plots at two sites (1.55 versus 1.4 at 3NRAF and 1.38 versus 1.26 at 3NRCB) while LBFLFK plots had a slightly higher (not significant) S-W index at 3NRBRK than Kumily (0.6 versus 0.33). Overall, no significant differences were found in the diversity and abundance of arthropods measured through visual assessment (Table H.6).

Table H.6. Mean Diversity and Abundance of Taxa Evaluated by Visual Observations as Measured by the Shannon-Weaver Index

Site	Growth Stage	Control (Kumily)	LBFLFK	Reference Variety Range	Control minus LBFLFK
		Mean (SE) min–max	Mean (SE) min–max	min–max	Difference (SE) (p-value)[1]
3NRAF	BBCH 12–16	0.32 (0.37) 0–0.64	0.17 (0.35) 0–0.69	0.15–0.35	NA
	BBCH 60–61	1.23 (0.22) 0.79–1.6	0.92 (0.22) 0.56–1.2	1–1.3	0.31 (0.22) (0.2181)
	BBCH 69	1.55 (0.069) 1.1–1.9	1.4 (0.069) 1.1–1.6	1.4–1.4	0.15 (0.069) (0.1114)
	BBCH 83–87	1.04 (0.11) 0.94–1.2	0.9 (0.11) 0.65–1.3	1–1.1	0.14 (0.11) (0.2942)
3NRBRK	BBCH 12–16	0.34 (0.23) 0–0.64	0.33 (0.23) 0–0.67	0.42–0.71	0.015 (0.23) (0.951)
	BBCH 60–61	0.88 (0.12) 0.68–1.1	1.01 (0.12) 0.73–1.2	0.96–1.2	-0.13 (0.12) (0.3622)
	BBCH 69	0.33 (0.38) 0–0.69	0.6 (0.55) 0–1.3	0–0.11	NA
	BBCH 83–87	0.15 (0.24) 0–0.6	0.41 (0.24) 0.18–0.96	0.22–0.4	-0.26 (0.24) (0.3056)
3NRCB	BBCH 12–16	0.97 (0.035) 0.9–1.1	0.93 (0.035) 0.91–0.97	0.94–0.97	0.043 (0.035) (0.3166)
	BBCH 60–61	0.29 (0.093) 0–0.55	0.31 (0.093) 0.18–0.47	0.21–0.42	-0.012 (0.093) (0.9019)
	BBCH 69	1.38 (0.12) 1.2–1.6	1.26 (0.12) 1.1–1.4	1.3–1.3	0.12 (0.12) (0.351)
	BBCH 83–87	1.27 (0.042) 1.2–1.3	1.2 (0.042) 1.1–1.3	1.2–1.3	0.073 (0.042) (0.179)

The Shannon-Weaver index is a measure of diversity with lower values indicating reduced diversity
Data are an average of four replications with 25 subsamples per replication.
NA indicates not suitable for statistical analysis.
[1] Difference test p-value: * indicates $p \leq 0.05$.

Table H.7. Taxa Evaluated by Visual Observation at Each Growth Stage

Site	Taxa	Growth Stage	Control (Kumily)	LBFLFK	Reference Variety Range	p-value[2] of Difference Between Control and LBFLFK
			Mean (SE)[1]	Mean (SE)[1]	min–max	
3NRAF	Apoidea (bees)	BBCH 12–16	0 (0)	0 (0)	0–0	NA
	Apoidea (bees)	BBCH 60–61	1.25 (0.96)	0.25 (0.5)	0–2	NA
	Apoidea (bees)	BBCH 69	6.36 (1.38)	3.67 (1.38)	2–7	0.0891
	Apoidea (bees)	BBCH 83–87	0 (0)	0 (0)	0–1	NA
	Araneae (spiders)	BBCH 12–16	0.75 (0.96)	1 (2)	0–1	NA
	Araneae (spiders)	BBCH 60–61	17.25 (1.19)	15.25 (1.19)	16–18	0.4832
	Araneae (spiders)	BBCH 69	8.3 (1.23)	12.45 (1.23)	10–13	0.0453*
	Araneae (spiders)	BBCH 83–87	7.36 (1.24)	10.72 (1.24)	11–14	0.0835
3NRBRK	Diptera (flies)	BBCH 12–16	7.81 (1.43)	2.23 (1.43)	8–15	< 0.0001*
	Diptera (flies)	BBCH 60–61	15.5 (1.2)	14.5 (1.2)	10–16	0.7151
	Diptera (flies)	BBCH 69	2.57 (1.01)	3.42 (1.01)	4–8	< 0.0001*
	Diptera (flies)	BBCH 83–87	7.71 (1.26)	7.93 (1.26)	10–11	0.9059
	Plutellidae (diamondback moths)	BBCH 12–16	1.25 (1.83)	1.5 (1.83)	2–3	0.7633
	Plutellidae (diamondback moths)	BBCH 60–61	1 (1.41)	1 (1.41)	0–2	NA
	Plutellidae (diamondback moths)	BBCH 69	0.25 (0.5)	0 (0)	0–0	NA
	Plutellidae (diamondback moths)	BBCH 83–87	0 (0)	0 (0)	0–0	NA

Site	Taxa	Growth Stage	Control (Kumily)	LBFLFK	Reference Variety Range	p-value[2] of Difference Between Control and LBFLFK
			Mean (SE)[1]	Mean (SE)[1]	min–max	
3NRCB	Apoidea (bees)	BBCH 12–16	0 (0)	0 (0)	0–0	NA
	Apoidea (bees)	BBCH 60–61	1.25 (1.5)	1.25 (0.96)	1–2	NA
	Apoidea (bees)	BBCH 69	1.5 (1.29)	1.25 (0.96)	1–2	NA
	Apoidea (bees)	BBCH 83–87	0 (0)	0 (0)	0–0	NA
	Coccinellidae (lady beetles)	BBCH 12–16	2.5 (1.29)	2.25 (0.5)	2–2	NA
	Coccinellidae (lady beetles)	BBCH 60–61	1 (0.82)	0.5 (0.58)	0–1	NA
	Coccinellidae (lady beetles)	BBCH 69	12.46 (1.22)	13.21 (1.22)	10–13	0.7675
	Coccinellidae (lady beetles)	BBCH 83–87	37.34 (1.12)	45.55 (1.12)	35–41	0.0709
	Diptera (flies)	BBCH 12–16	6 (0.82)	7 (2.31)	5–6	NA
	Diptera (flies)	BBCH 60–61	26.15 (1.17)	17.43 (1.17)	22–28	0.0086*
	Diptera (flies)	BBCH 69	12.65 (1.2)	18.85 (1.2)	15–18	0.0274*
	Diptera (flies)	BBCH 83–87	23.49 (1.16)	22.51 (1.16)	19–22	0.77

NA indicates not suitable for statistical analysis.
[1] Data are on a natural log scale and are an average of four replications with 25 subsamples per replication.
[2] Difference test p-value: * indicates p≤ 0.05.

H.5.1.2. Analysis of specific arthropods found by visual observation

The most common plant-dwelling pest taxon noted with visual methods was the diamondback moth (Lepidoptera: Plutellidae), making up 33% of the total, followed by the leaf beetle (Coleoptera: Chrysomelidae). The major taxon of beneficial predators was the lady beetle (Coccinellidae), which comprised 28% of the total observed arthropods. A low population of pollinators such as hoverflies and bees was also observed in visual assessments across all plots and at different growth stages (Table H.7).

At location 3NRAF, bees (Apoidea) and spiders (Araneae) were observed in numbers that were suitable for statistical analyses. At location 3NRBRK, flies (Diptera) and diamondback moths (Plutellidae) were observed in numbers that were suitable for statistical analyses. At location 3NRCB, bees (Apoidea), lady beetles (Coccinellidae), and flies (Diptera) were observed in numbers that were suitable for statistical analyses.

Some statistical differences in the number of flies (Diptera) were observed between LBFLFK and Kumily plots. During BBCH 12–16, the mean number of flies observed at site 3NRBRK was statistically ($p \leq 0.05$) lower in the LBFLFK plots than Kumily plots (Table H.7). However, at BBCH 69, the number of flies was statistically higher in the LBFLFK plots than Kumily plots (Table H.7). In both cases, the mean number of flies for both the LBFLFK and Kumily plots was below the range of references (Table H.7).

Another statistical difference for fly abundance was noted during BBCH 60–61 at site 3NRCB, in which the mean number of flies observed was lower in LBFLFK plots than Kumily plots (Table H.7). The number of flies in the LBFLFK plots was also lower than the range of references while the number of flies in the Kumily plots was within the range of references. However, at the same location at BBCH 69, the mean abundance of flies was statistically higher ($p \leq 0.05$) in the LBFLFK plots than in the Kumily plots (Table H.7). At this growth stage, the mean fly number in LBFLFK plots was above the reference variety range, but the mean fly number for Kumily plots was below the range.

The only other significant difference in taxa noted by visual observation was for spiders (Araneae), which were significantly higher in LBFLFK plots compared to Kumily plots during BBCH 69 (flowering stage) at site 3NRAF. No other statistical differences in arthropod abundance were observed through visual assessment at any other site or growth stage. The lack of consistency between sampling times within the field sites suggests that the statistical differences in the abundance of flies were not biologically relevant. No significant differences were found in the abundance of other arthropods (Apoidea, Plutellidae, and Coccinellidae) counted visually between LBFLFK and Kumily plots.

Organism abundance in LBFLFK and Kumily was generally within the range of reference plots.

H.5.2. Flying and foliage-dwelling arthropods: sticky traps

H.5.2.1. Diversity and abundance

Sticky traps were used to detect groups of flying and foliage-dwelling arthropods. Sticky trap capture in the field trials included common canola pests, beneficial invertebrate predators, and other functional groups such as pollinators. The S-W index and the statistical comparisons between sticky trap captures in LBFLFK and Kumily plots by site, taxa, and growth stage are presented in Table H.8 and Table H.9, respectively. The S-W index of sticky trap captures increased during the period of plant development, reaching a maximum diversity at BBCH 69 (flowering stage) in both LBFLFK and Kumily plots at all three sites. For sticky trap capture, the S-W index values were statistically the same in LBFLFK and Kumily at all four growth stages (sampling times) at each field site (Table H.8). Therefore, S-W index values for sticky trap capture reveal the presence of a similar diversity and abundance of arthropods in LBFLFK and Kumily.

H.5.2.2. Analysis of specific flying and foliage-dwelling arthropods found on sticky traps

The major taxa of flying and foliage-dwelling arthropods as measured by sticky traps captures included Diptera, Coleoptera, Lepidoptera, Hymenoptera, Neuroptera, and Hemiptera. Numerous insect species in different genera and/or families of canola pests and of beneficial insect predators were identified. The most common pest insects captured in sticky traps included flea beetles (Chrysomelidae), diamondback moths (Plutellidae), and tarnished plant bugs (Miridae). The major beneficial predators included lady beetles (Coccinellidae), lacewings (Order: Neuroptera), and spiders (Order: Araneae). Dipteran insects such as flies were the most collected taxon on sticky traps, representing 73% of total captures. In addition, pollinators such as bees (Apoidea) and hoverflies (Syrphidae) were also captured through sticky traps.

At site 3NRAF, spiders (Araneae), flea beetles (Chrysomelidae), flies (Diptera), and diamondback moths (Plutellidae) were found in an abundance that was suitable for statistical analysis. At site 3NRBRK, the number of bees (Apoidea), flea beetles (Chrysomelidae), lady beetles (Coccinellidae), and flies (Diptera) could be statistically analyzed. At site 3NRCB, bees, lady beetles (Coccinellidae), flies (Diptera), and lacewings (Neuroptera) were found in a number that could be statistically analyzed.

A few statistically significant differences between LBFLFK and Kumily plots were found. At site 3NRAF during BBCH 12–16, the mean numbers of flies (Diptera) and diamondback moths (Plutellidae) were significantly ($p \leq 0.05$) lower in the LBFLFK plots than the Kumily plots (Table H.9) and slightly lower than the range of references. However, such differences were not observed at other growth stages at this site. No other site showed significant differences in the mean capture of any organisms in sticky traps at the BBCH 12–16 growth stage.

During BBCH 60–61, the mean number of flea beetles (Chrysomelidae) at site 3NRAF was significantly ($p \leq 0.05$) higher in LBFLFK plots compared to those of Kumily, and the mean number of flea beetles in LBFLFK plots was slightly higher than the maximum of the range of references (Table H.9). Also at this growth stage, a smaller number of flies (Diptera) were caught by sticky traps in the LBFLFK plots at site 3NRBRK relative to the Kumily plots, and this value was under the range of references (Table H.9). No other site showed a significant difference in the number of organisms caught with sticky traps at BBCH 60–61.

During BBCH 69, the mean number of flies (Diptera) caught by sticky traps at site 3NRCB was higher in the LBFLFK plots than in the Kumily plots and was higher than the maximum of the range of references (Table H.9). No other site showed a significant difference in the number of organisms caught with sticky traps at BBCH 69.

During BBCH 83–87, the mean capture of flea beetles (Chrysomelidae) at site 3NRAF was higher in the LBFLFK plots than the Kumily plots, and this mean was slightly higher than the maximum of the range of references (Table H.9). No other differences were detected between LBFLFK and Kumily plots at BBCH 83–87.

In summary, the organisms captured through sticky traps did not show a consistent trend of statistical differences between LBFLFK and Kumily plots during the various plant development stages of canola at any specific site.

Table H.8. Mean Diversity and Abundance of Taxa Captured in Sticky Traps as Measured by the Shannon-Weaver Index

Site	Stage	Control (Kumily)	LBFLFK	Reference Variety Range	Control minus LBFLFK
		Mean (SE) min–max	Mean (SE) min–max	min–max	Difference (SE)[1] (p-value)
3NRAF	BBCH 12–16	0.94 (0.24) 0.51–1.1	0.86 (0.24) 0.29–1.2	0.83–0.91	0.082 (0.24) (0.7469)
	BBCH 60–61	1.22 (0.13) 0.96–1.6	0.98 (0.13) 0.93–1	0.98–1.2	0.24 (0.13) (0.1266)
	BBCH 69	1.5 (0.084) 1.2–1.7	1.55 (0.084) 1.4–1.8	1.2–1.4	-0.05 (0.084) (0.5934)
	BBCH 83–87	1.24 (0.063) 1.1–1.4	1.27 (0.063) 1.2–1.3	1.1–1.4	-0.035 (0.063) (0.6011)
3NRBRK	BBCH 12–16	0.21 (0.026) 0.071–0.32	0.23 (0.026) 0.16–0.33	0.2–0.28	-0.018 (0.026) (0.5436)
	BBCH 60–61	0.54 (0.11) 0.41–0.75	0.45 (0.11) 0.29–0.59	0.43–0.55	0.087 (0.11) (0.4424)
	BBCH 69	0.85 (0.11) 0.55–1	0.84 (0.11) 0.74–0.95	0.51–0.95	0.015 (0.11) (0.8995)
	BBCH 83–87	0.65 (0.088) 0.48–0.76	0.7 (0.088) 0.51–0.93	0.49–0.6	-0.05 (0.088) (0.6079)
3NRCB	BBCH 12–16	0.75 (0.025) 0.7–0.83	0.77 (0.025) 0.74–0.8	0.73–0.76	-0.02 (0.025) (0.4795)
	BBCH 60–61	0.66 (0.077) 0.51–0.78	0.68 (0.077) 0.53–0.75	0.54–0.68	-0.01 (0.077) (0.9011)
	BBCH 69	0.72 (0.067) 0.65–0.84	0.66 (0.067) 0.47–0.94	0.7–0.84	0.07 (0.067) (0.3757)
	BBCH 83–87	0.68 (0.07) 0.56–0.78	0.52 (0.07) 0.44–0.68	0.63–0.75	0.15 (0.07) (0.0747)

Shannon-Weaver index is a measure of diversity; values near zero indicate lower diversity; data are the average of four replications with 5 subsamples per replication.
[1] Difference test p-value: * indicates p≤ 0.05.

Table H.9. Taxa Evaluated by Sticky Trap Capture at Each Growth Stage

Site	Taxa	Growth Stage	Control (Kumily)	LBFLFK	Reference Variety Range	p-value[2] of Difference Between Control and LBFLFK
			Mean (SE)[1]	Mean (SE)[1]	min–max	
3NRAF	Araneae (spiders)	BBCH 12–16	2.25 (1.7)	2.75 (1.5)	1–3	NA
	Araneae (spiders)	BBCH 60–61	0.75 (0.96)	0 (0)	0–0	NA
	Araneae (spiders)	BBCH 69	0 (0)	0.25 (0.5)	0–0	NA
	Araneae (spiders)	BBCH 83–87	5.71 (1.32)	7.19 (1.32)	9–13	0.4062
	Chrysomelidae (flea beetles)	BBCH 12–16	5.99 (1.34)	5.27 (1.34)	6–8	0.6602
	Chrysomelidae (flea beetles)	BBCH 60–61	72.08 (1.08)	102.51 (1.08)	75–91	<0.0001*
	Chrysomelidae (flea beetles)	BBCH 69	2.25 (2.63)	1.75 (2.36)	1–2	NA
	Chrysomelidae (flea beetles)	BBCH 83–87	1.49 (1.51)	6.38 (1.51)	2–6	<0.0001*
	Diptera (flies)	BBCH 12–16	44.9 (1.14)	19.83 (1.14)	28–41	<0.0001*
	Diptera (flies)	BBCH 60–61	23.5 (1.16)	21.5 (1.16)	22–32	0.5511
	Diptera (flies)	BBCH 69	42.94 (1.12)	40.21 (1.12)	61–69	0.5476
	Diptera (flies)	BBCH 83–87	22.28 (1.16)	25.25 (1.16)	20–25	0.3862
	Plutellidae (diamondback moths)	BBCH 12–16	3.75 (2.12)	0.5 (2.12)	1–2	0.0074*
	Plutellidae (diamondback moths)	BBCH 60–61	10.65 (1.2)	13.58 (1.2)	4–14	0.1917
	Plutellidae (diamondback moths)	BBCH 69	1.93 (1.58)	2.9 (1.58)	2–3	0.3728
	Plutellidae (diamondback moths)	BBCH 83–87	0 (0)	0 (0)	0–0	NA

Site	Taxa	Growth Stage	Control (Kumily)	LBFLFK	Reference Variety Range	p-value[2] of Difference Between Control and LBFLFK
			Mean (SE)[1]	Mean (SE)[1]	min–max	
3NRBRK	Apoidea (bees)	BBCH 12–16	5.25 (1.35)	5.75 (1.35)	4–8	0.7631
	Apoidea (bees)	BBCH 60–61	5.99 (1.36)	3.77 (1.36)	6–10	0.1304
	Apoidea (bees)	BBCH 69	3.75 (1.39)	5.75 (1.39)	2–6	0.1978
	Apoidea (bees)	BBCH 83–87	11.14 (1.25)	8.47 (1.25)	4–7	0.2213
	Chrysomelidae (flea beetles)	BBCH 12–16	2.58 (1.01)	2.58 (1.01)	1–3	0.9998
	Chrysomelidae (flea beetles)	BBCH 60–61	0.75 (0.96)	0.75 (0.5)	0–2	NA
	Chrysomelidae (flea beetles)	BBCH 69	4.38 (1.37)	4.62 (1.37)	2–6	0.8716
	Chrysomelidae (flea beetles)	BBCH 83–87	1.75 (0.96)	2.75 (1.71)	2–4	NA
	Coccinellidae (lady beetles)	BBCH 12–16	3.5 (1.45)	3.75 (1.45)	5–6	0.8527
	Coccinellidae (lady beetles)	BBCH 60–61	2.75 (1.72)	1.25 (1.72)	2–2	0.1438
	Coccinellidae (lady beetles)	BBCH 69	11.43 (1.23)	12.43 (1.23)	2–14	0.683
	Coccinellidae (lady beetles)	BBCH 83–87	2.5 (2.65)	1.5 (1)	0–1	NA
	Diptera (flies)	BBCH 12–16	1200 (124.3)	950 (124.3)	861–1085	0.282
	Diptera (flies)	BBCH 60–61	104.16 (1.07)	83.62 (1.07)	93–114	0.0025*
	Diptera (flies)	BBCH 69	54.99 (1.1)	59.99 (1.1)	48–50	0.3512
	Diptera (flies)	BBCH 83–87	56.38 (1.1)	47.19 (1.1)	34–54	0.0701

Site	Taxa	Growth Stage	Control (Kumily)	LBFLFK	Reference Variety Range	p-value[2] of Difference Between Control and LBFLFK
			Mean (SE)[1]	Mean (SE)[1]	min–max	
3NRCB	Apoidea (bees)	BBCH 12–16	0.25 (0.5)	0.5 (0.58)	0–0	NA
	Apoidea (bees)	BBCH 60–61	1 (0)	0.75 (0.5)	0–1	NA
	Apoidea (bees)	BBCH 69	0 (0)	0.75 (0.5)	0–1	NA
	Apoidea (bees)	BBCH 83–87	0 (0)	0 (0)	0–0	NA
	Coccinellidae (lady beetles)	BBCH 12–16	2.75 (1.46)	4.75 (1.46)	1–2	0.1491
	Coccinellidae (lady beetles)	BBCH 60–61	1 (0)	1.25 (0.96)	0–1	NA
	Coccinellidae (lady beetles)	BBCH 69	2 (0.82)	1.75 (0.96)	2–3	NA
	Coccinellidae (lady beetles)	BBCH 83–87	15.5 (1.2)	15.5 (1.2)	14–17	0.9999
	Diptera (flies)	BBCH 12–16	77.05 (1.08)	70.44 (1.08)	78–104	0.2711
	Diptera (flies)	BBCH 60–61	37.25 (1.12)	38.75 (1.12)	36–46	0.7308
	Diptera (flies)	BBCH 69	77.02 (1.08)	89.6 (1.08)	71–83	0.0496*
	Diptera (flies)	BBCH 83–87	13.25 (1.22)	12.5 (1.22)	14–16	0.7676
	Neuroptera (lacewings)	BBCH 12–16	1.25 (0.5)	2.5 (1.73)	1–2	NA
	Neuroptera (lacewings)	BBCH 60–61	3.94 (1.39)	5.17 (1.39)	3–7	0.412
	Neuroptera (lacewings)	BBCH 69	11 (1.24)	10.25 (1.24)	8–12	0.7449
	Neuroptera (lacewings)	BBCH 83–87	2.25 (0.5)	1.5 (0.58)	1–2	NA

NA indicates not suitable for statistical analysis.
[1] Data are on a natural log scale and are an average of four replications with 25 subsamples per replication.
[2] Difference test p-value: * indicates p≤ 0.05.

H.5.3. Ground surface-dwelling arthropods: pitfall traps

Pitfall traps were used to detect groups of ground surface-dwelling arthropods. Pitfall trap capture in the field trials included common canola pests, beneficial invertebrate predators, and other functional groups. The major surface-dwelling arthropod taxa as measured by pitfall trap captures were Coleopteran families (such as Carabidae, Chrysomelidae, and Coccinellidae), Lepidopteran families (such as Plutellidae, Pieridae, and Noctuidae), Diptera families, Hemipteran families (such as Miridae and Aphididae), Hymenopteran families (consisting of Apoidea and Formicidae), class Collembola, and orders Thysanoptera and Araneae. Indirect decomposers including springtails (Collembola) were the most collected taxon with pitfall traps (35.7% of the total), followed by predatory ground beetles (Coleoptera: Carabidae; 29% of total).

H.5.3.1. Diversity and abundance

The S-W index and the statistical comparisons between pitfall trap captures in LBFLFK and Kumily plots by site, taxa, and growth stage are presented in Table H.10 and Table H.11, respectively. The S-W index of pitfall trap captures increased as plant development progressed, generally reaching a maximum at BBCH 69 (flowering stage) in both LBFLFK and Kumily plots at all three sites. For pitfall trap capture, the S-W index values were statistically the same in LBFLFK and Kumily at all four growth stages (sampling times) at each field site (Table H.10). Therefore, S-W index values for pitfall trap capture reveal similar diversity and abundance of arthropods in LBFLFK and Kumily plots.

Table H.10. Mean Diversity and Abundance of Taxa Captured by Pitfall Traps as Measured by the Shannon-Weaver Index

Site	Stage	LBFLFK	Control (Kumily)	Reference Variety Range	Control minus LBFLFK
		Mean (SE) min–max	Mean (SE) min–max	min–max	Difference (SE) (p-value)[1]
3NRAF	BBCH 12–16	0.64 (0.048) 0.48–0.78	0.75 (0.048) 0.69–0.82	0.67–0.71	0.11 (0.048) (0.104)
	BBCH 60–61	1.1 (0.08) 1–1.2	1.11 (0.08) 0.95–1.4	1–1.1	0.013 (0.08) (0.8864)
	BBCH 69	1.71 (0.084) 1.5–1.8	1.78 (0.084) 1.7–1.8	1.7–1.7	0.07 (0.084) (0.4383)
	BBCH 83–87	1.21 (0.16) 0.91–1.3	1.46 (0.16) 1.2–1.7	1.4–1.5	0.26 (0.16) (0.1558)
3NRBRK	BBCH 12–16	0.92 (0.075) 0.76–1	0.81 (0.075) 0.55–0.99	0.74–0.87	-0.11 (0.075) (0.2455)
	BBCH 60–61	0.87 (0.11) 0.74–1.1	0.88 (0.11) 0.66–0.99	0.65–0.79	0.01 (0.11) (0.9311)
	BBCH 69	0.95 (0.12) 0.64–1.1	0.87 (0.12) 0.7–0.97	0.91–1	-0.08 (0.12) (0.5387)
	BBCH 83–87	0.55 (0.24) 0.26–0.84	0.59 (0.24) 0–0.95	0.46–0.66	0.037 (0.24) (0.8833)
3NRCB	BBCH 12–16	0.67 (0.026) 0.65–0.68	0.62 (0.026) 0.54–0.68	0.63–0.65	-0.048 (0.026) (0.1608)
	BBCH 60–61	0.54 (0.061) 0.43–0.63	0.63 (0.061) 0.5–0.69	0.61–0.64	0.09 (0.061) (0.2339)
	BBCH 69	0.67 (0.038) 0.65–0.69	0.65 (0.038) 0.54–0.69	0.65–0.68	-0.023 (0.038) (0.5727)
	BBCH 83–87	0.62 (0.038) 0.52–0.69	0.68 (0.038) 0.67–0.68	0.62–0.68	0.052 (0.038) (0.2169)

Shannon-Weaver index is a measure of diversity; values near zero indicate lower diversity; data are the average of four replications with 5 subsamples per replication.
[1] Difference test p-value: * indicates p≤ 0.05.

Table H.11. Taxa Evaluated by Pitfall Trap Capture at Each Growth Stage

Site	Taxa	Growth Stage	Control (Kumily) Mean (SE)[1]	LBFLFK Mean (SE)[1]	Reference Variety Range min–max	p-value[2] of Difference Between Control and LBFLFK
3NRAF	Araneae (spiders)	BBCH 12–16	0.5 (1)	0.25 (0.5)	0–2	NA
	Araneae (spiders)	BBCH 60–61	0.75 (0.5)	1 (1.41)	0–1	NA
	Araneae (spiders)	BBCH 69	5.5 (1.36)	5.25 (1.36)	4–11	0.8788
	Araneae (spiders)	BBCH 83–87	7.84 (1.28)	6.5 (1.28)	6–8	0.45
	Diptera (flies)	BBCH 12–16	0.25 (0.5)	0 (0)	0–0	NA
	Diptera (flies)	BBCH 60–61	2.75 (1.53)	2.75 (1.53)	4–6	0.9999
	Diptera (flies)	BBCH 69	14.56 (1.22)	11.35 (1.22)	12–24	0.205
	Diptera (flies)	BBCH 83–87	2.99 (1.01)	2.53 (1.01)	3–4	<0.0001*
	Collembola (springtails)	BBCH 12–16	82.16 (1.08)	93.19 (1.08)	76–118	0.0923
	Collembola (springtails)	BBCH 60–61	102.11 (1.07)	106.84 (1.07)	87–100	0.5117
	Collembola (springtails)	BBCH 69	20.7 (1.17)	19.99 (1.17)	28–40	0.8179
	Collembola (springtails)	BBCH 83–87	0.75 (0.96)	0 (0)	0–3	NA
	Thysanoptera (thrips)	BBCH 12–16	0 (0)	0 (0)	0–0	NA
	Thysanoptera (thrips)	BBCH 60–61	14.69 (1.18)	15.95 (1.18)	6–20	0.6176
	Thysanoptera (thrips)	BBCH 69	41.54 (1.11)	42.25 (1.11)	36–82	0.8728
	Thysanoptera (thrips)	BBCH 83–87	3.81 (1.01)	1.2 (1.01)	7–10	<0.0001*
3NRBRK	Carabidae (ground beetles)	BBCH 12–16	25.64 (1.16)	16.26 (1.16)	21–28	0.0026*
	Carabidae (ground beetles)	BBCH 60–61	16.09 (1.2)	12.49 (1.2)	19–24	0.1684
	Carabidae (ground beetles)	BBCH 69	1.75 (1.56)	4.5 (1.56)	4–6	0.034*
	Carabidae (ground beetles)	BBCH 83–87	2.5 (1.55)	2.75 (1.55)	1–3	0.8273
	Collembola (springtails)	BBCH 12–16	8.89 (1.26)	8.19 (1.26)	6–11	0.7238
	Collembola (springtails)	BBCH 60–61	7.5 (1.38)	3.5 (1.38)	4–7	0.0185*
	Collembola (springtails)	BBCH 69	3.75 (1.46)	3.25 (1.46)	4–5	0.7057
	Collembola (springtails)	BBCH 83–87	1 (2.38)	0.5 (2.38)	1–3	0.4235

Site	Taxa	Growth Stage	Control (Kumily) Mean (SE)[1]	LBFLFK Mean (SE)[1]	Reference Variety Range min–max	p-value[2] of Difference Between Control and LBFLFK
3NRCB	Araneae (spiders)	BBCH 12–16	9.5 (1.26)	8.75 (1.26)	8–11	0.7256
	Araneae (spiders)	BBCH 60–61	13.25 (1.19)	22 (1.19)	12–14	0.0035*
	Araneae (spiders)	BBCH 69	6.75 (1.29)	8.75 (1.29)	8–8	0.311
	Araneae (spiders)	BBCH 83–87	6.75 (1.29)	8.75 (1.29)	6–7	0.311
	Carabidae (ground beetles)	BBCH 12–16	4.8 (1.36)	5.52 (1.36)	5–6	0.646
	Carabidae (ground beetles)	BBCH 60–61	7.42 (1.3)	6.92 (1.3)	6–7	0.7927
	Carabidae (ground beetles)	BBCH 69	9.75 (1.24)	13.25 (1.24)	10–13	0.146
	Carabidae (ground beetles)	BBCH 83–87	5.75 (1.36)	4.75 (1.36)	4–6	0.5377

NA indicates not suitable for statistical analysis.
[1] Data are on a natural log scale and are an average of four replications with 25 subsamples per replication.
[2] Difference test p-value: * indicates p≤ 0.05.

H.5.3.2. Analysis of specific ground surface-dwelling arthropods found in pitfall traps

At site 3NRAF, spiders (Araneae), flies (Diptera), springtails (Collembola), and thrips (Thysanoptera) were captured in numbers that were suitable for statistical analysis. At site 3NRBRK, ground beetles (Carabidae) and springtails (Collembola) were observed in numbers suitable for analysis. At site 3NRCB, the abundance of spiders (Araneae) and ground beetles (Carabidae) were suitable for statistical analysis.

A few statistically significant differences between LBFLFK and Kumily plots were found. At site 3NRBRK during BBCH 12–16, the mean number of ground beetles (Carabidae) found in pitfall traps was higher in the Kumily plots than those of LBFLFK, and the LBFLFK mean was below the minimum of the range of references (Table H.11). No other significant differences between LBFLFK and Kumily plots were present at BBCH 12–16.

At BBCH 60–61, two significant differences were found. Springtails (Collembola) were significantly less abundant in LBFLFK plots than Kumily plots at site 3NRBRK (Table H.11). The number of springtails (Collembola) in LBFLFK plots was below the minimum value of the range of references while the number of springtails in Kumily plots was above the range of references. Additionally, at site 3NRCB, spiders (Araneae) were significantly more abundant in LBFLFK than Kumily plots, and the mean number of spiders was higher in LBFLFK plots than the range of references (Table H.11).

At BBCH 69, ground beetles (Carabidae) at site 3NRBRK were significantly more abundant in LBFLFK than Kumily plots, but the LBFLFK value was within the range of references (Table H.11). No other significant differences were found in organism number at BBCH 69.

At BBCH 83–87, the number of both flies (Diptera) and thrips (Thysanoptera) caught by pitfall traps at site 3NRAF was significantly lower in LBFLFK than Kumily plots, and both the LBFLFK and Kumily plots had lower abundances of these organisms than the range of references (Table H.11). No other significant differences were found in organism number during BBCH 83–87.

In summary, most of the pitfall trap LBFLFK plot captures were not significantly different from the captures in the Kumily plots. Where significant differences were found, they were not consistent over time or across locations, suggesting that the differences were not biologically relevant.

H.5.4. Earthworm total number and weight

The population density of earthworms was measured at two developmental stages (BBCH 60–61 and BBCH 83–87). The results of the statistical analysis are summarized in Table H.12. Earthworm samples collected in these field trials were not identified to family, genus, or species level. A total of 608 earthworms representing one subclass (Oligochaeta) were collected at all trial locations.

At BBCH 60–61, mean captures for earthworms ranged from 0.68–3.01 individuals per sampled area per plot of LBFLFK and Kumily. Total mean weight of the earthworms sampled per plot measured between 0.21 and 2.25 g (Table H.12). At BBCH 83–87, earthworm numbers averaged between 0.75 and 26.71. Total mean weight of earthworms sampled per plot ranged from 0.6–20.85 g (Table H.12). For both sampling periods, no significant differences in earthworm counts or weights between LBFLFK and Kumily plots were present at any trial site, and values for LBFLFK generally were within the range of references.

Table H.12. Earthworm Abundance at Each Growth Stage

Site	Measurement	Growth Stage	Control (Kumily) Mean (SE)[1]	LBFLFK Mean (SE)[1]	Reference Variety Range min–max	p-value[2] of Difference Between Control and LBFLFK
3NRAF	Number	BBCH 60–61	1.82 (1.97)	0.68 (1.97)	1–2	0.1474
	Number	BBCH 83–87	4 (5.48)	3.25 (4.57)	0–5	NA
	Weight (g)	BBCH 60–61	0.94 (0.49)	0.21 (0.49)	0.55–0.75	0.2335
	Weight (g)	BBCH 83–87	2.01 (2.85)	1.36 (1.92)	0–1.6	NA
3NRBRK	Number	BBCH 60–61	3.01 (1.51)	2.15 (1.51)	3–6	0.4164
	Number	BBCH 83–87	26.71 (1.16)	20.53 (1.16)	17–22	0.0709
	Weight (g)	BBCH 60–61	2.25 (0.29)	1.75 (0.29)	2.5–4.88	0.1817
	Weight (g)	BBCH 83–87	20.85 (3.13)	11.93 (3.13)	12–16	0.0648
3NRCB	Number	BBCH 60–61	0.75 (0.5)	0.75 (0.5)	0–1	NA
	Number	BBCH 83–87	0.75 (0.96)	0.75 (0.96)	0–1	NA
	Weight (g)	BBCH 60–61	0.75 (0.36)	0.72 (0.36)	0.45–1.2	0.9469
	Weight (g)	BBCH 83–87	0.6 (0.77)	0.8 (0.96)	0.4–0.75	NA

NA indicates not suitable for statistical analysis.
[1] Data are on a natural log scale and are an average of four replications with 25 subsamples per replication.
[2] Difference test p-value: * indicates p≤ 0.05.

H.6. Summary of ecological interactions assessment

The diversity and abundance of enumerated taxa varied at the different plant growth stages and field trial locations assessed. However, no statistically significant differences were consistently present between plots of LBFLFK and the parental control variety Kumily in the diversity and abundance of organisms as measured by S-W index. In quantitative assessments of pest, beneficial, generalist, and pollinator organism abundance, no consistent statistically significant differences ($p \leq 0.05$) were detected between LBFLFK and Kumily. Some differences were identified for certain insects and other arthropods at certain locations for a given observation, but these differences were not considered biologically relevant as they were not consistent across locations and plant development stages, and no clear trends were found. The results of this study provide supporting evidence that invertebrate communities were not significantly affected by exposure to LBFLFK compared to Kumily. These findings demonstrate that no adverse effects of event LBFLFK on invertebrate communities, including beneficial predators, insect pests, pollinators, and earthworms, are expected.

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Appendix I. Compositional Analysis of Harvested Seed from EPA+DHA Canola Event LBFLFK – Materials, Methods, By-Site Analyses

This appendix supplements the information provided in the main text of the petition. It provides additional information on the inputs to the comparative composition study, the methods that were used to generate the data, the statistical models that were used, the results of by-site statistics comparisons that were performed, and the resources that were used to provide additional context on the range of nutritional components in canola.

I.1. Materials

EPA+DHA canola event LBFLFK and Kumily served as test and control entries, respectively, for two seasons to compare nutritional components of harvested canola seed. Six commercial canola varieties were included as study references. These test, control, and reference lines are listed in Table I.1 and Table I.2. The field trials used to generate the harvested grain are the same that were used to provide comparative data on the agronomic and phenotypic performance of LBFLFK.

Table I.1. Test, Control, and Reference Substances – Winter 2014/15

Line/ Variety	Purpose	Seed Lot ID	Herbicide Treatment
LBFLFK	Test substance	2047830031	Standard herbicide
LBFLFK	Test substance	2047830031	Standard herbicide + Beyond® herbicide
Kumily	Control substance	2047837301	Standard herbicide
Q2	Reference substance	910018225702	Standard herbicide
46A65	Reference substance	910018225703	Standard herbicide
IMC105	Reference substance	910018225707	Standard herbicide
IMC302	Reference substance	910018225705	Standard herbicide
Wizzard	Reference substance	910018225704	Standard herbicide
Orinoco	Reference substance	910018225706	Standard herbicide

Table I.2. Test, Control, and Reference Substances – Spring 2015

Line/ Variety	Purpose	Seed Lot ID	Herbicide Treatment
LBFLFK	Test substance	910019041494	Standard herbicide
LBFLFK	Test substance	910019041494	Standard herbicide + Beyond® herbicide
Kumily	Control substance	910019041495	Standard herbicide
Q2	Reference substance	910018225702	Standard herbicide
46A65	Reference substance	910018225703	Standard herbicide
IMC105	Reference substance	910018225707	Standard herbicide
IMC302	Reference substance	910018225705	Standard herbicide
Wizzard	Reference substance	910018225704	Standard herbicide
Orinoco	Reference substance	910018225706	Standard herbicide

I.1.1. Field production of the materials

A first season of field trials in 2014–2015 was conducted at six southern United States (U.S.) sites. Each trial was designed as a randomized complete block with four replications of eight entries (Table I.1). Each treatment plot consisted of 18 rows, and each row was approximately 5 m (16 feet) long. Row spacing was approximately 20 cm (8 inches). Each plot was separated by a fallow area of approximately 1.6 m (5 feet), and treatment plots were separated by plots of male-sterile canola to reduce cross-pollination. LBFLFK plots were randomized into two treatments as part of the design: sprayed with an application of Beyond®[1] herbicide (active ingredient imazamox, an imidazolinone) at 34–37 g a.i./ha at the 3–4 leaf stage, and the other treatment not sprayed with Beyond® herbicide. All plots were also treated with the same standard non-selective herbicide program. During harvest, the middle six rows of the plot were threshed and bagged separately for compositional analysis. Field samples were transported under ambient conditions to a test site for cleaning, subsampling for composition, and storage under ambient conditions. Subsamples for composition were milled frozen and then transported to the analytical laboratory under dry ice. Of the six harvested trial sites, five (located in Texas and Georgia) were used for compositional analysis (Table I.3).

Table I.3. Field Trial Locations – Winter 2014/15

Field Trial ID	City, State
3SRBLY1	Beasley, TX
3SRJV	Jeffersonville, GA
3SRKT	Kendleton, TX
3SROM	Odem, TX
3SRRH	Rio Hondo, TX
3SRWN1[1]	Washington, LA

[1] Samples from 3SRWN1 were not analyzed for compositional analysis.

A second season of field trials in 2015 was conducted at eight northern U.S. sites. Each trial was designed as a randomized complete block with four replications of eight entries (Table I.2). Each treatment plot consisted of approximately 18 rows, and each row was approximately 6 m (20 ft) long. Each plot was again separated by a fallow area of approximately 1.6 m (5 ft) with treatment plots separated by plots of male-sterile canola. LBFLFK plots were randomized into two treatments as part of the design: sprayed with an application of Beyond® herbicide at 35–36 g a.i./ha at the 3–4 leaf stage, and the other treatment not sprayed with Beyond® herbicide. All plots were treated with the same non-selective standard herbicide program. During harvest, the middle six rows of the plot were threshed and bagged separately for compositional analysis. Field samples were transported under ambient conditions to a test site for cleaning, subsampling for composition, and storage under ambient conditions. Subsamples for composition were

[1] Beyond® is a registered trademark of BASF Corp.

milled frozen and then transported to the analytical laboratory under dry ice. Of the eight harvested trial sites, seven (in Iowa, Minnesota at two locations, Montana, North Dakota, South Dakota, and Washington), were used for compositional analysis (Table I.4).

Table I.4. Field Trial Locations – Spring 2015

Field Trial ID	City, State
3NRLS	Lime Springs, IA
3NRGE	Geneva, MN
3NRSC[1]	Sartell, MN
3NRCB	Campbell, MN
3NRNW-1	Northwood, ND
3NRMA-2	Malta, MT
3NREP	Ephrata, WA
3NRBRK	Brookings, SD

[1] Samples from 3NRSC were not analyzed for compositional analysis.

I.2. Methods

Analytical methods described below were used for both field seasons.

Moisture

Summary: Samples were dried in an oven at 130°C for two hours, removed from oven, cooled in a desiccator, and re-weighed. Moisture loss was calculated as the difference between the initial and dried weight.

Limit of Quantification (LOQ): 0.2%

References:

American Oil Chemists' Society (AOCS) Ba 2a-38 (AOCS, 2017d)
AOCS Ac 2-41 (AOCS, 2017b)
Association of Analytical Communities (AOAC) 925.10 (AOAC, 2016a)

Crude fat

Summary: Samples were weighed, placed in a Soxhlet extraction tube, and attached to a condenser. Samples were extracted for 5 hours using diethyl ether, dried in a forced draft oven for 30 minutes, cooled to room temperature, and weighed. Fat was calculated as a percentage of the sample.

Limit of Quantification (LOQ): 0.1%

References:

AOAC 945.16 (AOAC, 2016d)
AOCS Ba 3-38 (AOCS, 2017e)
AOCS Ac 3-44 (AOCS, 2017c)
AOCS Aa 4-38 (AOCS, 2017a)

Crude Protein

Summary: Samples were entered into the combustion chamber of a protein analyzer in which the gas from the combustion was analyzed for nitrogen content and used to calculate protein. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25.

Limit of Quantification (LOQ): 0.1%

References:

AOAC 979.09 (AOAC, 2016i)
AOCS Ba 4e-93 (AOCS, 2017f)
AOCS Ba 4f-00 (AOCS, 2017g)

Ash

Summary: Samples were weighed into a dry crucible, ashed in a muffle furnace at 600°C, and the weight of the ash determined.

Limit of Quantification (LOQ): 0.4%

References:

AOAC 945.38c (AOAC, 2016e)
AOAC 942.05 (AOAC, 2016c)

Carbohydrates

Summary: Carbohydrates were calculated as the difference between 100 – (moisture + protein + fat + ash).

Limit of Quantification (LOQ): N/A

References:

21CFR101.9 (US FDA, 2017)
USDA Handbook No. 74 (Merrill and Watt, 1973)

Crude fiber

Summary: Two grams of sample was weighed and fat extraction performed by placing the sample in a Soxhlet extraction tube, which was attached to a condenser for a minimum of 1 hour. The sample was digested, using a sulfuric acid solution and a sodium hydroxide solution, and filtered. The sample was dried at 130°C for a minimum of 1 hour. The weight of the residue minus the ash from the residue determined the crude fiber.

Limit of Quantification (LOQ): 0.2%

References:

AOCS Ba 6-84 (AOCS, 2017h)
AOAC 962.09 (AOAC, 2016f)

Neutral detergent fiber

Summary: Sample was digested with neutral detergent. The weight of the fiber residue determined the neutral detergent fiber result, which consisted predominantly of hemicellulose, cellulose, and lignin.

Limit of Quantification (LOQ): 0.03%

Reference:

Ankom Technologies: NDF for Ankom 2000 Fiber Analyzer (Ankom Technology, 2017b)

Acid detergent fiber

Summary: Sample was digested with acid detergent. The weight of the residue minus the ash from the residue determined the acid detergent fiber result, which consisted predominantly of cellulose and lignin.

Limit of Quantification (LOQ): 0.03%

Reference:

ANKOM Technology Method 10-21-05 (Ankom Technology, 2017a)

Amino acids by acid hydrolysis

Summary: Samples were hydrolyzed in 6 N HCl at 110°C for 24 hours. Quantification was performed via ion exchange chromatography with a post-column ninhydrin reaction and ultraviolet–visible spectroscopy (UV/Vis) detection.

Limit of Quantification (LOQ):

Amino Acid	LOQ
Alanine	0.01%
Arginine	0.05%
Aspartic acid	0.02%
Glutamic acid	0.02%
Glycine	0.01%
Histidine	0.01%
Hydroxyproline	0.06%
Isoleucine	0.02%
Leucine	0.02%
Total Lysine	0.01%
Phenylalanine	0.03%
Proline	0.05%
Serine	0.01%
Threonine	0.02%
Tyrosine	0.04%
Valine	0.02%

Reference:

AOAC 982.30, modified (AOAC, 2016j)

Amino acids by performic acid oxidation

Summary: Cystine and cysteine were first converted to cysteic acid and methionine to methionine sulfone by performic acid oxidation. The sample was hydrolyzed to release the cysteic acid and methionine sulfone from the protein. Quantification was performed via ion exchange chromatography with OPA (o-phthalaldehyde) post-column reaction and detection was done using a fluorescence detector.

Limit of Quantification (LOQ):

Amino Acid	LOQ
Cystine	0.01%
Methionine	0.01%

Reference:

AOAC 994.12, modified (AOAC, 2016n)

Tryptophan by alkaline hydrolysis

Summary: Samples were subjected to an alkaline digestion with lithium hydroxide at 110°C for 22 hours. Quantification was performed via reverse-phase chromatography with UV/Vis detection.

Limit of Quantification (LOQ): 0.01%

Reference:

AOAC 988.15, modified (AOAC, 2016m)

Tocopherols

Summary: The samples were saponified with ethanolic KOH in the presence of an antioxidant (ascorbic acid). The mixture was extracted with a petroleum ether/ethyl acetate solution. The combined organic phases were washed with water and dried over sodium sulfate. The solvent was exchanged to isooctane before injection on a high-performance liquid chromatography (HPLC) system equipped with a silica column and fluorescence detector.

Limit of Quantification (LOQ):

Isomer	LOQ
alpha-tocopherol	0.1 mg/100 g
beta-tocopherol	0.1 mg/100 g
gamma-tocopherol	0.1 mg/100 g
delta-tocopherol	0.1 mg/100 g

Reference:

AOAC 971.30 with HPLC quantification, modified (AOAC, 2016h)

Vitamin K1

Summary: Vitamin K was extracted from samples using dimethyl sulfoxide and hexane. The extracts were cleaned using a solid phase extraction cartridge. Vitamin K was eluted by methylene chloride, dried under a stream of nitrogen, reconstituted in 2-propanol, and analyzed on the HPLC with fluorescence detection.

Limit of Quantification (LOQ): 0.000625 mg/100 g

Reference:

AOAC 999.15, modified (AOAC, 2016p)

Phenolic acids

Summary: Samples were saponified and extracted in basic conditions in methanol/water. The extracts were acidified and analyzed using liquid chromatography with ultraviolet detection (LC-UV).

Limit of Quantification (LOQ):

Phenolic Acid	LOQ
Sinapine	0.007%
Ferulic acid	10 µg/g
Coumaric acid	10 µg/g

Reference:

Hagerman and Nicholson (1982)

Glucosinolates

Summary: Ground samples, together with an internal standard (sinigrin), were extracted with hot methanol (70% volume/volume in water). The anionic glucosinolates were loaded onto an ion-exchange column. After treatment by sulfatase, the desulfoglucosinolates were eluted by water and quantitated by reverse-phase ultra-performance liquid chromatography (UPLC) and UV detection.

Limit of Quantification (LOQ):

Glucosinolate	LOQ
Progoitrin	0.05 µmol/g
Glucoalyssin	0.05 µmol/g
Glucobrassicin	0.05 µmol/g
Glucobrassicanapin	0.05 µmol/g
Glucoiberin	0.05 µmol/g
Gluconapin	0.05 µmol/g
Gluconapoleiferin	0.05 µmol/g
Gluconasturtiin	0.05 µmol/g
Glucoraphanin	0.05 µmol/g
Neoglucobrassicin	0.05 µmol/g
Epi-progoitrin	0.05 µmol/g
4-Hydroxyglucobrassicin	0.05 µmol/g

Reference:

International Organization for Standardization (ISO) 9167-1:1992 (International Organization for Standardization, 1992)

Tannins

Summary: Samples were weighed into filter paper, placed in a soxhlet extraction tube, and attached to a condenser. Samples were defatted for 5 hours using diethyl ether and evaporated overnight in a fume hood. Condensed tannin molecules reacted with vanillin to form a red adduct whose absorbance was determined at 500 nanometers. The sample absorbance was compared to a standard curve that was generated from the vanillin reaction with catechin standard.

Limit of Quantification (LOQ): 0.2%

Reference:

Price et al. (1978)

Phytic acid

Summary: Sample aliquot was extracted with Na_2SO_4 solution for a minimum of 3 hours. Phytic acid (phytate) was precipitated with $FeCl_3$ and the precipitant then ashed. The phosphorus content in the precipitate was determined by the inductively coupled plasma optical emission spectrometry method. The phosphorus content was expressed in phytic acid equivalents.

Limit of Quantification (LOQ): 0.14%

Reference:

Ellis et al. (1977)

Minerals

Summary: Samples were digested by dry ashing. The digests were analyzed by inductively coupled plasma optimal emission spectroscopy against a standard curve of National Institute of Standards and Technology traceable standards to determine the mineral content.

Limit of Quantification (LOQ):

Element	LOQ
Calcium	0.004%
Phosphorous	0.004%
Magnesium	0.001%
Potassium	0.004%
Sodium	0.002%
Iron	0.0002%
Zinc	0.001%
Copper	0.0001%
Manganese	0.00005%

References:

AOAC 965.17, modified (AOAC, 2016g)
AOAC 927.02, modified (AOAC, 2016b)
AOAC 984.27, modified (AOAC, 2016k)
AOAC 985.01, modified (AOAC, 2016l)

Fatty acids

Summary: Fat was extracted from samples using petroleum ether. The extracted fat was reacted with boron-trifluoride/methanol reagent to convert fatty acids present in any form into their methyl ester forms. Samples were extracted into hexane and injected onto a capillary column gas chromatograph. Standards of known composition were used to identify the fatty acids present, and the amount of each individual fatty acid was reported as a percentage of the total sample weight.

Limit of Quantification (LOQ):

Fatty Acid	LOQ	Fatty Acid	LOQ
C14:0	0.01%	C20:3n-6	0.01%
C16:0	0.02%	C20:3n-9	0.01%
C16:1n-7	0.01%	C20:4n-3	0.01%
C16:1n-9	0.01%	C20:4n-6	0.01%
C16:3n-3	0.01%	C20:5n-3	0.01%
C17:0	0.01%	C22:0	0.01%
C17:1	0.01%	C22:1n-9	0.01%
C18:0	0.02%	C22:2n-6	0.01%
C18:1n-7	0.02%	C22:4n-3	0.01%
C18:1n-9	0.02%	C22:4n-6	0.01%
C18:2n-6	0.02%	C22:5n-3	0.01%
C18:2n-9	0.02%	C22:5n-6	0.01%
C18:3n-3	0.01%	C22:6n-3	0.01%
C18:3n-6	0.01%	C24:0	0.01%
C18:4n-3	0.01%	C24:1n-9	0.01%
C20:0	0.01%	C16:1 trans	0.01%
C20:1n-9	0.01%	C18:1 trans	0.02%
C20:2n-6	0.01%	C18:2 trans	0.02%
C20:2n-9	0.01%	Total trans fatty acids	0.01%
C20:3n-3	0.01%		

References:

AOCS Ce 2-66 (AOCS, 2017j)
AOAC 996.06, mod. (AOAC, 2016o)
AOCS Ce 1-62 (AOCS, 2017i)

Phytosterols

Summary: Fat was extracted from the samples using petroleum ether. The extracted fat was saponified. The saponified extract was washed on to a neutral alumina solid phase extraction cartridge. The unsaponifiable material including the sterols was eluted using diethyl ether. The sterol fraction of the unsaponifiable material was isolated using a normal phase high pressure liquid chromatograph. The sterols were derivatized to silyl esters using chlorotrimethylsilane and injected onto a capillary column gas chromatograph. Identification of sterols was performed using an internal quality control sample of known sterol composition. Quantification of sterols was performed using the response relative to the response of the cholestenol internal standard.

Limit of Quantification (LOQ):

Phytosterol	LOQ
24-Methylene cholesterol	0.0004%
Beta-sitosterol	0.0004%
Brassicasterol	0.0004%
Campestanol	0.0004%
Campesterol	0.0004%
Cholesterol	0.0004%
Clerosterol	0.0004%
Delta-5 avenasterol	0.0004%
Delta-5,23 stigmastadienol	0.0004%
Detla-5,24 stigmastadienol	0.0004%
Delta-7 avenasterol	0.0004%
Delta-7 stigmastenol	0.0004%
Sitostanol	0.0004%
Stigmasterol	0.0004%
Total phytosterols	0.0004%

References:

ISO 12228 (International Organization for Standardization, 2014)
AOCS Ch 6-91 (AOCS, 2017k)

I.3. Statistical analysis

Statistical methodology applied to the 2014/15 and the 2015 season are described below. Differences in the management of the data were limited to the data transformations applied to some analytes for the data from the 2015 season. All other statistical approaches were the same for each season.

I.3.1. Preliminary assessment of the data

Grain composition data were received from Eurofins Scientific, Inc., Nutrition Analysis Center (Des Moines, IA) for the 112 analytes listed in Table I.5. An additional component, total glucosinolates, was calculated as the sum of 4-hydroxyglucobrassicin, epi-progoitrin, glucobrassicin, glucobrassicanapin, gluconapin, gluconapoleiferin, and progoitrin. Glucosinolates that had values less than the limit of quantitation (LOQ) for most replicates were not included in this sum. Moisture and fatty acid content were given in terms of fresh weight and % of the total fatty acids; data from all other analytes were reported in terms of dry weight. Data points recorded as below the assay LOQ were assigned a value equal to half the LOQ for that analyte before mean calculations. If the resulting mean was less than LOQ, the mean was reported as < LOQ, but otherwise the calculated mean value was reported. If a pairwise comparison involved a mean that was < LOQ, an estimation of standard errors and subsequent significance tests were not carried out.

Table I.5. Measured Harvested Canola Components

Amino Acids[1]		
Alanine	Leucine	Threonine
Arginine	Isoleucine	Tryptophan
Aspartic Acid	Methionine	Tyrosine
Cystine	Phenylalanine	Valine
Glutamic Acid	Proline	Hydroxyproline
Glycine	Serine	Total Lysine
Histidine		
Antinutrients[1]		
Progoitrin	Gluconapoleiferin	4-Hydroxyglucobrassicin
Glucoalyssin	Gluconasturtiin	Phytic Acid
Glucobrassicin	Glucoraphanin	Tannins
Glucobrassicanapin	Neoglucobrassicin	Sinapine
Glucoiberin	Epi-Progoitrin	Coumaric Acid
Gluconapin	Total Glucosinolates[4]	Ferulic Acid
Fatty Acids[2]		
C14:0	C18:2n-9	C20:4n-6
C16:0	C18:2 trans	C20:5n-3
C16:1n-7	C18:3n-3	C22:0
C16:1n-9	C18:3n-6	C22:1n-9
C16:1 trans	C18:4n-3	C22:2n-6
C16:3n-3	C20:0	C22:4n-3
C17:0	C20:1n-9	C22:4n-6
C17:1	C20:2n-6	C22:5n-3
C18:0	C20:2n-9	C22:5n-6
C18:1n-7	C20:3n-3	C22:6n-3
C18:1n-9	C20:3n-6	C24:0
C18:1 trans	C20:3n-9	C24:1n-9
C18:2n-6	C20:4n-3	Total trans fatty acids
Minerals[1]		
Calcium	Phosphorus	Potassium
Copper	Magnesium	Sodium
Iron	Manganese	Zinc
Proximates and Fibers[1]		
Acid detergent fiber	Ash	Moisture[3]
Crude fiber	Crude fat	Protein
Neutral detergent fiber		
Sterols[1]		
24-Methylene cholesterol	Cholesterol	Delta-7 avenasterol
Beta-sitosterol	Clerosterol	Delta-7 stigmastenol
Brassicasterol	Delta-5 avenasterol	Sitostanol
Campestanol	Delta-5,23 stigmastadienol	Stigmasterol
Campesterol	Delta-5,24 stigmastadienol	Total phytosterols
Vitamins[1]		
Vitamin K1	Beta-tocopherol	Delta-tocopherol
Alpha-tocopherol	Gamma-tocopherol	Total tocopherols

[1] Data are reported on a dry weight basis.
[2] Data are reported as percent of total fatty acids.
[3] Data are reported as percent of fresh weight.
[4] Data are obtained by calculation.

I.3.2. Detection of outliers and data transformations

Using the R package lmerTest (Kuznetsova et al., 2015), a linear mixed model was applied to all measurements suitable for statistical analysis to detect potential outliers in the dataset by screening Studentized residuals (Belsley et al., 1980). The model effects included entry, site, replication within-site, and entry-by-site interaction. A residual was the difference between any value and its predicted value from a statistical model. The Studentized residuals were scaled to have estimated variance = 1. Data points with Studentized residuals outside of the ± 6 standard deviation range were considered for exclusion, as extreme outliers, from the final analyses (Table I.6 and Table I.7).

Table I.6. Composition Values Identified as Outliers – Winter 2014/15

Component	Site	Plot	Entry	Material	Value	Unit[1]	Residual
Alpha-tocopherol	3SROM	309	E01	LBFLFK	46.2	mg/100 g	8.89
Ash	3SRKT	201	E01	LBFLFK	27.2	%	12.22
Delta-7 stigmastenol	3SRRH	304	E08	IMC302	0.02	%	7.73
Delta-tocopherol	3SRKT	111	E09	Wizzard	4.07	mg/100 g	11.63
Glucoalyssin	3SRBLY1	309	E03	Kumily	0.058	µmol/g	-7.21
Glucobrassicin	3SRJV	205	E09	Wizzard	0.74	µmol/g	6.39
Glucobrassicin	3SRRH	401	E01	LBFLFK	0.063	µmol/g	-7.94
Gluconapin	3SRJV	203	E05	Q2	7.14	µmol/g	10.06
Iron	3SRKT	201	E01	LBFLFK	0.78	%	12.73
Neoglucobrassicin	3SRJV	205	E09	Wizzard	0.47	µmol/g	6.70
Neutral detergent fiber	3SRKT	201	E01	LBFLFK	26.8	%	7.23
Total tocopherols	3SROM	309	E01	LBFLFK	73	mg/100 g	6.01
C18:3n-3	3SRBLY1	202	E05	Q2	4.52	%	-6.82
C20:0	3SRRH	408	E09	Wizzard	< 0.04	%	-6.68

[1] Unit in dry weight except for fatty acids.

Table I.7. Composition Values Identified as Outliers – Spring 2015

Component	Site	Plot	Entry	Material	Value	Unit	Studentized Residual
Alpha-tocopherol	3NRNW-1	102	E05	IMC105	3.704[1]	%	9.00
Total tocopherols	3NRNW-1	102	E05	IMC105	64.238	%	6.50
Vitamin K1	3NRBRK	204	E01	LBFLFK	-2.440[1]	%	-5.61
Gluconapin	3NREP	308	E02	Kumily	0.025[2]	%	-5.98

[1] Natural log-transformed data.
[2] Half of the LOQ (0.05).

Plots of residuals against predicted values and site were also examined to confirm points identified as outliers and to check the need for a data transformation.

For the 2014/15 season, no transformations were suggested after Kolmogorov-Smirnov normality tests and a visual inspection of residual plots (Q-Q plots, histograms, and scatter plots). For the 2015 season, log transformations were applied to the analytes in Table I.8.

Table I.8. Grain Component Analytes Subjected to Transformation Prior to ANOVA – Spring 2015

Analytes	Transformation
Beta-tocopherol	natural log
Vitamin K1	natural log
Glucoalyssin	natural log
Progoitrin	natural log
Delta-tocopherol	natural log
Glucobrassicin	natural log
Alpha-tocopherol	natural log
Glucobrassicanapin	natural log
C20:1n-9	natural log

I.3.3. Detection of heterogeneity of error variance among sites

Graphs of residuals against predicted values and site (data not shown) were examined to assess the assumption of homogeneous error variances. Apparent site-related variance heterogeneity in graphs was also assessed by comparing Akaike information criterion values (Sakamoto et al., 1986) for the linear mixed model with homogenous error variances and heterogeneous error across sites.

I.3.4. Range determination

Ranges reported were based on the minimum and maximum of by-site and by-entry means. The means at each site were calculated based on the four replication plots at each site. The lowest mean out of the six reference varieties was set to be the lower limit of the reference range, and the highest mean out of six reference varieties was set to be the upper limit of the reference range. The same method was applied to obtain a separate range for each test entry and the control. Identified outliers were not included in the calculation.

I.3.5. Data not suitable for ANOVA

Composition analytes that were highly discrete or that contained numerous zeroes or numerous values < LOQ were identified based on the three criteria listed below. If the measured response met any of the 3 criteria, it was considered not suitable for analysis of variance (ANOVA).

a. The variable had less than or equal to 6 distinct values.
b. More than 40% of the data points were the same value.
c. More than 40% of the within-site-and-genotype variances of the variable were 0.

In some cases, discreteness was also declared if the observations met the above criteria at most of the sites. For the compositional measurements that were determined not suitable for statistical analysis (Table I.9 and Table I.10), results were summarized in terms of across-site means and ranges. For these data that were unsuitable for ANOVA, missing p-values were reported as not appropriate or not suitable for statistical analysis.

Table I.9. Grain Components Not Suitable for Statistical Analysis – Winter 2014/15

Fatty Acids		
C14:0	C18:3n-6	C20:5n-3
C16:1n-9	C18:4n-3	C22:1n-9
C16:1 trans	C20:2n-6	C22:2n-6
C16:3n-3	C20:2n-9	C22:4n-3
C17:0	C20:3n-3	C22:4n-6
C17:1	C20:3n-6	C22:5n-3
C18:1 trans	C20:3n-9	C22:5n-6
C18:2n-9	C20:4n-3	C22:6n-3
C18:2 trans	C20:4n-6	
Other Analytes		
Campestanol	Glucoiberin	Delta-7 avenasterol
Cholesterol	Gluconapoleiferin	Epi-progoitrin
Clerosterol	Glucoraphanin	Tannins
Copper	Manganese	Zinc
Delta-5,23 stigmastadienol	Coumaric acid	Sitostanol
24-methylene cholesterol	Delta-5,24 stigmastadienol	

Table I.10. Grain Components Not Suitable for Statistical Analysis – Spring 2015

Fatty Acids		
C14:0	C18:3n-6	C20:5n-3
C16:1n-9	C18:4n-3	C22:1n-9
C16:1 trans	C20:2n-6	C22:2n-6
C16:3n-3	C20:2n-9	C22:4n-3
C17:0	C20:3n-3	C22:4n-6
C17:1	C20:3n-6	C22:5n-3
C18:1 trans	C20:3n-9	C22:5n-6
C18:2n-9	C20:4n-3	C22:6n-3
C18:2 trans	C20:4n-6	
Other Analytes		
Campestanol	Delta-7 avenasterol	Manganese
Cholesterol	Copper	Sodium
Clerosterol	Glucoiberin	Stigmasterol
Delta-5,23 stigmastadienol	Glucoraphanin	Tannins
Delta-5,24 stigmastadienol	Iron	Zinc
24-methylene cholesterol	Delta-5 avenasterol	Delta-7 stigmastenol
Epi-progoitrin	Gluconapoleiferin	Neoglucobrassicin
Sitostanol		

Additionally, because the EPA+DHA canola trait influences the fatty acid composition of event LBFLFK compared to other canola varieties, there are fatty acid composition data values that were consistently found above the LOQ in LBFLFK samples but not in the other entries. Therefore, these data were not suitable for ANOVA, and no statistical comparisons were performed. Summary statistics (means and ranges) were recorded for the LBFLFK entries (Table I.11 and Table I.12).

Table I.11. Fatty Acid Values in LBFLFK with More than 50% of Observations below the Limit of Quantification in All Entries Other than LBFLFK – Winter 2014/15

Component (% of total fatty acids)	LBFLFK (sprayed)[1]	LBFLFK (non-sprayed)[2]
	Mean (SE) min–max	Mean (SE) min–max
C16:1 trans	0.068 (0.0031) 0.065–0.073	0.066 (0.0038) 0.06–0.07
C16:3n-3[3]	0.025 (0.02)[3] < LOQ–0.061	< LOQ
C18:2n-9	0.9 (0.089) 0.82–1.03	0.91 (0.071) 0.84–1.01
C18:2 trans	< LOQ	< LOQ
C18:3n-6	1.75 (0.42) 1.12–2.17	1.7 (0.44) 0.97–2.09
C18:4n-3	0.26 (0.039) 0.2–0.29	0.25 (0.044) 0.18–0.29
C20:2n-9	0.22 (0.042) 0.17–0.28	0.23 (0.038) 0.19–0.26
C20:3n-3	0.064 (0.0063) 0.057–0.073	0.062 (0.0076) 0.052–0.073
C20:3n-6	3.56 (0.79) 2.25–4.19	3.56 (0.77) 2.29–4.19
C20:3n-9	0.062 (0.012) 0.048–0.07	0.064 (0.0099) 0.052–0.077
C20:4n-3	1.77 (0.39) 1.15–2.11	1.8 (0.37) 1.27–2.12
C20:4n-6	2.26 (0.36) 1.89–2.72	2.19 (0.39) 1.66–2.62
C20:5n-3	7.21 (1.26) 4.98–7.94	7.21 (1.34) 4.83–7.96
C22:1n-9	< LOQ	< LOQ
C22:2n-6	< LOQ	< LOQ
C22:4n-3	0.51 (0.12) 0.32–0.64	0.51 (0.1) 0.36–0.61
C22:4n-6	0.46 (0.11) 0.29–0.56	0.44 (0.11) 0.27–0.54
C22:5n-3	2.94 (0.53) 2.05–3.44	2.93 (0.46) 2.16–3.38
C22:5n-6	0.089 (0.027) 0.051–0.12	0.085 (0.022) 0.048–0.11
C22:6n-3	1.02 (0.18) 0.73–1.18	1.02 (0.18) 0.71–1.15

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] This analyte was only detected > LOQ in one replicate (plot sample) of an LBFLFK sample.

Table I.12. Fatty Acid Values in LBFLFK with More than 50% of Observations below the Limit of Quantification in All Entries Other than LBFLFK – Spring 2015

Component (% of total fatty acids)	LBFLFK (sprayed)[1] Mean (SE) Min–Max	LBFLFK (non-sprayed)[2] Mean (SE) Min–Max
C16:1 trans	0.057 (0.0043) 0.05–0.06	0.053 (0.0022) 0.05–0.055
C16:3n-3	< LOQ	< LOQ
C18:1 trans	0.12 (0.019) 0.1–0.15	0.13 (0.022) 0.1–0.15
C18:2n-9	1.12 (0.15) 0.96–1.38	1.12 (0.15) 0.96–1.4
C18:2 trans	< LOQ	< LOQ
C18:3n-6	1.6 (0.16) 1.44–1.82	1.62 (0.16) 1.46–1.85
C18:4n-3	0.26 (0.038) 0.21–0.33	0.26 (0.039) 0.22–0.34
C20:2n-9	0.33 (0.06) 0.27–0.43	0.33 (0.062) 0.26–0.44
C20:3n-3	0.067 (0.0093) 0.06–0.082	0.066 (0.0089) 0.06–0.08
C20:3n-6	4.06 (0.38) 3.65–4.53	4.08 (0.31) 3.74–4.5
C20:3n-9	0.079 (0.016) 0.06–0.1	0.077 (0.016) 0.057–0.1
C20:4n-3	1.92 (0.27) 1.54–2.37	1.92 (0.25) 1.55–2.35
C20:4n-6	1.87 (0.25) 1.62–2.19	1.87 (0.25) 1.57–2.23
C20:5n-3	6.27 (0.46) 5.47–6.98	6.26 (0.49) 5.32–6.93
C22:1n-9	< LOQ	< LOQ
C22:2n-6	< LOQ	< LOQ
C22:4n-3	0.68 (0.12) 0.54–0.9	0.72 (0.1) 0.6–0.91
C22:4n-6	0.45 (0.042) 0.38–0.5	0.45 (0.046) 0.38–0.51
C22:5n-3	2.75 (0.15) 2.51–3	2.74 (0.17) 2.44–2.97
C22:5n-6	0.072 (0.017) 0.05–0.1	0.072 (0.015) 0.055–0.098
C22:6n-3	0.77 (0.12) 0.59–0.96	0.76 (0.11) 0.61–0.95

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

I.3.6. Statistical analysis of continuous measurements

The data obtained for compositional analysis for each field season were subjected to statistical analysis using R version 3.2.2 (R Core Team, 2015). Across-site analyses were carried out for composition analytes that were considered suitable for ANOVA. Data were subjected to mixed model ANOVA based on the model in equation (1) using R package lmerTest (Kuznetsova et al., 2015):

$$Y_{ijk} = U + T_i + S_j + B(S)_{jk} + ST_{ij} + e_{ijk} \quad (1)$$

In this model, Y_{ijk} is the observed response for entry i at site j block k, U is the overall mean, T_i is the entry effect, S_j is the site effect, $B(S)_{jk}$ is the effect of block within location, ST_{ij} is the entry-by-site interaction effect, and e_{ijk} is the residual error. Entry effect was considered as fixed while the effects of site, block within site, and entry-by-site interaction were considered as random. In the ANOVA, the reference varieties were not included to avoid inflation of the entry-by-site interaction.

Two versions of the model in equation (1) were employed depending on whether site-related variance heterogeneity was evident for a given response variable. For most variables, the standard model with the e_{ijk} assumed to have constant variance was used, but where appropriate, the more general model with variance e_{ijk} differing by site was used with R package nlme (Pinheiro et al., 2016).

Significance of the overall entry effect was evaluated using the mixed model F-test, and comparison of the across-site mean for each LBFLFK entry with the mean for the parent Kumily was conducted using a t-test. For convenience, contrasts between each LBFLFK entry and Kumily are referred to as “difference tests” in tables within the results section. Across-site means for each LBFLFK entry were also compared with ranges based on the smallest and largest of the by-site and by-entry means for the set of six reference varieties.

Individual site analyses were performed if a significant entry-by-site interaction occurred. The analysis was implemented using R package lmerTest (Kuznetsova et al., 2015) based on the mixed model in equation (2):

$$Y_{ij} = U + T_i + B_j + e_{ij} \quad (2)$$

where Y_{ij} is the observed response for entry i at block j, U is the overall mean, T_i is the entry effect, B_j is the effect of block, and e_{ij} is the residual error. The entry effect was considered as fixed while the effect of block was considered as random. Results for the comparison of each LBFLFK entry with Kumily are reported for each site.

A significance level of α = 0.05 (confidence level 95%) was used for all statistical tests.

I.4. By-site analysis of compositional analytes

Analytes measured from the winter 2014/15 and spring 2015 trials that had significant F-test p-values for entry-by-site interactions in the across-site analysis are presented in Table I.13 and Table I.14, respectively. The by-site analyses for the winter 2014/15 trials are presented in Table I.15 to Table I.27 in the order listed in Table I.13. The by-site analyses for the spring 2015 trials are presented in Table I.28 to Table I.40 in the order listed in Table I.14.

Table I.13. Compositional Analytes with Significant Entry-by-Site Interaction – Winter 2014/15

Component	Entry-by-Site ProbF[1]
Neutral detergent fiber	0.004
C16:1n-7	0.0004
C18:0	< 0.0001
C18:1n-9	< 0.0001
C18:2n-6	< 0.0001
C18:3n-3	< 0.0001
C20:1n-9	< 0.0001
C22:0	< 0.0001
C24:1n-9	0.0007
Total trans fatty acids	0.0025
Glucobrassicin	< 0.0001
Delta-5 avenasterol	< 0.001
Delta-7 stigmastenol	< 0.001

[1] F-test p-value of entry-by-site interaction in combined site analysis.

Table I.14. Compositional Analytes with Significant Entry-by-Site Interaction – Spring 2015

Component	Entry-by-Site ProbF[1]
C16:1n-7	< 0.001
C18:0	< 0.036
C18:1n-7	< 0.013
C18:1n-9	< 0.001
C18:2n-6	< 0.001
C18:3n-3	< 0.001
C20:1n-9	< 0.001
C22:0	< 0.001
C24:0	< 0.001
C24:1n-9	< 0.001
Coumaric acid	0.011
Brassicasterol	< 0.001
Total phytosterols	0.022

[1] F-test p-value of entry-by-site interaction in combined site analysis.

Table I.15. Neutral Detergent Fiber – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3SRBLY1	18.4 (0.42) 17.9–19.2	16.65 (0.42) 15.4–18.1	1.75 (0.59) (0.016)*	15.53–17.38	17.05 (0.42) 16.2–17.8	1.35 (0.59) (0.048)*
3SRJV	16.38 (0.33) 15.8–16.8	15.85 (0.33) 15.1–16.2	0.52 (0.47) (0.296)	14.97–17.3	16.95 (0.33) 15.8–18.1	-0.57 (0.47) (0.255)
3SRKT	17.07 (0.45) 16.7–17.3	18.61 (0.45) 17.3–20.2	-1.53 (0.58) (0.043)*	15.37–18.02	16.82 (0.45) 16–17.9	0.25 (0.53) (0.658)
3SROM	16.1 (0.39) 15.5–16.5	16.52 (0.39) 15.4–17.9	-0.42 (0.55) (0.469)	15.88–17.18	17.55 (0.39) 17.1–17.9	-1.45 (0.55) (0.039)*
3SRRH	16.65 (0.36) 15.8–17.4	16.65 (0.36) 16–17.2	0 (0.4) (1)	15.47–17.3	16.77 (0.36) 16–17.8	-0.12 (0.4) (0.764)

All data are in units of % dry weight.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.16. C16:1n-7 – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3SRBLY1	0.29 (0.0048) 0.28–0.31	0.2 (0.0048) 0.19–0.2	0.095 (0.0068) (< 0.001)*	0.21–0.24	0.19 (0.0048) 0.19–0.2	0.1 (0.0068) (< 0.001)*
3SRJV	0.28 (0.01) 0.27–0.3	0.22 (0.01) 0.2–0.27	0.058 (0.014) (0.003)*	0.2–0.25	0.21 (0.01) 0.2–0.21	0.072 (0.014) (0.001)*
3SRKT	0.32 (0.0029) 0.31–0.33	0.21 (0.0029) 0.21–0.21	0.1 (0.0041) (< 0.001)*	0.22–0.28	0.21 (0.0029) 0.21–0.21	0.1 (0.0041) (< 0.001)*
3SROM	0.34 (0.0026) 0.34–0.35	0.22 (0.0026) 0.22–0.23	0.12 (0.0026) (< 0.001)*	0.24–0.29	0.22 (0.0026) 0.21–0.22	0.13 (0.0026) (< 0.001)*
3SRRH	0.32 (0.0059) 0.31–0.32	0.2 (0.0059) 0.19–0.23	0.11 (0.0075) (< 0.001)*	0.24–0.33	0.2 (0.0059) 0.19–0.2	0.12 (0.0075) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.17. C18:0 – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3SRBLY1	2.06 (0.023) 2.02–2.1	2.68 (0.023) 2.65–2.77	-0.62 (0.033) (< 0.001)*	1.85–2.18	2.62 (0.023) 2.58–2.68	-0.56 (0.033) (< 0.001)*
3SRJV	1.99 (0.03) 1.95–2	2.62 (0.03) 2.53–2.71	-0.64 (0.041) (< 0.001)*	1.96–2.17	2.38 (0.03) 2.32–2.47	-0.4 (0.041) (< 0.001)*
3SRKT	1.96 (0.015) 1.91–2.01	2.49 (0.015) 2.46–2.51	-0.53 (0.019) (< 0.001)*	1.78–2.08	2.51 (0.015) 2.49–2.54	-0.55 (0.019) (< 0.001)*
3SROM	1.96 (0.02) 1.93–1.98	2.55 (0.02) 2.5–2.59	-0.59 (0.028) (< 0.001)*	1.84–2.22	2.51 (0.02) 2.45–2.56	-0.55 (0.028) (< 0.001)*
3SRRH	1.86 (0.026) 1.84–1.88	2.37 (0.026) 2.32–2.4	-0.51 (0.036) (< 0.001)*	1.84–2.1	2.43 (0.026) 2.35–2.54	-0.57 (0.036) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.18. C18:1n-9 – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3SRBLY1	55.51 (0.26) 55.32–55.67	25.59 (0.26) 25.46–25.78	29.92 (0.37) (< 0.001)*	58.49–74.38	25.39 (0.26) 24.54–26.62	30.11 (0.37) (< 0.001)*
3SRJV	55.08 (0.33) 54.3–55.47	31.04 (0.33) 30.33–31.52	24.04 (0.46) (< 0.001)*	56.53–75.19	32.26 (0.33) 31.61–33.47	22.82 (0.46) (< 0.001)*
3SRKT	53.98 (0.25) 53.44–54.46	24.82 (0.25) 24.49–25.2	29.16 (0.27) (< 0.001)*	55.61–74.16	24.71 (0.25) 24.15–25.55	29.27 (0.27) (< 0.001)*
3SROM	53.64 (0.21) 53.01–54.19	22.53 (0.21) 22.17–22.78	31.11 (0.3) (< 0.001)*	57.02–76.02	22.62 (0.21) 22.29–23.3	31.02 (0.3) (< 0.001)*
3SRRH	54.86 (0.46) 53.97–55.53	23.52 (0.46) 22.35–24.41	31.34 (0.43) (< 0.001)*	55.59–73.48	24.72 (0.46) 23.46–26.18	30.14 (0.43) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.19. C18:2n-6 – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3SRBLY1	19.03 (0.27) 18.77–19.27	27.41 (0.27) 26.99–27.87	-8.38 (0.2) (< 0.001)*	6.8–20.61	27.31 (0.27) 26.45–28.12	-8.28 (0.2) (< 0.001)*
3SRJV	19.27 (0.21) 18.95–19.75	29.52 (0.21) 29.08–30.29	-10.24 (0.3) (< 0.001)*	6.64–23.41	28.12 (0.21) 27.59–28.38	-8.85 (0.3) (< 0.001)*
3SRKT	20.11 (0.24) 19.76–20.54	27.35 (0.24) 27.04–27.73	-7.24 (0.18) (< 0.001)*	6.78–21.83	27.21 (0.24) 26.58–28.1	-7.1 (0.18) (< 0.001)*
3SROM	21 (0.18) 20.58–21.32	29.41 (0.18) 28.94–29.94	-8.42 (0.25) (< 0.001)*	5.68–20.81	29.48 (0.18) 29.11–29.92	-8.48 (0.25) (< 0.001)*
3SRRH	20.97 (0.34) 20.59–21.51	30.28 (0.34) 29.38–31.77	-9.31 (0.48) (< 0.001)*	7.15–23.45	29.86 (0.34) 29.48–30.27	-8.89 (0.48) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.20. C18:3n-3 – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3SRBLY1	7.62 (0.082) 7.38–7.85	4.97 (0.082) 4.9–5.03	2.64 (0.12) (< 0.001)*	2.15–7.28	4.87 (0.082) 4.73–5.04	2.75 (0.12) (< 0.001)*
3SRJV	8.38 (0.087) 8.05–8.6	5.65 (0.087) 5.49–5.85	2.73 (0.11) (< 0.001)*	1.97–7.47	6.16 (0.087) 6.05–6.24	2.22 (0.11) (< 0.001)*
3SRKT	8 (0.063) 7.81–8.24	5.11 (0.063) 5.05–5.18	2.89 (0.089) (< 0.001)*	2.14–8.39	5.06 (0.063) 4.99–5.15	2.94 (0.089) (< 0.001)*
3SROM	6.91 (0.041) 6.82–7.05	4.28 (0.041) 4.19–4.34	2.63 (0.05) (< 0.001)*	1.69–6.63	4.32 (0.041) 4.27–4.4	2.6 (0.05) (< 0.001)*
3SRRH	6.55 (0.066) 6.36–6.84	4.12 (0.066) 4.06–4.16	2.43 (0.092) (< 0.001)*	1.94–6.28	4.14 (0.066) 4.11–4.16	2.41 (0.092) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.21. C20:1n-9 – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3SRBLY1	1 (0.0081) 0.96–1.02	0.65 (0.0081) 0.64–0.65	0.35 (0.011) (< 0.001)*	0.99–1.25	0.64 (0.0081) 0.63–0.64	0.36 (0.011) (< 0.001)*
3SRJV	0.96 (0.0072) 0.94–0.97	0.72 (0.0072) 0.71–0.73	0.24 (0.01) (< 0.001)*	0.95–1.24	0.73 (0.0072) 0.71–0.75	0.22 (0.01) (< 0.001)*
3SRKT	1 (0.0091) 0.97–1.03	0.66 (0.0091) 0.63–0.67	0.34 (0.0098) (< 0.001)*	0.99–1.31	0.65 (0.0091) 0.64–0.66	0.34 (0.0098) (< 0.001)*
3SROM	0.98 (0.0062) 0.98–0.99	0.59 (0.0062) 0.57–0.6	0.39 (0.0087) (< 0.001)*	0.96–1.34	0.59 (0.0062) 0.57–0.6	0.39 (0.0087) (< 0.001)*
3SRRH	0.93 (0.01) 0.9–0.96	0.59 (0.01) 0.56–0.61	0.34 (0.014) (< 0.001)*	0.93–1.29	0.61 (0.01) 0.61–0.62	0.32 (0.014) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.22. C22:0 – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3SRBLY1	0.35 (0.0055) 0.33–0.37	0.26 (0.0055) 0.25–0.26	0.092 (0.0077) (< 0.001)*	0.26–0.39	0.25 (0.0055) 0.24–0.26	0.1 (0.0077) (< 0.001)*
3SRJV	0.3 (0.0035) 0.29–0.31	0.24 (0.0035) 0.24–0.25	0.052 (0.0046) (< 0.001)*	0.24–0.35	0.25 (0.0035) 0.24–0.25	0.05 (0.0046) (< 0.001)*
3SRKT	0.35 (0.0058) 0.33–0.37	0.26 (0.0058) 0.25–0.27	0.085 (0.0078) (< 0.001)*	0.28–0.39	0.26 (0.0058) 0.26–0.27	0.085 (0.0078) (< 0.001)*
3SROM	0.37 (0.0032) 0.37–0.37	0.26 (0.0032) 0.25–0.27	0.1 (0.0046) (< 0.001)*	0.28–0.42	0.26 (0.0032) 0.25–0.26	0.11 (0.0046) (< 0.001)*
3SRRH	0.35 (0.0033) 0.34–0.36	0.26 (0.0033) 0.25–0.26	0.095 (0.0047) (< 0.001)*	0.28–0.44	0.26 (0.0033) 0.25–0.26	0.095 (0.0047) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.23. C24:1n-9 – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3SRBLY1	0.13 (0.0033) 0.12–0.14	0.087 (0.0033) 0.084–0.09	0.043 (0.0046) (< 0.001)*	0.1–0.14	0.084 (0.0033) 0.075–0.092	0.046 (0.0046) (< 0.001)*
3SRJV	0.1 (0.0026) 0.1–0.11	0.078 (0.0026) 0.072–0.083	0.027 (0.003) (< 0.001)*	0.084–0.12	0.08 (0.0026) 0.076–0.087	0.025 (0.003) (< 0.001)*
3SRKT	0.14 (0.0033) 0.13–0.15	0.091 (0.0033) 0.082–0.1	0.048 (0.0044) (< 0.001)*	0.12–0.14	0.091 (0.0033) 0.088–0.093	0.049 (0.0044) (< 0.001)*
3SROM	0.15 (0.0031) 0.14–0.15	0.09 (0.0031) 0.08–0.099	0.058 (0.0041) (< 0.001)*	0.096–0.14	0.089 (0.0031) 0.081–0.093	0.058 (0.0041) (< 0.001)*
3SRRH	0.13 (0.0034) 0.12–0.14	0.084 (0.0034) 0.075–0.088	0.049 (0.0049) (< 0.001)*	0.11–0.16	0.088 (0.0034) 0.083–0.092	0.044 (0.0049) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.24. Total Trans Fatty Acids – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3SRBLY1	0.14 (0.018) 0.1–0.19	0.32 (0.018) 0.29–0.34	-0.17 (0.025) (0.001)*	0.081–0.11	0.37 (0.018) 0.31–0.4	-0.22 (0.025) (0.001)*
3SRJV	0.11 (0.021) 0.096–0.12	0.24 (0.021) 0.21–0.3	-0.13 (0.027) (0.003)*	0.08–0.11	0.26 (0.021) 0.2–0.33	-0.15 (0.027) (0.001)*
3SRKT	0.097 (0.013) 0.09–0.1	0.35 (0.013) 0.29–0.39	-0.25 (0.019) (< 0.001)*	0.077–0.13	0.34 (0.013) 0.34–0.34	-0.24 (0.019) (< 0.001)*
3SROM	0.093 (0.0079) 0.085–0.097	0.35 (0.0079) 0.34–0.36	-0.25 (0.009) (< 0.001)*	0.083–0.12	0.37 (0.0079) 0.36–0.41	-0.28 (0.009) (< 0.001)*
3SRRH	0.13 (0.013) 0.096–0.16	0.36 (0.013) 0.35–0.39	-0.24 (0.019) (< 0.001)*	0.096–0.11	0.36 (0.013) 0.33–0.41	-0.24 (0.019) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.25. Glucobrassicin – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3SRBLY1	0.26 (0.025) 0.24–0.27	0.5 (0.025) 0.44–0.58	-0.25 (0.035) (< 0.001)*	0.14–0.5	0.5 (0.025) 0.44–0.58	-0.24 (0.035) (< 0.001)*
3SRJV	0.11 (0.011) 0.11–0.12	0.22 (0.011) 0.19–0.25	-0.11 (0.015) (< 0.001)*	< LOQ–0.24	0.18 (0.011) 0.15–0.21	-0.072 (0.015) (0.001)*
3SRKT	0.25 (0.02) 0.22–0.29	0.43 (0.02) 0.36–0.47	-0.18 (0.028) (< 0.001)*	0.15–0.45	0.54 (0.02) 0.51–0.58	-0.3 (0.028) (< 0.001)*
3SROM	0.37 (0.036) 0.33–0.45	0.84 (0.036) 0.77–0.89	-0.47 (0.051) (< 0.001)*	0.14–0.69	0.76 (0.036) 0.65–0.87	-0.4 (0.051) (< 0.001)*
3SRRH	0.34 (0.031) 0.32–0.39	0.96 (0.031) 0.89–1	-0.62 (0.047) (< 0.001)*	0.18–0.9	0.83 (0.031) 0.76–0.93	-0.49 (0.044) (< 0.001)*

All data are in units of µmol/g dry weight.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.26. Delta-5 Avenasterol – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3SRBLY1	0.007 (0.0003) 0.006–0.008	0.0055 (0.0003) 0.005–0.006	0.0015 (0.0003) (0.004)*	0.0045–0.01	0.006 (0.0003) 0.006–0.006	0.001 (0.0003) (0.022)*
3SRJV	0.02 (0.0002) 0.02–0.02	0.009 (0.0002) 0.008–0.01	0.011 (0.0003) (< 0.001)*	0.0092–0.03	0.01 (0.0002) 0.01–0.01	0.01 (0.0003) (< 0.001)*
3SRKT	0.0085 (0.0005) 0.008–0.01	0.006 (0.0005) 0.005–0.007	0.0025 (0.0006) (0.007)*	0.0057–0.018	0.0065 (0.0005) 0.006–0.008	0.002 (0.0006) (0.018)*
3SROM	0.0085 (0.0002) 0.008–0.009	0.0062 (0.0002) 0.006–0.007	0.0022 (0.0003) (< 0.001)*	0.0042–0.01	0.006 (0.0002) 0.006–0.006	0.0025 (0.0003) (< 0.001)*
3SRRH	0.008 (0.0002) 0.008–0.008	0.0055 (0.0002) 0.005–0.006	0.0025 (0.0002) (< 0.001)*	0.0052–0.01	0.0055 (0.0002) 0.005–0.006	0.0025 (0.0002) (< 0.001)*

All data are in units of % dry weight.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.27. Delta-7 Stigmastenol – By-Site Summary Statistics – Winter 2014/15

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3SRBLY1	0.0022 (0.0005) 0.001–0.004	0.0068 (0.0005) 0.006–0.008	-0.0045 (0.0006) (< 0.001)*	0.0018–0.0072	0.006 (0.0005) 0.005–0.007	-0.0037 (0.0006) (0.001)*
3SRJV	0.0025 (0.0004) 0.002–0.003	0.0062 (0.0004) 0.005–0.008	-0.0038 (0.0006) (< 0.001)*	0.0027–0.0088	0.0048 (0.0004) 0.004–0.005	-0.0023 (0.0006) (0.004)*
3SRKT	0.0018 (0.0002) 0.001–0.002	0.0042 (0.0002) 0.004–0.005	-0.0025 (0.0003) (< 0.001)*	0.002–0.0057	0.005 (0.0002) 0.005–0.005	-0.0032 (0.0003) (< 0.001)*
3SROM	0.0035 (0.0002) 0.003–0.004	0.0098 (0.0002) 0.009–0.01	-0.0062 (0.0003) (< 0.001)*	0.0032–0.01	0.01 (0.0002) 0.01–0.01	-0.0065 (0.0003) (< 0.001)*
3SRRH	0.0025 (0.0005) 0.002–0.003	0.007 (0.0005) 0.006–0.008	-0.0045 (0.0007) (< 0.001)*	0.0027–0.01	0.0065 (0.0005) 0.005–0.008	-0.004 (0.0007) (< 0.001)*

All data are in units of % dry weight.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.28. C16:1n-7 – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3NRBRK	0.38 (0.0043) 0.37–0.39	0.23 (0.0043) 0.22–0.24	0.15 (0.0061) (< 0.001)*	0.25–0.28	0.23 (0.0043) 0.22–0.24	0.15 (0.0061) (< 0.001)*
3NRCB	0.29 (0.0024) 0.29–0.3	0.19 (0.0024) 0.19–0.19	0.1 (0.0033) (< 0.001)*	0.2–0.24	0.18 (0.0024) 0.18–0.19	0.11 (0.0033) (< 0.001)*
3NREP	0.25 (0.0028) 0.24–0.26	0.17 (0.0028) 0.17–0.17	0.08 (0.0039) (< 0.001)*	0.18–0.21	0.17 (0.0028) 0.16–0.17	0.082 (0.0039) (< 0.001)*
3NRGE	0.28 (0.0046) 0.27–0.3	0.18 (0.0046) 0.18–0.19	0.098 (0.0061) (< 0.001)*	0.21–0.23	0.19 (0.0046) 0.19–0.2	0.087 (0.0061) (< 0.001)*
3NRLS	0.28 (0.002) 0.28–0.29	0.19 (0.002) 0.19–0.19	0.092 (0.0026) (< 0.001)*	0.19–0.23	0.19 (0.002) 0.18–0.19	0.095 (0.0026) (< 0.001)*
3NRMA-2	0.28 (0.0074) 0.27–0.3	0.19 (0.0074) 0.17–0.21	0.087 (0.01) (< 0.001)*	0.2–0.23	0.19 (0.0074) 0.17–0.2	0.09 (0.01) (< 0.001)*
3NRNW-1	0.29 (0.0031) 0.28–0.3	0.19 (0.0031) 0.19–0.2	0.098 (0.0044) (< 0.001)*	0.2–0.23	0.19 (0.0031) 0.19–0.2	0.098 (0.0044) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.29. C18:0 – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3NRBRK	2.17 (0.028) 2.15–2.18	2.69 (0.028) 2.61–2.79	-0.52 (0.039) (< 0.001)*	1.98–2.23	2.63 (0.028) 2.58–2.68	-0.46 (0.039) (< 0.001)*
3NRCB	2.08 (0.014) 2.06–2.11	2.69 (0.014) 2.64–2.72	-0.61 (0.017) (< 0.001)*	1.82–2.04	2.66 (0.014) 2.64–2.68	-0.58 (0.017) (< 0.001)*
3NREP	2.27 (0.014) 2.24–2.31	2.8 (0.014) 2.78–2.81	-0.53 (0.02) (< 0.001)*	1.87–2.14	2.77 (0.014) 2.74–2.81	-0.51 (0.02) (< 0.001)*
3NRGE	2.33 (0.034) 2.24–2.42	3.06 (0.034) 3.02–3.14	-0.72 (0.047) (< 0.001)*	1.9–2.1	3 (0.034) 2.94–3.08	-0.67 (0.047) (< 0.001)*
3NRLS	2.24 (0.058) 2.1–2.37	2.96 (0.058) 2.86–3.15	-0.72 (0.063) (< 0.001)*	1.92–2.18	2.92 (0.058) 2.83–3.01	-0.68 (0.063) (< 0.001)*
3NRMA-2	2.17 (0.071) 2.04–2.38	2.74 (0.071) 2.7–2.78	-0.57 (0.087) (0.001)*	1.88–2.19	2.7 (0.071) 2.54–2.98	-0.53 (0.087) (0.001)*
3NRNW-1	1.95 (0.011) 1.94–1.96	2.52 (0.011) 2.49–2.55	-0.57 (0.016) (< 0.001)*	1.73–1.94	2.52 (0.011) 2.49–2.54	-0.57 (0.016) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.30. C18:1n-7 – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3NRBRK	4.13 (0.037) 4.04–4.22	3.98 (0.037) 3.89–4.08	0.15 (0.033) (0.003)*	3.14–3.47	3.98 (0.037) 3.95–4.01	0.14 (0.033) (0.004)*
3NRCB	3.35 (0.012) 3.31–3.37	3.36 (0.012) 3.34–3.38	-0.015 (0.0097) (0.174)	2.75–3.19	3.32 (0.012) 3.29–3.35	0.03 (0.0097) (0.022)*
3NREP	3.3 (0.018) 3.26–3.35	3.21 (0.018) 3.17–3.25	0.087 (0.026) (0.008)*	2.73–3.2	3.21 (0.018) 3.17–3.25	0.087 (0.026) (0.008)*
3NRGE	3.39 (0.036) 3.31–3.54	3.31 (0.036) 3.26–3.38	0.077 (0.05) (0.158)	2.82–3.24	3.36 (0.036) 3.33–3.41	0.028 (0.05) (0.598)
3NRLS	3.28 (0.053) 3.15–3.36	3.34 (0.053) 3.22–3.48	-0.058 (0.026) (0.07)	2.57–3.15	3.29 (0.053) 3.17–3.44	-0.015 (0.026) (0.586)
3NRMA-2	3.64 (0.084) 3.49–3.78	3.59 (0.084) 3.37–3.82	0.048 (0.12) (0.697)	2.94–3.46	3.57 (0.084) 3.34–3.8	0.07 (0.12) (0.568)
3NRNW-1	3.44 (0.03) 3.39–3.5	3.41 (0.03) 3.38–3.48	0.025 (0.042) (0.568)	2.85–3.27	3.38 (0.03) 3.31–3.49	0.06 (0.042) (0.188)

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.31. C18:1n-9 – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3NRBRK	49.59 (0.39) 48.63–50.24	23.31 (0.39) 22.75–24	26.28 (0.46) (< 0.001)*	55.21–74.13	23 (0.39) 22.17–24.45	26.59 (0.46) (< 0.001)*
3NRCB	54.71 (0.18) 54.3–55.09	25.32 (0.18) 24.81–25.59	29.4 (0.18) (< 0.001)*	57.76–75.13	25.47 (0.18) 25.03–26.01	29.24 (0.18) (< 0.001)*
3NREP	56.69 (0.33) 56.3–56.9	28.18 (0.33) 28.01–28.33	28.51 (0.47) (< 0.001)*	57.82–75.48	27.98 (0.33) 26.75–29.05	28.71 (0.47) (< 0.001)*
3NRGE	56.05 (0.29) 55.37–56.4	27.27 (0.29) 26.66–27.7	28.77 (0.4) (< 0.001)*	57.87–75.94	26.8 (0.29) 25.9–27.68	29.25 (0.4) (< 0.001)*
3NRLS	56.66 (0.29) 55.96–57.31	26.18 (0.29) 25.63–26.95	30.48 (0.4) (< 0.001)*	58.34–76.18	25.93 (0.29) 25.54–26.64	30.73 (0.4) (< 0.001)*
3NRMA-2	55.11 (0.22) 54.51–55.45	27.25 (0.22) 26.55–27.72	27.86 (0.28) (< 0.001)*	57.45–74.97	27.34 (0.22) 27.02–27.91	27.77 (0.28) (< 0.001)*
3NRNW-1	55.02 (0.17) 54.63–55.31	27.32 (0.17) 27.02–27.43	27.7 (0.24) (< 0.001)*	58.08–76.44	27.38 (0.17) 26.69–27.73	27.64 (0.24) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.32. C18:2n-6 – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3NRBRK	21.89 (0.19) 21.65–22.45	27.06 (0.19) 27.03–27.12	-5.17 (0.23) (< 0.001)*	6.89–23.23	27.5 (0.19) 26.91–28.05	-5.62 (0.23) (< 0.001)*
3NRCB	19.89 (0.089) 19.7–20.12	27.52 (0.089) 27.34–27.67	-7.64 (0.13) (< 0.001)*	6.61–22.38	27.74 (0.089) 27.53–27.95	-7.85 (0.13) (< 0.001)*
3NREP	18.39 (0.15) 18.25–18.65	28.26 (0.15) 27.98–28.39	-9.87 (0.21) (< 0.001)*	6.49–22.91	28.2 (0.15) 27.76–28.67	-9.81 (0.21) (< 0.001)*
3NRGE	18.73 (0.24) 18.44–19.17	29.61 (0.24) 29.05–30.69	-10.88 (0.35) (< 0.001)*	5.94–21.41	29.86 (0.24) 29.62–30.03	-11.13 (0.35) (< 0.001)*
3NRLS	18.48 (0.32) 18.15–18.76	28.84 (0.32) 28.06–29.68	-10.36 (0.45) (< 0.001)*	5.81–21.5	29.14 (0.32) 28.53–29.84	-10.66 (0.45) (< 0.001)*
3NRMA-2	17.98 (0.3) 17.52–18.4	26.09 (0.3) 25.31–26.85	-8.12 (0.3) (< 0.001)*	6.6–21.22	25.97 (0.3) 25.27–26.48	-7.99 (0.3) (< 0.001)*
3NRNW-1	19.68 (0.17) 19.42–20	27.84 (0.17) 27.6–28.36	-8.16 (0.23) (< 0.001)*	5.81–21.93	28.05 (0.17) 27.62–28.52	-8.38 (0.23) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.33. C18:3n-3 – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3NRBRK	8.99 (0.055) 8.83–9.06	5.92 (0.055) 5.71–6.07	3.07 (0.063) (< 0.001)*	2.21–8.48	6.06 (0.055) 6.02–6.12	2.92 (0.063) (< 0.001)*
3NRCB	7.72 (0.047) 7.57–7.81	4.88 (0.047) 4.77–4.99	2.84 (0.037) (< 0.001)*	2.09–7.49	4.9 (0.047) 4.8–4.97	2.82 (0.037) (< 0.001)*
3NREP	7.83 (0.034) 7.8–7.85	5.12 (0.034) 5.06–5.22	2.71 (0.048) (< 0.001)*	2.27–7.93	5.08 (0.034) 4.99–5.17	2.75 (0.048) (< 0.001)*
3NRGE	7.49 (0.12) 7.22–7.93	5.28 (0.12) 5.22–5.39	2.21 (0.16) (< 0.001)*	1.97–7.56	5.21 (0.12) 4.92–5.45	2.28 (0.16) (< 0.001)*
3NRLS	7.17 (0.1) 6.91–7.4	5.09 (0.1) 4.86–5.32	2.08 (0.13) (< 0.001)*	2.06–7.42	5.01 (0.1) 4.84–5.18	2.16 (0.13) (< 0.001)*
3NRMA-2	9.08 (0.19) 8.72–9.57	6.08 (0.19) 5.69–6.44	3 (0.23) (< 0.001)*	2.4–8.52	5.94 (0.19) 5.47–6.51	3.13 (0.23) (< 0.001)*
3NRNW-1	7.83 (0.052) 7.76–8.04	5.31 (0.052) 5.21–5.36	2.52 (0.061) (< 0.001)*	1.99–7.67	5.34 (0.052) 5.23–5.45	2.5 (0.061) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.34. C20:1n-9 – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control/LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control/LBFLFK (non-sprayed)
	Mean min–max	Mean min–max	Ratio (p-value)[3]	min–max	Mean min–max	Ratio (p-value)[3]
3NRBRK	1.07[4] 1.05–1.08	0.68[4] 0.68–0.68	1.58[4] (< 0.001)*	1.05–1.4	0.69[4] 0.69–0.7	1.56[4] (< 0.001)*
3NRCB	1.03[4] 1.03–1.04	0.68[4] 0.68–0.68	1.53[4] (< 0.001)*	1.04–1.33	0.68[4] 0.68–0.69	1.51[4] (< 0.001)*
3NREP	1.01[4] 0.99–1.03	0.71[4] 0.71–0.71	1.42[4] (< 0.001)*	1–1.45	0.72[4] 0.71–0.73	1.41[4] (< 0.001)*
3NRGE	1[4] 0.96–1.03	0.7[4] 0.69–0.71	1.44[4] (< 0.001)*	1–1.27	0.69[4] 0.68–0.69	1.45[4] (< 0.001)*
3NRLS	1.01[4] 0.98–1.04	0.69[4] 0.68–0.7	1.47[4] (< 0.001)*	1.01–1.32	0.69[4] 0.68–0.7	1.48[4] (< 0.001)*
3NRMA-2	1.07[4] 0.97–1.12	0.73[4] 0.72–0.74	1.46[4] (< 0.001)*	1.05–1.37	0.73[4] 0.72–0.74	1.46[4] (< 0.001)*
3NRNW-1	1.01[4] 0.97–1.04	0.71[4] 0.7–0.72	1.42[4] (< 0.001)*	1.04–1.3	0.71[4] 0.71–0.71	1.42[4] (< 0.001)*

All data are in units of % of total fatty acids.

[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.

[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.

[3] Difference test p-value: * indicates p≤ 0.05.

[4] Data were log-transformed prior to statistical analysis, and means presented here are back-transformed. The comparison of the control to LBFLFK is presented as the ratio of the two back-transformed means (same as difference of log-transformed means). Back-transformed SE is not provided.

Table I.35. C22:0 – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3NRBRK	0.41 (0.0055) 0.4–0.44	0.3 (0.0055) 0.3–0.31	0.11 (0.0074) (< 0.001)*	0.31–0.45	0.3 (0.0055) 0.3–0.31	0.11 (0.0074) (< 0.001)*
3NRCB	0.35 (0.0032) 0.35–0.35	0.26 (0.0032) 0.25–0.27	0.087 (0.0042) (< 0.001)*	0.27–0.37	0.26 (0.0032) 0.26–0.27	0.085 (0.0042) (< 0.001)*
3NREP	0.29 (0.0017) 0.29–0.3	0.22 (0.0017) 0.22–0.22	0.075 (0.0024) (< 0.001)*	0.23–0.31	0.22 (0.0017) 0.22–0.22	0.075 (0.0024) (< 0.001)*
3NRGE	0.34 (0.0054) 0.32–0.36	0.26 (0.0054) 0.25–0.26	0.085 (0.0075) (< 0.001)*	0.27–0.36	0.26 (0.0054) 0.26–0.27	0.077 (0.0075) (< 0.001)*
3NRLS	0.34 (0.0046) 0.34–0.35	0.25 (0.0046) 0.24–0.27	0.09 (0.0057) (< 0.001)*	0.26–0.37	0.25 (0.0046) 0.25–0.25	0.092 (0.0057) (< 0.001)*
3NRMA-2	0.33 (0.0026) 0.33–0.34	0.25 (0.0026) 0.24–0.25	0.085 (0.0026) (< 0.001)*	0.27–0.35	0.24 (0.0026) 0.24–0.25	0.087 (0.0026) (< 0.001)*
3NRNW-1	0.32 (0.0032) 0.31–0.33	0.26 (0.0032) 0.25–0.26	0.065 (0.0035) (< 0.001)*	0.26–0.34	0.26 (0.0032) 0.25–0.26	0.062 (0.0035) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.36. C24:0 – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3NRBRK	0.24 (0.0022) 0.24–0.25	0.15 (0.0022) 0.15-0.15	0.095 (0.0026) (< 0.001)*	0.2–0.31	0.15 (0.0022) 0.15–0.16	0.092 (0.0026) (< 0.001)*
3NRCB	0.19 (0.0032) 0.18–0.2	0.13 (0.0032) 0.13–0.13	0.058 (0.0035) (< 0.001)*	0.17–0.27	0.12 (0.0032) 0.12–0.13	0.062 (0.0035) (< 0.001)*
3NREP	0.17 (0.0014) 0.16–0.17	0.11 (0.0014) 0.11–0.11	0.058 (0.002) (< 0.001)*	0.15–0.24	0.11 (0.0014) 0.11–0.11	0.058 (0.002) (< 0.001)*
3NRGE	0.2 (0.0063) 0.18–0.23	0.13 (0.0063) 0.13–0.14	0.07 (0.009) (< 0.001)*	0.18–0.26	0.14 (0.0063) 0.13–0.14	0.068 (0.009) (< 0.001)*
3NRLS	0.2 (0.0029) 0.2–0.21	0.13 (0.0029) 0.12–0.14	0.075 (0.0041) (< 0.001)*	0.17–0.27	0.13 (0.0029) 0.13–0.13	0.075 (0.0041) (< 0.001)*
3NRMA-2	0.18 (0.0025) 0.18–0.19	0.12 (0.0025) 0.11–0.12	0.065 (0.0035) (< 0.001)*	0.16–0.25	0.11 (0.0025) 0.11–0.12	0.07 (0.0035) (< 0.001)*
3NRNW-1	0.16 (0.0029) 0.15–0.17	0.11 (0.0029) 0.11–0.11	0.05 (0.0041) (< 0.001)*	0.15–0.24	0.1 (0.0029) 0.1–0.11	0.055 (0.0041) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.37. C24:1n-9 – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3NRBRK	0.19 (0.0028) 0.18–0.2	0.11 (0.0028) 0.11–0.11	0.08 (0.0039) (< 0.001)*	0.14–0.18	0.11 (0.0028) 0.11–0.12	0.077 (0.0039) (< 0.001)*
3NRCB	0.13 (0.0022) 0.13–0.14	0.083 (0.0022) 0.079–0.087	0.05 (0.0032) (< 0.001)*	0.11–0.14	0.084 (0.0022) 0.081–0.091	0.048 (0.0032) (< 0.001)*
3NREP	0.094 (0.0014) 0.09–0.096	0.066 (0.0014) 0.064–0.069	0.028 (0.002) (< 0.001)*	0.084–0.11	0.064 (0.0014) 0.06–0.068	0.03 (0.002) (< 0.001)*
3NRGE	0.12 (0.0045) 0.098–0.13	0.078 (0.0045) 0.075–0.08	0.039 (0.006) (0.001)*	0.1–0.12	0.082 (0.0045) 0.072–0.09	0.035 (0.006) (0.001)*
3NRLS	0.12 (0.0019) 0.11–0.12	0.074 (0.0019) 0.07–0.077	0.041 (0.0027) (< 0.001)*	0.094–0.12	0.072 (0.0019) 0.071–0.074	0.043 (0.0027) (< 0.001)*
3NRMA-2	0.13 (0.0041) 0.11–0.14	0.082 (0.0041) 0.078–0.084	0.045 (0.0036) (< 0.001)*	0.11–0.14	0.083 (0.0041) 0.075–0.088	0.044 (0.0036) (< 0.001)*
3NRNW-1	0.12 (0.0031) 0.11–0.13	0.08 (0.0031) 0.08–0.081	0.04 (0.0043) (< 0.001)*	0.1–0.13	0.083 (0.0031) 0.078–0.092	0.037 (0.0043) (< 0.001)*

All data are in units of % of total fatty acids.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.38. Coumaric Acid – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3NRBRK	16.89 (0.93) 12.95–19.19	14.17 (0.93) 13.32–14.69	2.72 (1.03) (0.038)*	11.26–38.6	14.2 (0.93) 12.86–16.46	2.69 (1.03) (0.040)*
3NRCB	14.17 (1.52) 13.24–16.08	11.8 (1.52) 10.81–12.63	2.37 (2.16) (0.300)	10.22–41.86	< LOQ < LOQ–15.31	NA
3NREP	18.5 (1.45) 15.75–20.42	< LOQ	NA	17.36–56.5	10.88 (1.45) < LOQ–15.8	7.62 (1.87) (0.007)*
3NRGE	21.03 (1.98) 16.72–24.54	13.19 (1.98) < LOQ–17.87	7.84 (2.79) (0.020)*	13.24–45.92	15.41 (1.98) 13.61–18.06	5.62 (2.79) (0.075)
3NRLS	18.78 (1.86) 15.36–22.65	12.84 (1.86) 11.89–14.12	5.94 (1.95) (0.023)*	12.12–49.87	< LOQ–15.39	NA
3NRMA-2	13.81 (2.29) 11.84–15.07	< LOQ	NA	14.79–34.01	14.18 (2.29) < LOQ–23.84	-0.37 (3.13) (0.9098)
3NRNW-1	15.04 (1.09) 13.11–15.76	< LOQ	NA	11.07–33.92	< LOQ < LOQ–11.27	NA

NA indicates not suitable for statistical analysis.
All data are in units of µmol/g dry weight.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.39. Brassicasterol – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3NRBRK	0.12 (0.0005) 0.12–0.12	0.074 (0.0005) 0.073–0.076	0.046 (0.0007) (< 0.001)*	0.055–0.098	0.074 (0.0005) 0.072–0.075	0.046 (0.0007) (< 0.001)*
3NRCB	0.13 (0.0003) 0.13–0.13	0.076 (0.0003) 0.076–0.077	0.053 (0.0003) (< 0.001)*	0.06–0.11	0.076 (0.0003) 0.075–0.077	0.054 (0.0003) (< 0.001)*
3NREP	0.12 (0.0016) 0.11–0.12	0.071 (0.0016) 0.069–0.073	0.046 (0.0023) (< 0.001)*	0.063–0.11	0.071 (0.0016) 0.069–0.073	0.046 (0.0023) (< 0.001)*
3NRGE	0.12 (0.0016) 0.12–0.12	0.069 (0.0016) 0.064–0.073	0.051 (0.0021) (< 0.001)*	0.06–0.11	0.071 (0.0016) 0.068–0.074	0.049 (0.0021) (< 0.001)*
3NRLS	0.1 (0.0022) 0.097–0.11	0.065 (0.0022) 0.061–0.07	0.037 (0.0028) (< 0.001)*	0.052–0.09	0.064 (0.0022) 0.061–0.068	0.037 (0.0028) (< 0.001)*
3NRMA-2	0.12 (0.0017) 0.11–0.12	0.07 (0.0017) 0.066–0.072	0.048 (0.0024) (< 0.001)*	0.061–0.1	0.072 (0.0017) 0.07–0.074	0.045 (0.0024) (< 0.001)*
3NRNW-1	0.12 (0.002) 0.12–0.13	0.076 (0.002) 0.073–0.08	0.049 (0.0024) (< 0.001)*	0.061–0.11	0.074 (0.002) 0.072–0.078	0.051 (0.0024) (< 0.001)*

All data are in units of % dry weight.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

Table I.40. Total Phytosterols – By-Site Summary Statistics – Spring 2015

Site	Control (Kumily)	LBFLFK (sprayed)[1]	Control minus LBFLFK (sprayed)	Reference Variety Range	LBFLFK (non-sprayed)[2]	Control minus LBFLFK (non-sprayed)
	Mean (SE) min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]	min–max	Mean (SE) min–max	Difference (SE) (p-value)[3]
3NRBRK	1.02 (0.011) 0.99–1.04	0.91 (0.011) 0.89–0.95	0.11 (0.014) (< 0.001)*	0.86–1.08	0.89 (0.011) 0.88–0.92	0.13 (0.014) (< 0.001)*
3NRCB	0.92 (0.0047) 0.92–0.93	0.86 (0.0047) 0.85–0.87	0.06 (0.0067) (< 0.001)*	0.86–1.01	0.85 (0.0047) 0.84–0.86	0.072 (0.0067) (< 0.001)*
3NREP	0.82 (0.0057) 0.81–0.83	0.75 (0.0057) 0.73–0.77	0.071 (0.0071) (< 0.001)*	0.81–0.94	0.75 (0.0057) 0.74–0.75	0.071 (0.0071) (< 0.001)*
3NRGE	0.89 (0.016) 0.88–0.9	0.75 (0.016) 0.7–0.8	0.14 (0.022) (0.001)*	0.85–0.99	0.78 (0.016) 0.75–0.81	0.1 (0.022) (0.004)*
3NRLS	0.78 (0.02) 0.75–0.81	0.71 (0.02) 0.68–0.78	0.068 (0.023) (0.024)*	0.74–0.9	0.71 (0.02) 0.66–0.75	0.069 (0.023) (0.023)*
3NRMA-2	0.9 (0.013) 0.87–0.92	0.78 (0.013) 0.72–0.8	0.12 (0.015) (< 0.001)*	0.85–0.98	0.81 (0.013) 0.79–0.82	0.096 (0.015) (0.001)*
3NRNW-1	0.89 (0.012) 0.86–0.92	0.84 (0.012) 0.82–0.88	0.056 (0.015) (0.009)*	0.85–1.01	0.82 (0.012) 0.81–0.84	0.072 (0.015) (0.003)*

All data are in units of % dry weight.
[1] LBFLFK (sprayed) received standard herbicide treatments for weed control plus Beyond® herbicide spray (35 g a.i./ha) at the 3–4 leaf stage.
[2] LBFLFK (non-sprayed) received standard herbicide treatments for weed control but no Beyond® herbicide spray.
[3] Difference test p-value: * indicates p≤ 0.05.

I.5. Literature ranges

Table I.41. Peer-Reviewed Literature and ILSI Crop Composition Database Ranges for Canola Grain Composition Reference Range Comparisons

Grain Component	Peer-Reviewed Literature		ILSI CCDB[1]
	Range (reference)	Fraction (seed, oil, or meal)	Range
Proximates and Fibers (% dry weight)			
Protein	17.4–23.0 (Pritchard et al., 2000)[2] 32.4–34.5 (Lajolo et al., 1991)[3]	seed meal	15.6–35.7
Crude fat	39.3–43.3 (Pritchard et al., 2000)[2]	seed	24.6–55.2
Ash	3.29–3.62 (Lajolo et al., 1991)[3]	meal	2.8–8.7
Crude fiber	9.9–13.2 (Mailer et al., 2008)[4] 23.7–27.5 (Lajolo et al., 1991)[3]	meal meal	11.2–37.8
Neutral detergent fiber	31.2–39.4 (Mailer et al., 2008)[4] 12.7–16.0 (Lajolo et al., 1991)[3]	meal meal	10.93–53.7
Acid detergent fiber	16.6–20.5 (Mailer et al., 2008)[4] 10.7–14.1 (Lajolo et al., 1991)[3]	meal meal	8.94–42.3
Amino Acids (% dry weight)			
Alanine	1.64–1.89 (Lajolo et al., 1991)[3]	meal	0.73–1.43
Arginine	2.02–2.26 (Lajolo et al., 1991)[3]	meal	0.97–2.10
Aspartic acid	2.67–2.94 (Lajolo et al., 1991)[3]	meal	1.15–2.62
Cystine	0.47–0.69 (Lajolo et al., 1991)[3]	meal	0.19–0.96
Glutamic acid	5.83–6.50 (Lajolo et al., 1991)[3]	meal	2.37–7.31
Glycine	1.81–2.02 (Lajolo et al., 1991)[3]	meal	0.86–1.75
Histidine	0.99–1.09 (Lajolo et al., 1991)[3]	meal	0.47–1.05
Hydroxyproline	-	-	NR
Isoleucine	1.10–1.39 (Lajolo et al., 1991)[3]	meal	0.65–1.35
Leucine	2.56–2.65 (Lajolo et al., 1991)[3]	meal	1.14–2.35
Total Lysine	1.94–2.13 (Lajolo et al., 1991)[3]	meal	1.07–2.09[5]
Methionine	0.69–0.89 (Lajolo et al., 1991)[3]	meal	0.19–0.71
Phenylalanine	1.26–1.45 (Lajolo et al., 1991)[3]	meal	0.69–1.52
Proline	1.96–2.68 (Lajolo et al., 1991)[3]	meal	1.01–2.13
Serine	1.77–1.97 (Lajolo et al., 1991)[3]	meal	0.66–1.53
Threonine	1.90–1.99 (Lajolo et al., 1991)[3]	meal	0.72–1.38
Tryptophan	-	-	0.166–0.442
Tyrosine	0.89–1.05 (Lajolo et al., 1991)[3]	meal	0.41–0.93
Valine	1.34–1.71 (Lajolo et al., 1991)[3]	meal	0.82–1.7
Fatty Acids (% wt/wt total fatty acids)			
C14:0	ND (0.05)–0.2 (Codex Alimentarius, 1999)[6]	oil	< LOQ (0.04)–0.09
C16:0	4.3–4.5 (Pritchard et al., 2000)[2] 0.4 (Lajolo et al., 1991)[3] 2.5–7.0 (Codex Alimentarius, 1999)[6]	seed meal oil	3.55–5.7
C16:1n-7	-	-	0.16–0.4

Grain Component	Peer-Reviewed Literature		ILSI CCDB[1]
	Range (reference)	Fraction (seed, oil, or meal)	Range
C16:1n-9	ND (0.05)–0.6 (Codex Alimentarius, 1999)[6]	oil	NR
C16:3n-3	-	oil	NR
C17:0	ND (0.05)–0.3 (Codex Alimentarius, 1999)[6]	oil	< LOQ (0.03)–0.14
C17:1	ND (0.05)–0.3 (Codex Alimentarius, 1999)[6]	oil	< LOQ (0.04)–0.16
C18:0	1.9–2.1 (Pritchard et al., 2000)[2] 0.8–3.0 (Codex Alimentarius, 1999)[6]	seed oil	1.5–2.77
C18:1n-7	-	-	NR
C18:1n-9	62.3–74.4 (Werteker et al., 2010) 58.5–60.7 (Pritchard et al., 2000)[2] 57.2–60.6 (Lajolo et al., 1991)[3] 51.0–70.0 (Codex Alimentarius, 1999)[6]	oil seed meal oil	53.19–69.45
C18:2n-6	11.0–21.0 (Werteker et al., 2010) 19.2–20.9 (Pritchard et al., 2000)[2]	oil seed	14.13–25.68
C18:2n-9	15.0–30.0 (Codex Alimentarius, 1999)[6]	oil	NR
C18:3n-3	5.1–8.3 (Werteker et al., 2010) 9.8–11.6 (Pritchard et al., 2000)[2] 5.0–14.0 (Codex Alimentarius, 1999)[6]	oil seed oil	NR
C18:3n-6	-	-	< LOQ (ND)
C18:4n-3	-	-	NR
C20:0	0.2–1.2 (Codex Alimentarius, 1999)[6]	oil	< LOQ (0.49)–0.86
C20:1n-9	1.2– 1.3 (Pritchard et al., 2000)[2] 0.1–4.3 (Codex Alimentarius, 1999)[6]	seed oil	1.00–1.82[7]
C20:2n-6	ND (0.05)–0.1 (Codex Alimentarius, 1999)[6, 7]	oil	< LOQ (0.04)–0.86[7]
C20:2n-9			
C20:3n-3	-	-	< LOQ (ND)[7]
C20:3n-6	-	-	
C20:3n-9	-	-	
C20:4n-3	-	-	NR
C20:4n-6	-	-	< LOQ (ND)
C20:5n-3	-	-	NR
C22:0	ND (0.05)–0.6 (Codex Alimentarius, 1999)[6]	oil	0.19–0.46
C22:1n-9	0.3–0.5 (Pritchard et al., 2000)[2] ND (0.05)–2.0 (Codex Alimentarius, 1999)[6]	seed oil	< LOQ (0.07)–1.43[7]
C22:2n-6	ND (0.05)–0.1 (Codex Alimentarius, 1999)[6]	oil	NR
C22:4n-3	-	-	NR
C22:4n-6	-	-	NR

Grain Component	Peer-Reviewed Literature		ILSI CCDB[1]
	Range (reference)	Fraction (seed, oil, or meal)	Range
C22:5n-3	-	-	NR
C22:5n-6	-	-	NR
C22:6n-3	-	-	NR
C24:0	ND (0.05)–0.3 (Codex Alimentarius, 1999)[6]	oil	< LOQ (0.09)–0.26
C24:1n-9	ND (0.05)–0.4 (Codex Alimentarius, 1999)[6]	oil	< LOQ (0.08)–0.4[7]
C16:1 trans fatty acids	-	-	NR
C18:1 trans fatty acids	-	-	NR
C18:2 trans fatty acids	-	-	NR
Total trans fatty acids	-	-	NR
Vitamins (mg/100g dry weight)			
Vitamin K1			0.04–0.56[10]
alpha-tocopherol	10.0–38.6 (Codex Alimentarius, 1999)[6]	oil	0.96–17.96
beta-tocopherol	ND–14.0 (Codex Alimentarius, 1999)[6]	oil	< LOQ (0.133)–0.288
delta-tocopherol	ND–2.2 (Codex Alimentarius, 1999)[6]	oil	< LOQ (0.143)–0.151
gamma-tocopherol	18.9–75.3 (Codex Alimentarius, 1999)[6]	oil	2.5–27.4
Total tocopherols	43.0–268 (Codex Alimentarius, 1999)[6] 57–69 (Vlahakis and Hazebroek, 2000)[9]	oil oil	3.582–38.939
Minerals (% dry weight)			
Calcium	0.50–0.59 (Lajolo et al., 1991)[3]	meal	0.248–1.41
Phosphorous	1.27–1.55 (Lajolo et al., 1991)[3]	meal	0.41–1.85
Magnesium	0.78–0.86 (Lajolo et al., 1991)[3]	meal	0.26–0.53
Potassium	1.03–1.41 (Lajolo et al., 1991)[3]	meal	0.46–1.4
Sodium	ND (Lajolo et al., 1991)[3]	meal	< LOQ (0.0001)–0.136
Iron	0.05–0.12 (Lajolo et al., 1991)[3]	meal	0.0034–0.0531
Zinc	0.005–0.007 (Lajolo et al., 1991)[3]	meal	0.0022–0.0155
Copper	0.002–0.029 (Lajolo et al., 1991)[3]	meal	< LOQ (0.0001)–0.001
Manganese	0.006–0.008 (Lajolo et al., 1991)[3]	meal	0.0015–0.0108
Antinutrients			
Phytic acid (% dry weight)	2.9–3.4 (Lajolo et al., 1991)[3]	meal	0.94–3.88

Grain Component	Peer-Reviewed Literature		ILSI CCDB[1]
	Range (reference)	Fraction (seed, oil, or meal)	Range
Tannins–soluble condensed (% dry weight)	-	-	0.05–0.34
Sinapine (% dry weight)	1.29–1.52 (Mailer et al., 2008)[4]	meal	0.19–1.36
Ferulic acid (µg/g)	5.0–79 (Kozlowska et al., 1983)[8] 150 (Dabrowski and Sosulski, 1984)[8]	flour flour	NR
Coumaric acid (µg/g)	trace (5.0)–30 (Kozlowska et al., 1983)[8]	flour	NR
Glucosinolates (µmol/g dry weight)			
Progoitrin	11.26–21.91 (Lajolo et al., 1991)[3] 3.14–7.03 (Jankowski et al., 2015)	meal seed	0.11–9.73
Glucoalyssin	0.05–9.80 (Jankowski et al., 2015)	seed	< LOQ (0.07)–0.56
Glucobrassicin	0.07–0.40 (Jankowski et al., 2015)	seed	< LOQ (0.06)–1.84
Glucobrassicanapin	0.90–1.67 (Lajolo et al., 1991)[3] 0.18–0.56 (Jankowski et al., 2015)	meal seed	< LOQ (0.39)–1.8
Glucoiberin	-	-	< LOQ (ND)
Gluconapin	4.89–8.89 (Lajolo et al., 1991)[3] 1.44–4.04 (Jankowski et al., 2015)	meal seed	< LOQ (0.1)–6.84
Gluconapoleiferin	0.66–1.19 (Lajolo et al., 1991)[3] 0.17–0.32 (Jankowski et al., 2015)	meal seed	< LOQ (0.03)–0.62
Gluconasturtiin	trace (0.05)–0.19 (Jankowski et al., 2015)	seed	< LOQ (0.13)–1.65
Glucoraphanin	-	-	< LOQ (0.05)–0.96
Neoglucobrassicin	0.05–0.07 (Jankowski et al., 2015)	seed	< LOQ (0.02)–0.34
Epi-progoitrin	-	-	< LOQ (0.07)–0.53
4-Hydroxyglucobrassicin	1.34–5.09 (Jankowski et al., 2015)	seed	< LOQ (0.05)–10.4
Total glucosinolates	13–27 (Mailer et al., 2008)[4] 7.8–26.8 (Pritchard et al., 2000)[2]	meal seed	0.41–31.98
Phytosterols (% dry weight)			
24-Methylene cholesterol	-	-	NR
Beta-sitosterol	0.231–0.392 (Vlahakis and Hazebroek, 2000)[9] 0.399–0.512 (Codex Alimentarius, 1999)[6]	oil oil	0.03–0.21
Brassicasterol	0.053–0.106 (Vlahakis and Hazebroek, 2000)[9] 0.044–0.115 (Codex Alimentarius, 1999)[6]	oil oil	0.0057–0.0477
Campestanol	-	-	NR

Grain Component	Peer-Reviewed Literature		ILSI CCDB[1]
	Range (reference)	Fraction (seed, oil, or meal)	Range
Campesterol	0.152–0.308 (Vlahakis and Hazebroek, 2000)[9] 0.219–0.342 (Codex Alimentarius, 1999)[6]	oil oil	0.02–0.13
Cholesterol	ND–0.012 (Codex Alimentarius, 1999)[6]	oil	< LOQ (0.0004)–0.0028
Clerosterol	-	-	NR
Delta-5 avenasterol	0.022–0.058 (Codex Alimentarius, 1999)[6]	oil	NR
Delta-5,23 stigmastadienol	-	-	NR
Delta-5,24 stigmastadienol	-	-	NR
Delta-7 avenasterol	ND–0.007 (Codex Alimentarius, 1999)[6]	oil	NR
Delta-7 stigmastenol	ND–0.012 (Codex Alimentarius, 1999)[6]	oil	NR
Sitostanol	-	-	NR
Stigmasterol	ND (Vlahakis and Hazebroek, 2000)[9] 0.002–0.009 (Codex Alimentarius, 1999)[6]	oil oil	< LOQ (0.001)–0.0078
Total phytosterols	0.459– 0.807 (Vlahakis and Hazebroek, 2000)[9] 0.45–1.13 (Codex Alimentarius, 1999)[6]	oil oil	0.06–0.39

Dash (-) = not defined or no value; NR = Not Reported; < LOQ = Less than Limit of Quantitation. In cases where < LOQ is noted, the minimum reported value of the quantifiable data or ND (not detected) is shown in parentheses.

[1] ILSI (2016). Search criteria for canola seed, all locations, all years.

[2] Canola seed at 8.5% moisture.

[3] Canola meal at 10.5% moisture. Amino acids converted from g/16 g N to % (dry weight) based on the 6.25 N to protein conversion factor and the average reported protein content of 33.5% as in the following example for alanine:

$$\frac{4.90\ g\ Alanine}{16\ g\ N} * \frac{1\ g\ N}{6.25\ g\ Protein} * 33.5\ (\%)\ Protein = 1.64\ \%\ Alanine$$

[4] In oil-free, dry canola meal; for sinapine, g/kg converted to % dry weight.

[5] Reported as lysine.

[6] Rapeseed oil (low erucic acid); for vitamins, mg/kg converted to mg/100g; total phytosterols converted from mg/kg to % dry weight; other sterols converted from % of total sterols to % dry weight.

[7] Fatty acids reported as 20:1 eicosenoic, 20:2 eicosadienoic, 20:3 eicosatrienoic, 20:4 arachidonic, 22:1 erucic, 24:1 nervonic.

[8] In canola flour, mg/100g converted to µg/g.

[9] Canola oil; total tocopherols converted from ppm to mg/100g; phytosterols converted from ppm to %.

[10] Converted from mg/g to mg/100g.

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