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Subject: Additional information on Environmental Assessment and Impact on Agronomic Practices for 305423 Soybean (APHIS # 06-354-01P)

Dear Ms.Green,

As per our conversation dated February 7, 2008, Pioneer Hi-Bred International, Inc. is happy to provide USDA-APHIS with some additional information on Environmental Assessment and Impact on Agronomic Practices (Section X in the original petition), as they relate to the deregulation of 305423 soybean. The attached 10-page document includes additional information related to these topics, and the new information presented is highlighted in the text.

Please feel free to contact me at 515-334-6388 or padma.commuri@pioneer.com if you require any additional information.

Sincerely,

Padma Commuri
Registration Manager

X. ENVIRONMENTAL ASSESSMENT AND IMPACT ON AGRONOMIC PRACTICES

X-A. Environmental Assessment of the GM-HRA Protein

The *gm-hra* gene which is used solely as a selectable marker in 305423 soybean encodes a modified version of the acetolactate synthase (ALS) enzyme (refer to Section VII for the details on the protein biochemistry and mode of action). ALS proteins are ubiquitously present in nature, as ALS genes have been isolated from bacteria, fungi, algae and plants (Friden *et al.,* 1985; Falco *et al*., 1985; Reith and Munholland, 1995; Mazur *et al*., 1987). Moreover, naturally occurring mutations in plant ALS proteins that confer herbicide tolerance have also been identified (for a review, see Duggleby and Pang, 2000; Tan *et al*., 2006). Several crops where herbicide tolerant *als* genes are utilized to confer commercial level of herbicide tolerance have been commercialized and do not display any unexpected environmental consequences (for example, Clearfield®[1] wheat, Clearfield® sunflower, Clearfield® lentils, STS®[2] soybean). None of these ALS enzymes or herbicide tolerant crops is known to pose an environmental safety concern. This indicates a previous history of exposure to and safe use of proteins similar to GM-HRA.

The GM-HRA protein introduced into 305423 soybean has minimal modifications compared to the endogenous soybean ALS enzyme from which it was derived. It has only two amino acid differences from the corresponding endogenous soybean ALS protein, plus an additional five amino acids at the N-terminus derived from translation of 15 nucleotides from the *als* 5' UTR (refer to Section VII). In addition to being derived from a soybean protein and having a high degree of similarity to other ALS enzymes, the GM-HRA amino acid sequence does not have any homology to proteins that are toxic to humans or animals, as described in Section VII-D. Therefore, the GM-HRA protein is highly unlikely to pose a safety risk to beneficial organisms or the environment.

In conclusion, there are not likely to be any environmental effects due to the presence of the GM-HRA protein introduced into 305423 soybean.

X-B. Fate of Transgenic DNA in Humans and Animals

Transgenic DNA is no different from other DNA consumed as part of the normal diet. Genetically engineered organisms have been used in drug production (Thayer, 2005) and microbial fermentation (cheese) since the late 1970's (National Center for Biotechnology Education, 2006, Maryanski, 1995). More than 1.4 billion cumulative acres of engineered food and feed crops have been grown and consumed worldwide over the past seven years (ISAAA, 2006). The FDA has not reported any significant concerns with bioengineered food and feed currently on the market. The EPA has exempted from a tolerance DNA that encodes currently registered plant incorporated protectants because of a lack of toxicity (Federal Register, 2001).

Studies in humans and animals following the fate of DNA once consumed have shown that the majority of DNA is degraded in the gastrointestinal tract. There is evidence that DNA can move from the gastrointestinal tract lumen to other areas of the body, but this is considered to be a normal occurrence and no risks have been identified as a result of absorption (Einspanier *et al*., 2001; Duggan *et al*., 2003).

[1] Clearfield® is a registered trademark of BASF.
[2] STS® is a registered trademark of DuPont or its affiliates.

X-C. Weediness Potential of 305423 Soybean

Commercial soybean varieties in the United States are not considered weeds and are not effective in invading established ecosystems. Soybean has been grown throughout the world without any report that it is a serious weed. Cultivated soybean is unlikely to become a weed. Soybean seed rarely displays any dormancy characteristics and only under certain environmental conditions grows as a volunteer in the year following cultivation. If this should occur, volunteers do not compete well with the succeeding crop and can easily be controlled mechanically or chemically. The soybean plant has no weedy tendencies and is non-invasive in natural habitats in the United States. It does not grow in unmanaged habitats.

Soybeans are not an overwintering crop: they are not frost tolerant and do not survive freezing winter conditions (OECD, 2000). Studies by Kodama and co-authors (1994, 1995) indicate that increases in levels of trienoic fatty acids such as hexadecatrienoic acid (C16:3) and linolenic acid (C18:3) can enhance cold tolerance in model plants such as *Arabidopsis thaliana* and tobacco. In the case of 305423 soybean, levels of linolenic acid are significantly decreased. Therefore, we would not expect 305423 soybean plants to exhibit any enhanced cold tolerance. Moreover, in-season and post-trial monitoring of 305423 soybean experiments confirmed there were no unexpected changes relative to weediness and seed ability to survive over winter.

There is little probability that 305423 soybean could become a problem weed. Although 305423 soybean contains the *gm-hra* gene, it is only used as a selectable marker gene in 305423 soybean and does not confer commercial levels of herbicide tolerance in this transgenic event. Normal agronomic practices and weed control measures can therefore be used. Various characteristics that might impart weediness potential were evaluated for 305423 and control soybean in comparative studies (Section VIII). No differences were seen in characteristics such as seed germination, emergence, seedling vigor, seed shattering, yield, and disease/insect susceptibility. Assessment of these data detected no biologically significant differences between 305423 and control soybean indicative of a selective advantage that would result in increased weediness potential. Furthermore, post-harvest monitoring of field trial plots containing 305423 soybean has shown no differences in survivability or persistence of 305423 soybean as compared to control or conventional soybeans. The ecological fitness of 305423 soybean is therefore similar to that of conventional soybeans.

X-D. Gene Flow Assessment

Vertical Transfer of the Introduced Genetic Material

Due to the reproductive morphology (papilionaceous flower) of soybeans, this crop exhibits a high percentage of self-fertilization. Natural outcrossing levels in soybean range from less than 0.5% to about 1% (Carlson and Lersten, 1987). As a reflection of this low potential for cross-pollination, Certified Seed Regulations allow foundation seed to be grown adjacent to other soybean varieties as long as the distance is adequate to prevent mechanical mixing of the harvested seeds (see 7 CFR §201.76, http://www.access.gpo.gov/nara/cfr/waisidx_01/7cfr201_01.html). Hence, the probability of gene transfer from 305423 soybean to other commercial soybean varieties is very low.

Cultivated soybean can cross only with other members of its subgenus, *Soja* (reviewed in OECD, 2000). However, the potential for such gene flow to wild soybean relatives is limited by geographic isolation. Wild soybean species are native to China, Korea, Japan, Taiwan and the former USSR, and do not exist naturally in the United States. These species are not naturalized in North America, and although they could occasionally be grown in research plots, there are no reports of their escape from such plots to unmanaged habitats. There is therefore no potential for gene flow from cultivated 305423 soybean plants to wild soybean relatives in the United States.

Horizontal Transfer of the Introduced Genetic Material

There is no known mechanism for, or definitive demonstration of, DNA transfer from plants to microbes (Nap *et al.*, 1992; Redenbaugh *et al.*, 1994). Many genomes have been sequenced from bacteria that are closely associated with plants (*e.g*. *Agrobacterium* and *Rhizobium*), and there is no evidence that these organisms contain genes derived from plants (Kaneko *et al.*, 2002, Wood *et al*., 2001). The occurrence of potential horizontal gene transfer in the environment (bacteria, pathogens, *etc*.) has been studied using soil, water, and mammalian digestive tract systems. These studies conclude that the risk of a possible transfer is irrelevant to an environmental risk assessment of transgenic soybean (Bogosian and Kane, 1991; Prins and Zadoks, 1994; Schluter *et al*., 1995; Jonas *et al.,* 2001). Where sequence data indicate that horizontal gene transfer may have occurred, these events are estimated to occur on an evolutionary time scale on the order of millions of years (Koonin, 2001; Brown, 2003). In addition, transgene DNA promoters and coding sequences are optimized for plant expression and not bacterial expression, and it is therefore very unlikely that a protein corresponding to the transgene would be produced. Even if such a transfer were to take place and protein produced, the DNA and protein would not present a human health or plant pest risk. The *gm-fad2-1* gene fragment and the *gm-hra* gene are endogenous to soybean.

X-E. Current Agronomic Practices for U.S. Soybean

E1. Soybean Production

The United States is the world's leading soybean producer and exporter. Farm value of U.S. soybean production in 2003/04 was $18.0 billion, the second-highest value among U.S.-produced crops, trailing only corn (USDA/ERS, 2006a). In 2004, soybean was planted on 75.1 million acres in 31 states, mostly in the Upper Midwest, Delta, and Southeast regions of the United States (USDA-NASS, 2006; USDA/ERS, 2006b). The average annual yields by state varied from 31 to 50 bushels/acre due to differences in rainfall, climate and soil productivity. However, yields in individual fields can be as high as 80 to 90 bushels/acre.

Soybeans are typically grown in the United States as row crops. Planting usually begins in late April or early-to-mid May, and harvesting generally occurs in late October to early November. Clean tillage has been the traditional method of field preparation, but no tillage and reduced tillage systems have become increasingly common. More than 60% of soybean acres are now grown under some form of conservation tillage (no-till, ridge-till or mulch-till). Conservation tillage practices provide the advantages of decreased soil compaction and fuel costs through reduction in use of heavy machinery, reduced soil erosion and better soil moisture conservation. Irrigation is not usually practiced (Van Doren and Reicosky, 1987); in 2002 only 7.5% of planted soybean acres were irrigated (USDA/ERS, http://www.ers.usda.gov/).

Most soybeans (67% in 2002, USDA/ERS (2002)) are grown in rotation following corn, with another 18% in rotation with other row crops and small grains; 14% are grown continuously. In areas with a longer growing season such as the southern United States, soybeans are also double cropped, after winter wheat is harvested (6% of planted acres in 2004, CTIC (2006)). Crop rotation aids in the management of diseases, insects and weeds and increases organic matter and soil fertility. In addition, crop rotation allows growers to diversify farm production to minimize market risks.

E2. Agricultural Chemical Use in Soybeans

Soybeans are quite intensively managed, as evidenced by the chemical usage data from the 2004 USDA-NASS Agricultural Chemical Usage Report (http://www.nass.usda.gov/). Eleven states (81% of the total US soybean acreage) were included in this report: Arkansas, Illinois, Indiana, Iowa, Kansas, Minnesota, Missouri, Nebraska, North Dakota, Ohio and South Dakota. Phosphate and potash were the most commonly applied fertilizers in soybean; they were used on average in 26% and 23% of the acreage, respectively, of the acreage in the states listed in the report. However, the

acreage treated with fertilizer varies greatly by state, with some treating up to 63% of acres, and others only 5%. Because phosphate and potash are stable in soils, many growers don't apply them every year. Phosphate and potash are often applied on the corn crop in a corn-soybean rotation, using the same application for both crops. Insecticides and fungicides were not widely used, with 4% and 1% of the acres treated, respectively. Herbicides were applied on 97% of the soybean acres. Glyphosate dominated herbicide usage, with application on 87% of planted acres. The next most used herbicides were chlorimuron-ethyl (7% of acres treated), sulfentrazone (6%), trifluralin (5%), and pendimethalin (4%).

Similar to other value-added soybeans (low linolenic, clear-hilum, non-GMO), 305423 soybean is intended to be marketed under an identity preservation program. Identity preservation is a stringent process by which a crop is grown, handled, delivered, and processed under controlled conditions that insure the purity and maintenance of a unique, value-added trait from the farm-gate to end use. Specially bred high oleic soybeans will be offered under a premium to growers who contract their crop with local participating elevators under specified production, delivery, and sampling standards. Other than this, management and production practices for growing high oleic 305423 soybeans are much the same as growing any variety of conventional soybeans. No impact on current soybean cultivation practices is expected from introduction of the high oleic 305423 soybean. With exception of the intended changes in fatty acid composition of the seed, 305423 soybean were shown to be substantially equivalent to conventional soybean varieties in agronomic, ecological and compositional characteristics and have the same levels of resistance to insects and diseases.

E3. Weeds in Soybean

Growers must control weeds that compete with their crops for water, nutrients and sunlight. Depending on the crop and specific production region, weeds can decrease crop yields from 35% - 100% as well as interfere with harvest. More than 200 weed species, including 32 confirmed herbicide resistant species, threaten soybean yields in the United States each year (Weed Science Society of America, www.weedscience.org).

Soybeans are very sensitive to weed competition in the first few weeks after emergence. Weed scientists recommend that growers eliminate weed competition within three to five weeks after emergence. This helps insure that soybeans do not suffer irreversible yield loss due to early competitive pressures from weeds (DeFelice and Butzen, 1997). Soybeans are very competitive with weeds once a canopy has developed, but early weeds can cause significant yield loss. Once weeds begin to impact soybean yield, each additional day they are allowed to compete can result in yield losses up to 1% per day (Hartzler, 2003). Narrow row planting has been helpful in increasing the competitive advantage over weeds, as the plants "shade out" later emerging weeds. However, because soybeans are relatively short, tall weeds can still shade the soybean plants, leading to yield reduction.

Common weed problems in soybean fields include annuals (summer annual grass and broadleaf weeds such as foxtails or common ragweed), winter annuals and biennials such as marestail and biennial wormwood, and perennials such as quackgrass and Johnsongrass. As growers shift to more no-till soybean production, weed specialists are seeing an increased frequency of winter annual, biennial and perennial weeds. The winter perennials are particularly competitive and difficult to control, as these weeds re-grow every year from rhizomes or root systems. Dense populations of winter annuals can physically interfere with soybean planting and in some cases even reduce soil drying to such an extent that it delays soybean planting

E4. Weed Management in Soybean

Before the introduction of herbicides, weed control in soybean was through mechanical cultivation. Soil-incorporated and preemergence herbicides began to replace tillage and cultivation practices for soybeans in the 1960's (Carpenter and Gianessi, 1999). In the 1980's, postemergence herbicides became available to soybean growers, and their use has been steadily increasing. In 1988, 44% of

soybean acres were treated with postemergence herbicides. By 2002, 91.8% of soybean acres were treated with postemergence herbicides (Carpenter and Gianessi, 1999; USDA/ERS, 2002).

The availability of postemergence herbicides together with herbicide tolerant crops has greatly facilitated the adoption of conservation tillage practices. Growers gained additional tools to control weeds; they could use a burndown treatment for early weed control and then apply one or two postemergence herbicides to control in-season weeds without tillage. This has ecological advantages in reducing soil erosion, lowering fuel and equipment costs, and allowing growers to plant larger acreages (since spraying is faster than tilling).

Narrow row spacing, which increases yields due to more efficient use of space, has also been made possible by postemergence herbicides. Mechanical cultivation is not possible with very narrow (~7.5 in) row spacing, but postemergence herbicides eliminate the need for mechanical cultivation. Narrow rows also improve weed control because the canopy closes more quickly and shades out later-emerging weeds.

Decisions about weed management may be the most complex ones that growers make, because each weed control option has trade-offs and affects the feasibility of using other options. Generally, growers must manage a wide array of broadleaf and grass weeds simultaneously. In selecting a weed management strategy, growers choose the most economical means to control weeds that does not decrease the quality or quantity of the crop. Therefore, growers will often use a combination of weed management techniques, including application of different herbicides, to effectively control weeds in their fields. The combination that a grower chooses to use depends upon factors such as: weed spectrum, level of infestation, soil type, cropping system, weather, and time and labor available for the treatment option. Data from the 2002 USDA-NASS Agricultural Chemical Usage Report indicate that, after herbicide usage, scouting was the most prevalent form of pest management practice for soybeans, with 92% of those surveyed scouting through general observation or deliberate scouting activities. Of those who completed scouting activities, 91% monitored for weeds. To avoid pest pressures (weeds, insects and diseases), 79% of growers rotated their crops. Of those surveyed, 55% used no-till/minimum till practices to manage pest pressures.

E5. Crop Rotation Practices

Most soybeans (67% in 2002, USDA/ERS, (2002)) are grown in rotation following corn, with another 18% in rotation with other row crops and small grains. About 14% are grown continuously. In areas with a longer-growing season such as the southern United States, soybeans are also double cropped, with soybeans planted after winter wheat is harvested (6% of planted acres in 2004, CTIC (2006)). Crop rotation aids in the management of diseases, insects and weeds and increases organic matter and soil fertility. In addition, crop rotation allows growers to diversify farm production to minimize market risks.

X-F. Impact of the Introduction of 305423 Soybean on Agronomic Practices

F1. Impact on Cultivation and Management Practices

No negative impact is expected from the introduction of 305423 soybean on current cultivation and management practices for soybean. We expect that 305423 soybean will displace a proportion of soybeans having the normal commodity soy oil profile, but no significant increase in planted soybean acres is anticipated. The 305423 soybean has been shown to be comparable to conventional soybean in phenotypic, ecological and compositional characteristics ((Sections VIII and IX). 305423 soybean is expected to be similar in its agronomic characteristics and have the same levels of resistance to insects and diseases as other commercial soybeans.

F2. Impact on Weed Control Practices

The commercialization of herbicide tolerant 305423 soybean is expected to have no impact on weed control practices. The 305423 soybean was not selected for commercial sulfonylurea or other ALS-inhibitor herbicide tolerance during the breeding and product development process. All conventional soybean lines, as well as 305423 soybean, have an inherent level of tolerance to certain sulfonylurea herbicides. Pioneer has no plans to market 305423 soybean as having a weed control advantage over conventional soybeans. Several ALS-inhibiting herbicides are currently registered for use in soybean and are used to control troublesome weeds. As with conventional soybeans, 305423 soybean should only be sprayed using sulfonylurea herbicides labeled for use on conventional soybeans. ALS inhibitor herbicides are typically applied at very low use rates, in the ounces/acre or less range and no increase in the usage of ALS-inhibiting herbicides is expected because of the introduction of 305423 soybean.

Approximately 87% of the U.S. soybean crop is already planted with glyphosate tolerant varieties and no increase in the use of glyphosate over soybeans due to the commercialization of 305423 soybean is expected.

F3. Impact on Insect Control Practices

305423 soybean has been shown to be no different than conventional soybean in agronomic characteristics, and ecological observations have shown no changes in susceptibility to insect damage (Section VIII). Therefore, there are no expected impacts on insect control practices for 305423 soybean.

F4. Impact on Crop Rotation Practices

We do not expect changes in crop rotation practices. There would be no expected increase in soybean-after-soybean plantings. ALS-inhibiting herbicides can have recrop restrictions. However, as the ALS-inhibiting herbicides used on 305423 soybean will be the same as the ones currently labeled for usage on conventional non-GM soybean, we would not expect any changes in the current crop rotation practices.

F5. Potential Impact on the Development of Herbicide Resistant Weeds

Growers have been dealing with the issue of herbicide-resistant weeds for decades. The commercialization of 305423 soybean is not going to result in any increased potential for development of resistance weeds since 305423 soybean is not marketed as a herbicide tolerant trait and there will be no increase in the usage of ALS inhibitor herbicides.

F6. Impact on Volunteer Management

We do not expect changes in volunteer management practices. 305423 soybean does not display any dormancy characteristics, and soybean in general grows as a volunteer in the year following cultivation only under certain environmental conditions. When this occurs, volunteers do not compete well with the succeeding crop and can easily be controlled mechanically or chemically with herbicides like glyphosate. Agronomic and phenotypic data confirm that similar to conventional soybean, 305423 soybean does not have any weedy characteristics.

F7. Potential Impact on Organic or Conventional Farming

Growers choose to grow organic, conventional or biotechnology-derived soybean primarily based on economic and market factors. Growers of organic soybean and conventional soybean for non-biotechnology markets are generally paid a premium for their products, justifying the additional production costs. Conventional and organic soybean seeds are readily available to growers who decide to plant them. In addition to the market segments that produce organic or conventional soybean, distinct value-added specialty soybeans (low linolenic, clear hilum or high protein) have also been grown and successfully marketed for specific food uses in domestic and export markets for many years (Cui *et al.*, 2004). The introduction of 305423 soybean offers growers another value-added specialty soybean option. Value-added specialty soybean products are grown under identity preservation programs with grower premiums similar to conventional and organic products (see Section X-E for details). Growers will continue to have the option of growing conventional, organic or biotechnology-derived soybeans based on market factors.

X-G. Weed Resistance Management

G1. Evolution of resistant weeds

Crop pests respond to the repeated use of any mechanism that attempts to control them by evolving biological tactics to escape control. The widespread use of herbicides can lead to weed populations that are no longer susceptible. The first documented case of a weed evolving resistance in response to repeated use of an herbicide occurred in the mid 1960's (Ryan, 1970). During the 1970's, growers in the U.S. and Europe began to realize that one class of herbicides (triazines) that had successfully controlled many different weeds was no longer effective against certain populations of as many as 30 different weed species (LeBaron and McFarland, 1990; Bandeen *et al*., 1982). By 1990, weed scientists had evidence that at least 81 weed species contained individuals (biotypes) that had evolved resistance to one or more herbicides; 15 different classes of herbicides were no longer effective against at least one weed species (Holt and LeBaron, 1990). Currently more than 305 biotypes of herbicide resistant weeds occur around the world. Data from the international survey of herbicide resistant weeds can be found at http://www.weedscience.org/in.asp. Introduction of 305423 soybean will not have any impact on the evolution of resistant weeds since 305423 soybean is not marketed as a herbicide tolerant trait and there will be no increase to the current levels of herbicide usage on soybeans.

G2. Herbicide Resistance and Integrated Weed Management

The concerns associated with the use of herbicides and herbicide resistant crops include the evolution of resistant weeds, misapplication of the herbicide, herbicide drift, crop injury, carryover between growing seasons, costs, and the need for timely application. Herbicides are important tools for growers, and they should be used properly to preserve their effectiveness. Any weed management option that reduces herbicide-imposed selection pressure will reduce the rate of resistance development to the herbicide. By adopting practices such as mixing herbicides with different modes of action and crop rotation, selection pressure for resistant weeds can be reduced and the usefulness of herbicides preserved. These approaches are often part of Integrated Weed Management (IWM) programs.

Integrated Weed Management (IWM) is modeled after the more familiar Integrated Pest Management (IPM) used to control insects and plant pathogens. Both IWM and IPM are based on ecological and evolutionary principles.

IWM utilizes a range of weed control methods, including the following:

a. Avoid using the same herbicide or herbicides with the same mode of action multiple times per year or year after year.
b. Use tank-mixtures consisting of different herbicide types with overlapping weed spectra.
c. Use crop rotations because different crops allow different cultural and tillage options that compete much differently with weeds.
d. When using herbicides, use full label rates and tank mix partners.
e. Use clean seed and clean equipment to minimize spread of weed seed.
f. Monitor fields after herbicide applications for appearance of resistant weeds.
g. Control weeds before they form seed.
h. Where practical, use cover crops, set-aside programs, and other methods to reduce weed seed in soil.

IWM relies on using a variety of control measures to slow the evolution of resistance to a single control measure; therefore IWM is maximized when growers have access to the widest possible array of weed control tools. Pioneer and DuPont through its Crop Protection Chemicals business actively promote IWM techniques through communication, research, education and participation in industry coalitions such as the Herbicide Resistance Action Committee (HRAC). Introduction of 305423 soybean will not impact the herbicide resistance of the crops, weed control options available to the growers remain the same and the integrated weed management practices applied will be the same as with the conventional soybeans, since 305423 soybean is not marketed as herbicide tolerant trait.

X-H. Potential Impacts on Raw or Processed Agricultural Commodities

Data submitted on agronomic performance, disease and insect susceptibility, and compositional analyses of 305423 soybean show no significant differences between 305423 soybean and non-transgenic control soybean that would be expected to cause either a direct or indirect plant pest effect on any raw or processed plant commodity. The 305423 soybean will also be reviewed by the FDA for use in food and feed. It is not anticipated that 305423 soybeans would be processed any differently from the way conventional soybeans are processed. Meal produced from 305423 soybean will be used in the same manner as conventional soybean meal. Oil derived from 305423 soybean will be a value-added oil intended as a replacement for hydrogenated fats containing trans fatty acids (Kinney and Knowlton, 1997). Based on the analyses above, we expect no significant impact on raw or processed agricultural commodities other than the intended fatty acid changes in identity-preserved oil products based on the introduction of 305423 soybean.

X-I. Potential Impact on Non-target Organisms, Including Beneficial Organisms and Threatened or Endangered Species

As already mentioned in Section X-H of our petition, with introduction of 305423 soybean, we would expect no effect on non-target organisms, including beneficial organisms and threatened or endangered species. The only novel protein expressed in 305423 soybean is GM-HRA. Based on the safety of the GM-HRA protein expressed in 305423 soybean described in Section X-A and the compositional analysis described in Section IX, we would expect no effect on non-target organisms, including beneficial organisms and threatened or endangered species. A wide variety of ALS proteins are already present in the environment. The GM-HRA protein is not a potential food allergen and exhibited no toxicity to mice in an acute feeding study (data not shown). In addition, the nutritional comparability of 305423 soybean was evaluated by comparing growth performance and carcass yield variables of broiler chickens fed diets containing 305423 soybean with those fed diets from non-transgenic soybeans. No statistically significant differences were observed in mortality, weight gain, mortality-adjusted feed efficiency, and carcass yields between broilers consuming diets produced with 305423 soybean fractions and those consuming diets produced with near isoline control soybean fractions (data not shown). Based on tests conducted in rodents (mouse acute study), and poultry (42-day broiler study), the GM-HRA protein is not toxic to mammals and the nutritional

wholesomeness of 305423 soybean is comparable to other conventional soybean varieties. Observations made during field testing have revealed no effects on invertebrate populations.

We expect that 305423 soybean will displace a proportion of soybeans having the normal commodity soy oil profile, but do not expect that 305423 soybean will cause new soybean acres to be planted in areas that are not already in agricultural use. Threatened or endangered species are generally found outside of agricultural fields. Any habitat disruption within fields will be comparable to any other cropping systems. Based on this information, we would not expect cultivation of 305423 soybean to have an effect on threatened or endangered species, or expect it to adversely change designated critical habitats compared to current agricultural practices

X-J. Potential Impact on Aquatic Organisms

As mentioned in Section X-I, a wide variety of ALS proteins are already present in the environment. The GM-HRA protein is the only novel protein expressed in 305423 soybean and is neither toxic nor allergenic. Based on the safety of the GM-HRA protein expressed in 305423 soybean described in Section X-A and the compositional analysis and nutritional wholesomeness described in Section IX and Section X-I, 305423 soybean is comparable to the conventional soybean and no new byproducts that might have an impact on the aquatic ecosystems are produced from 305423 soybean. Therefore, with introduction of 305423 soybean, we do not expect any negative affects on the well being of the aquatic organisms.

X-K. Potential Impact on Biodiversity

The 305423 soybean does not have an increased weediness potential, and unconfined cultivation of 305423 soybean should not lead to increased weediness of other sexually compatible relatives, as non-cultivated *Glycine* species are not found in the United States (Section X-C). Therefore, it is unlikely to have effects on non-target organisms common to the agricultural ecosystem or threatened or endangered species recognized by the U.S. Fish and Wildlife Service and the National Marine Fishery Services, and there is no apparent potential for significant impact to biodiversity.

X-L. Overall Environmental and Agronomic Practices Conclusions

A thorough characterization of 305423 soybean was performed, including molecular analysis, GM-HRA protein level analysis, phenotypic and ecological evaluation, and nutrient composition evaluation. Assessment of the data generated supports the conclusion of no increased plant pest potential, phenotypic comparability, and familiarity as they relate to ecological risk assessment.

Due to the previous history of exposure to and safe use of organisms containing proteins similar to GM-HRA, as well as the safety assessment on the GM-HRA protein, no environmental effects due to the presence of the GM-HRA protein introduced in 305423 soybean are expected. Likewise, there is no impact on public health or safety expected due to the DNA introduced in 305423 soybean.

The 305423 soybean has been shown to be agronomically and ecologically similar to conventional soybeans, which have no weedy tendencies and are non-invasive in natural habitats. No differences were seen in characteristics such as seed germination, dormancy, emergency, seedling vigor, seed shattering, yield, and disease/insect susceptibility. Assessment of these data detected no biologically significant differences between 305423 and control soybean indicative of a selective advantage that would result in increased weediness or outcrossing potential. On the basis of these data, it is concluded that there is no increased plant pest potential of 305423 soybeans.

Because of the agronomic similarity of 305423 soybean to conventional soybean, there is no significant impact expected on raw or processed agricultural commodities (other than the intended fatty acid changes in identity-preserved oil products), on non-target, beneficial organisms (including threatened and endangered species), or on biodiversity. Impacts on organic or conventional farming

are also expected to be minimal, as growers' decisions to plant biotechnology-derived, organic or conventional soybean are driven largely by market dynamics. Market dynamics, grower choice, and existing soybean production practices will not change due to the availability of 305423 soybean other than offering growers another value-added specialty soybean option.

With the introduction of high oleic 305423 soybean, we do not expect any change in agronomic practices.

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Petition for the Determination of Nonregulated Status for High Oleic 305423 Soybean

The undersigned submits this petition under 7 CFR Part 340.6 to request that the Administrator, Animal and Plant Health Inspection Service, make a determination that the article should not be regulated under 7 CFR Part 340.

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OECD Unique Identifier: DP-3Ø5423-1

Submitted December 18, 2006
Revised October 5, 2007

No CBI

Release of Information

Pioneer is submitting the information in this assessment for review by the USDA as part of the regulatory process. By submitting this information, Pioneer does not authorize its release to any third party except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C., Section 552; USDA complies with the provisions of FOIA and USDA's implementation regulations (7 CFR Part 1.4); and this information is responsive to the specific request. Except in accordance with the Freedom of Information Act, Pioneer does not authorize the release, publication or other distribution of this information (including website posting) without Pioneer's prior notice and consent.

Certification

The undersigned certifies that, to the best knowledge and belief 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 petitioners, which are unfavorable to the petition.

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Summary

Pioneer Hi-Bred International, Inc. (Pioneer) is submitting a Petition for Determination of Nonregulated Status for high oleic soybean event DP-3Ø5423-1. Event DP-3Ø5423-1 (hereafter referred to as 305423 soybean) was developed by Pioneer, a DuPont Company. Pioneer requests a determination from USDA - Animal and Plant Health Inspection Service (APHIS) that 305423 soybean and any crosses of this line with nonregulated soybean lines no longer be considered regulated articles under 7 CFR §340.

Pioneer has developed a transgenic soybean product that provides soybean seeds with increased levels of monounsaturated (oleic) fatty acid and decreased levels of polyunsaturated fatty acids (linoleic and linolenic) and to a lesser extent, palmitic acid. The high oleic oil derived from 305423 soybeans will be beneficial for both the food and industrial oil sectors. The food service industry and food processors will benefit from the availability of a highly stable vegetable oil suitable for frying applications without the need for hydrogenation. In the industrial sector, high oleic soybean oil will offer an ingredient that is stable to oxidation for the formulation of cost effective, renewable, environmentally friendly industrial fluids. Pioneer brand high oleic soybean varieties are intended to be used for the production of TREUS™[1] brand high oleic soybean oil in an identity preserved production system.

The 305423 soybean plants have been genetically modified by co-transformation, via microprojectile bombardment, with two separate cassettes: a 2924 base pair DNA fragment containing the *gm-fad2-1* cassette that is responsible for the unique oil profile and a 4512 base pair DNA fragment containing the *gm-hra* gene cassette conferring tolerance to sulfonylurea herbicides used as a selective agent following transformation.

The conversion of oleic acid into linoleic acid in soybean seeds is catalyzed by omega-6 desaturase encoded by the *FAD2-1* gene. The *gm-fad2-1* cassette contains a fragment of a soybean microsomal omega-6 desaturase gene 1 (*FAD2-1*) under the control of the soybean seed-preferred Kunitz trypsin inhibitor 3 (KTi3) promoter. The *gm-fad2-1* fragment corresponds to about 40% of the middle of the open reading frame of the *FAD2-1* gene and does not code for a functional protein. Transcription of the *gm-fad2-1* fragment acts to silence expression of the endogenous omega-6 desaturase, resulting in an increased level of oleic acid and decreased levels of linoleic and linolenic acids (hereafter referred to as high oleic phenotype). Seed-specific silencing of the *FAD2-1* gene was confirmed by gene expression analysis using Northern hybridization technique.

The *gm-hra* gene cassette contains a *gm-hra* gene that encodes a GM-HRA protein, a modified version of a soybean acetolactate synthase (GM-ALS) under the control of the soybean constitutive S-adenosyl-L-methionine synthetase (SAMS) promoter. GM-ALS is involved in branched chain amino acid biosynthesis in plastids and is inhibited by certain classes of herbicides (*e.g.*, sulfonylureas). Expression of the GM-HRA protein confers tolerance to acetolactate synthase inhibiting herbicides. The herbicide tolerant *gm-hra* gene was made by isolating the soybean *gm-als* gene and introducing specific point mutations known to confer tolerance to sulfonylurea herbicides. In 305423 soybean, the *gm-hra* gene is solely used as a selectable marker. Pioneer does not plan to promote 305423 soybean varieties as tolerant to commercially available sulfonylurea herbicides.

The only novel protein expressed in 305423 soybean is GM-HRA. The allergenic potential of the GM-HRA protein was assessed using a step-wise, weight of evidence approach utilizing guidance from the Codex Alimentarius Commission. Bioinformatic analyses of the GM-HRA amino acid sequence revealed no biologically significant identities to known or putative protein allergens or

[1] TREUS™ is a trademark of Pioneer Hi-Bred International, Inc.

toxins. The GM-HRA protein was non-glycosylated and was shown to hydrolyze rapidly (within 30 seconds) in both simulated gastric and simulated intestinal fluids. There was no evidence of acute toxicity in mice at a dose of 2000 mg purified protein preparation per kg of body weight (equivalent to approximately 582 mg of full-length GM-HRA protein per kg of body weight). These data support the food and feed safety of the GM-HRA protein.

Molecular characterization of 305423 soybean by Southern blot analysis showed several partial and complete copies of the *gm-fad2-1* cassette (in total, eight copies of the KTi3 promoter, seven copies of the *gm-fad2-1* fragment and five copies of the KTi3 terminator) and a single copy of the intact *gm-hra* cassette were inserted into 305423 soybean genome at a single genetic locus. Multiple copies of the *gm-fad2-1* fragment appear to be necessary for effective co-suppression of the endogenous gene. Southern blot analysis confirmed the absence of all functional elements from the plasmid backbone in 305423 soybean (the hygromycin resistance gene and the plasmid origin of replication) and the presence of a small non-functional fragment of the plasmid backbone DNA. Segregation data for three generations confirmed Mendelian inheritance of the *gm-fad2-1* and *gm-hra* genetic elements and the stability of the inserted DNA was further confirmed through three generations of breeding.

The 305423 soybean has been field tested since 2002 in the major soybean growing regions of the continental United States as well as Hawaii. All field tests have occurred under field permits granted by USDA - APHIS. Comprehensive agronomic performance and ecological observation assessments for 305423 soybean were conducted in replicated, multi-site field studies at a total of 13 North American locations over the 2005 and 2006 growing seasons. Characteristics such as emergence, seedling vigor, plant height, lodging, days to maturity, shattering, seed weight, yield, disease incidence and insect damage were measured. Seed germination and dormancy data were also collected in laboratory experiments. All field trials of 305423 soybean were observed for opportunistic disease or insect biotic stressors and for normal phenotypic characteristics. Analysis of agronomic and ecological data showed no biologically meaningful differences between 305423 soybean and control soybean lines, indicating no plant pest characteristics for 305423 soybean. These data support a conclusion of agronomic and phenotypic comparability of 305423 soybean to conventional soybean varieties with respect to the lack of increased weediness or plant pest potential.

Because of the agronomic similarity of 305423 soybean to conventional soybean and the demonstrated safety of the GM-HRA protein, there is no significant impact expected on raw or processed agricultural commodities (other than the intended fatty acid changes in identity-preserved oil products), on non-target, beneficial organisms (including threatened and endangered species), or on biodiversity. Impacts on organic or conventional farming are also expected to be minimal, as growers' decisions to plant biotechnology-derived, organic or conventional soybean are driven largely by market dynamics. Market dynamics, grower choice, and existing soybean production practices will not change due to the availability of 305423 soybean other than offering growers another value-added specialty soybean option.

Extensive nutrient composition analysis of forage and grain was conducted to compare the composition of 305423 soybean to that of a non-transgenic near isoline and four conventional soybean varieties. In total, data from 52 different analytical components (52 in grain and five of those in forage) are presented. Compositional analysis of 305423 soybean was used to evaluate any changes in the levels of key nutrients, isoflavones, or antinutrients. Along with the agronomic data included in this petition, the compositional analysis shows that 305423 soybean does not exhibit unexpected or unintended effects with respect to plant pest risk.

Based on the compositional evaluation, the grain and forage of 305423 soybean were comparable to conventional soybean except for the intended changes in fatty acid composition of the grain due the introduction of the *gm-fad2-1* gene fragment. The content of oleic acid was increased while the content of the linoleic acid, linolenic acid, and to a lesser extent, palmitic acid was decreased. A trace amount (0.3% of the total fatty acids) of the 9,15-linoleic acid isomer is

also present in 305423 soybean grain; however this compound is also found in many edible food sources at concentrations up to 5.4% of the total fatty acids. An increase in two minor fatty acids, heptadecanoic acid and heptadecenoic acid, was detected, but this is not unexpected as expression of the GM-HRA protein likely results in a slight shift in availability of the GM-HRA enzyme substrates, pyruvate and 2-ketobutyrate. These two compounds are also substrates for the enzyme complex that initiates oil biosynthesis. Levels of heptadecanoic and heptadecenoic acids are very low in 305423 soybean (0.8% and 1.2% of the total fatty acids, respectively). In addition to also being present in conventional soybean varieties, these two fatty acids are components of commonly consumed foods such as olive oil, beef, tofu and other foods.

Based on the food and feed safety assessment of 305423 soybean, we conclude there will be no significant adverse effects to animal or human health. A detailed assessment of the food and feed safety and nutritional value of 305423 soybean has been submitted to FDA.

Information presented herein demonstrates that 305423 soybean exhibits no plant pathogenic properties and is no more likely to become a weed of agriculture or be invasive of natural habitats than conventional soybean varieties. The GM-HRA protein does not exhibit properties of toxins or allergens and is therefore unlikely to have an impact on threatened or endangered species, beneficial organisms, animals or humans. Agronomic practices for 305423 soybean will be unchanged from those for existing soybean varieties.

Therefore, Pioneer requests that APHIS grant the request for a determination of nonregulated status for 305423 soybean and any crosses of this line with nonregulated soybean lines.

Abbreviations, Acronyms and Definitions

~	approximately
305423 soybean	soybean lines containing event DP-3Ø5423-1
ADF	acid detergent fiber
AHAS	acetohydroxyacid synthase
ALS	acetolactate synthase protein
als	acetolactate synthase gene
APHIS	Animal and Plant Health Inspection Service
b	base
bp	base pair
cDNA	complementary deoxyribonucleic acid
CFIA	Canadian Food Inspection Agency
Da	dalton
daf	days after flowering
dapA	dihydrodipicolinate synthase gene
DIG	digoxygenin
DNA	deoxyribonucleic acid
DP-3Ø5423-1	event DP-3Ø5423-1 conferring a high oleic phenotype to soybean lines
EAFS 3054.2.3	initial event name of DP-3Ø5423-1 used in USDA field data reports
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
E score	expectation score
EST	expressed sequence tag
FAD	fatty acid desaturase protein
FAD	fatty acid desaturase gene
FAD2-1	soybean omega-6 desaturase gene
FAD2-2	soybean omega-6 desaturase gene
FAD3	soybean omega-3 desaturase gene
FDA	Food and Drug Administration
FDR	false discovery rate
FID	flame ionization detector
Flp	flippase recombinase
FRT	Flp recombinase recombination site
GC	gas chromatography

GM-ALS	soybean acetolactate synthase protein
gm-fad2-1	soybean *FAD2-1* gene fragment
gm-hra	modified version of soybean acetolactate synthase gene
GM-HRA	modified version of soybean acetolactate synthase protein
HRP	horseradish peroxidase
hyg	hygromycin
IgG	immunoglobulin G
ILSI	International Life Sciences Institute
kb	kilobase pair (for DNA), or kilobase (for RNA)
kDa	kilodalton
KTi1, KTi2	Kunitz trypsin inhibitor 1 and 2 genes
KTi3	Kunitz trypsin inhibitor 3, gene or protein
LDL	low-density lipoprotein, also known as the "bad" cholesterol
LLOQ	lower limit of quantitation
MALDI-MS	matrix assisted laser desorption ionization mass spectrometry
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
NDF	neutral detergent fiber
OD	optical density
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
Ori (plasmid ori)	bacterial origin of replication
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing 0.05% (volume/volume) Tween 20
PCR	polymerase chain reaction
ppm	parts per million
P-value	probability value
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
SAMS	S-adenosyl-L-methionine synthetase
SAS	statistical analysis software
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSC	sodium chloride – sodium citratein buffer
sp.	species
TE	tris-EDTA buffer
Tris	tris(hydroxymethyl)aminomethane

USDA-APHIS	United States Department of Agriculture – Animal and Plant Health Inspection Service
USDA-NASS	United States Department of Agriculture – National Agricultural Statistics Service
UTR	untranslated region

Note: Abbreviations of units of measurement and of physical and chemical quantities are presented according to the standard format described in Instructions to Authors in the Journal of Biological Chemistry (http://www.jbc.org/).

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I. Rationale for the Development of 305423 Soybean

I-A. Basis for the Request for a Determination of Nonregulated Status under 7 CFR Part 340.6

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. 7701-7772) and the Plant Quarantine Act (7 U.S.C. 151-167), to prevent the introduction or dissemination of plant pests into or within the United States. Part 340 regulates introduction of organisms altered or produced through genetic engineering which are plant pests or which there is a reason to believe are plant pests. The APHIS regulations at 7 CFR §430.6 provide that an applicant may petition APHIS to evaluate submitted data on the genetically engineered crop to determine that a regulated article does not present a plant pest risk and therefore should no longer be regulated.

Pioneer Hi-Bred International, Inc. is submitting data for genetically engineered high oleic 305423 soybean and requests a determination from APHIS that event DP-3Ø5423-1 and crosses of this event with nonregulated soybean lines no longer be considered regulated articles under 7 CFR §340.

APHIS has previously granted nonregulated status to three soybean sub-lines (G94-1, G94-19, and G168) derived from a single transformation event 260-05 where the high oleic phenotype was conferred by introduction of the *FAD2-1* gene (Federal Register Docket No. 96-098-2 (1997)). Development of these soybean sub-lines demonstrated that altering the expression of the *FAD2-1* gene is a successful approach to optimize fatty acid profile of soybean oil.

I-B. Benefits of 305423 Soybean

The commercialization of high oleic 305423 soybean is expected to have a beneficial impact on the food and industrial oil sectors as it will provide them with soybean seeds with increased levels of monounsaturated (oleic) fatty acid and decreased levels of polyunsaturated fatty acids (linoleic and linolenic acid).

Modification of the fatty acid composition of oilseeds is aimed at improving the functional performance of vegetable oils and has been one of the major goals of many plant breeders over the years. Soybean oil has poor oxidative stability due to naturally occurring levels of polyunsaturated fatty acids that limit its use. Hydrogenation is a chemical process that is used to stabilize oils to oxidation by reducing the polyunsaturated fat content. Hydrogenation of soybean oil by processing facilities has undesirable consequences including the formation of *trans* fatty acid isomers and a characteristic “hydrogenated flavor” (Fernandez San Juan, 1995). Partially hydrogenated oils are used by food processors because they extend the shelf life and give foods desirable taste and texture. However, *trans* fatty acids have come under considerable attack because of the negative health aspects associated with their consumption. *Trans* fatty acids have been linked to heart disease and have been shown to have adverse effects on blood lipid levels by increasing total and LDL ("bad") cholesterol levels. Both the American Heart Association and the World Health Organization have recommended limiting intake of *trans* fatty acids. High oleic soybean oil derived from 305423 soybeans is an alternative to partially hydrogenated oils as it is naturally stable to oxidation as a result of its’ reduced polyunsaturated fat content. This is of considerable importance to the food industry and adds a significant choice for the consumer. Pioneer brand high oleic soybean varieties are intended to be used for the production of TREUS™[1] brand high oleic soybean oil, in an identity preserved production system.

[1] TREUS™ is a trademark of Pioneer Hi-Bred International, Inc.

Food service and food processors will benefit from the availability of a highly stable vegetable oil suitable for frying applications without the need for hydrogenation. Specifically, potential food market applications for high oleic soybean include spraying oils where oxidative stability is required and frying oils for snack foods where heat stability is of utmost importance. High oleic soybean oil is also ideal for use as a base oil in blending applications where it can address nutritional, taste, stability and other attributes desired for specific products. In addition, naturally oxidatively stable oil provides for less rancidity and longer shelf life for the oil itself and for the end-use products.

In the industrial sector, high oleic soybean oil will offer an ingredient that is stable to oxidation for the formulation of cost effective, renewable, environmentally friendly industrial fluids. A valuable application for high oleic soybean oil is its use as a lubricant for fragile environments (*e.g.*, marine or forest) where an oxidatively, heat stable and biodegradable oil is required.

The availability of high oleic 305423 soybean with increased levels of monounsaturated (oleic) acid and decreased levels of polyunsaturated acids (linoleic acid and linolenic acid) in soybean seed will therefore be beneficial for the food and industrial oil sectors.

I-C. Submissions to Other Regulatory Agencies

A safety and nutritional assessment for feed and food derived from 305423 soybean has been submitted to FDA in December, 2006. Submissions to Health Canada and to the Canadian Food Inspection Agency (CFIA) for food, feed and environmental approvals were made in March 2007. Throughout 2007, the necessary submissions for import approval in key international markets are planned.

II. The Biology of Soybean

Refer to the OECD Consensus Document on the Biology of *Glycine max* (L.) Merr. (Soybean), 2000, for information pertaining to the following aspects of soybean biology:

- general description, including taxonomy and morphology and use as a crop plant;
- agronomic practices;
- centers of origin;
- reproductive biology;
- cultivated *Glycine max* as a volunteer weed;
- ability to cross inter-species/genus, introgression into relatives, and interactions with other organisms;
- summary of the ecology of *Glycine max*.

Characterization of the Recipient Soybean Variety

A publicly available variety called Jack was used as the recipient line for generation of 305423 soybean. This variety was originally developed at the Illinois Agricultural Experimental Station and commercially released in 1989 (Nickell *et al*., 1990). Jack is classified as maturity group II and is best adapted to approximately 40 to 42 degrees of Northern latitude. It has white flowers, gray pubescence, brown pods at maturity, and seeds with dull yellow coat and yellow hila. Jack was developed and released because of its resistance to soybean cyst nematode (Races 3 and 4) and higher yield when compared with varieties of similar maturity. It is susceptible to phytophthora rot (Races 1, 4, and 7).

Jack is extensively used in soybean transformation because of its high embryogenic capacity (Stewart *et al*., 1996; Santarem *et al*., 1998; Yan *et al*., 2000). Somatic embryos can be induced from immature cotyledons, proliferated, and maintained in liquid medium until transformation.

III. Method of Development of 305423 Soybean

III-A. Description of the Transformation System

Two linear DNA fragments PHP19340A (2924 bp, containing the *gm-fad2-1* cassette) conferring the high oleic phenotype and PHP17752A (4512 bp, containing the *gm-hra* gene cassette) conferring the herbicide tolerant phenotype were used for transformation to generate 305423 soybean. Refer to Section IV for the detailed description of the DNA fragments. The fragments were obtained from the respective plasmids, PHP19340 and PHP17752, by digestion with *Asc* I restriction enzyme and purification using agarose gel electrophoresis.

The transgenic 305423 soybean was generated by biolistics-mediated transformation using the Biolistics PDS-1000/He particle gun manufactured by Bio-Rad (Hercules, CA) essentially as described by Klein *et al*. (1987). The targets for transformation were clumps of secondary somatic embryos derived from explants from small, immature soybean seeds of variety Jack. The secondary somatic embryos were excised from immature explants, transferred to a liquid soybean culture maintenance medium, and subcultured at regular intervals until prepared for bombardment.

Soybean somatic embryogenic cultures were used in transformation experiments from two to four months after initiation. On the day of transformation, microscopic gold particles were coated with a mixture of DNA of the two purified fragments, PHP19340A and PHP17752A, and accelerated into the embryogenic soybean cultures. Only the PHP19340A and PHP17752A linear DNA fragments were used, and no additional DNA (*e.g.,* carrier DNA) was incorporated into the transformation process.

Following transformation, the soybean tissue was transferred to flasks of fresh liquid culture maintenance medium for recovery. After seven days the liquid culture medium was changed to culture maintenance medium supplemented with chlorsulfuron as the selection agent. Chlorsulfuron belongs to a family of ALS-inhibiting herbicides, and therefore only soybean cells that had stably inherited the *gm-hra* transgene continued to grow.

After several weeks in the culture maintenance medium supplemented with chlorsulfuron, small islands of healthy, chlorsulfuron-tolerant green tissue became visible and started to grow out of pieces of dying somatic embryogenic tissue. Green embryogenic clumps were excised from associated pieces of dying or dead tissue and received regular changes of fresh liquid selection medium until the start of the regeneration process. Embryogenic tissue samples were analyzed to confirm the presence of the *gm-fad2-1* and *gm-hra* transgenes by Southern blot hybridization. Plants were regenerated and transferred to the greenhouse for seed production. Overall schematic of the development of 305423 soybean is presented in Figure 1. A diagram of the breeding process that followed the development of 305423 soybean and the genotypes of 305423 soybean and control soybean plants selected for various assessments presented further in this petition are shown in Figure 2.

The 305423 soybean is defined as the line containing event DP-3Ø5423-1. This event will be commercialized in many Pioneer soybean varieties. In the early breeding process the recipient line Jack was both selfed to increase the seed and then crossed to elite lines to start commercial product development. The event designation remains the same regardless of the germplasm or variety that the event is bred into.

Two Pioneer elite lines were used for breeding 305423 soybean: PHSB02 (also referred to as Elite 1) and PHSB01 (also referred to as Elite 2). Both elite lines are mid-maturity (group II) soybean varieties.

III-B. Selection of Comparators for 305423 Soybean

To ensure an accurate assessment of the impact of transgene insertion on various characteristics of 305423 soybean, a proper selection of comparators is important. Two types of soybean lines, control soybean and reference soybean, were used as comparators for 305423 soybean.

The control soybean lines should have a genetic background similar to that of 305423 soybean but lack the transgenic insert. For the majority of the assessments, the null segregant plants of the same generation as 305423 soybean plants from that same experiment were used as controls (Figure 2). The corresponding null segregant plants of 305423 soybean is an appropriate control for the generations obtained by/after crossing to an Elite line (Figure 2), because genetically they are almost identical to corresponding 305423 soybean plants except they do not carry the inserted transgenic DNA.

For Southern and Northern blot analyses, the soybean variety Jack was used as the control. Jack is the original transgene recipient variety that has undergone transformation with DNA fragments PHP19340A and PHP17752A to generate 305423 soybean (refer to Section III-A). T0 plants of 305423 soybean and all subsequent selfed generations derived are genetically almost identical to Jack soybean with the exception of the transgenic insert DNA. In addition to Jack control soybean, a Pioneer Elite variety was employed for the Southern analysis as another non-transgenic control for the F2 generation. Elite was one of the elite varieties used in breeding crosses to generate F2 line.

For the nutrient composition assessment and field agronomic characteristics in Experiment B, four commercial soybean reference varieties were also included as the comparators, along with the control null segregant plants of BC1F5 (Figure 2). Data from the reference varieties was used to help determine the normal variation seen in soybean and to develop the statistical tolerance intervals.

Figure 1. Schematic of the Development of 305423 Soybean

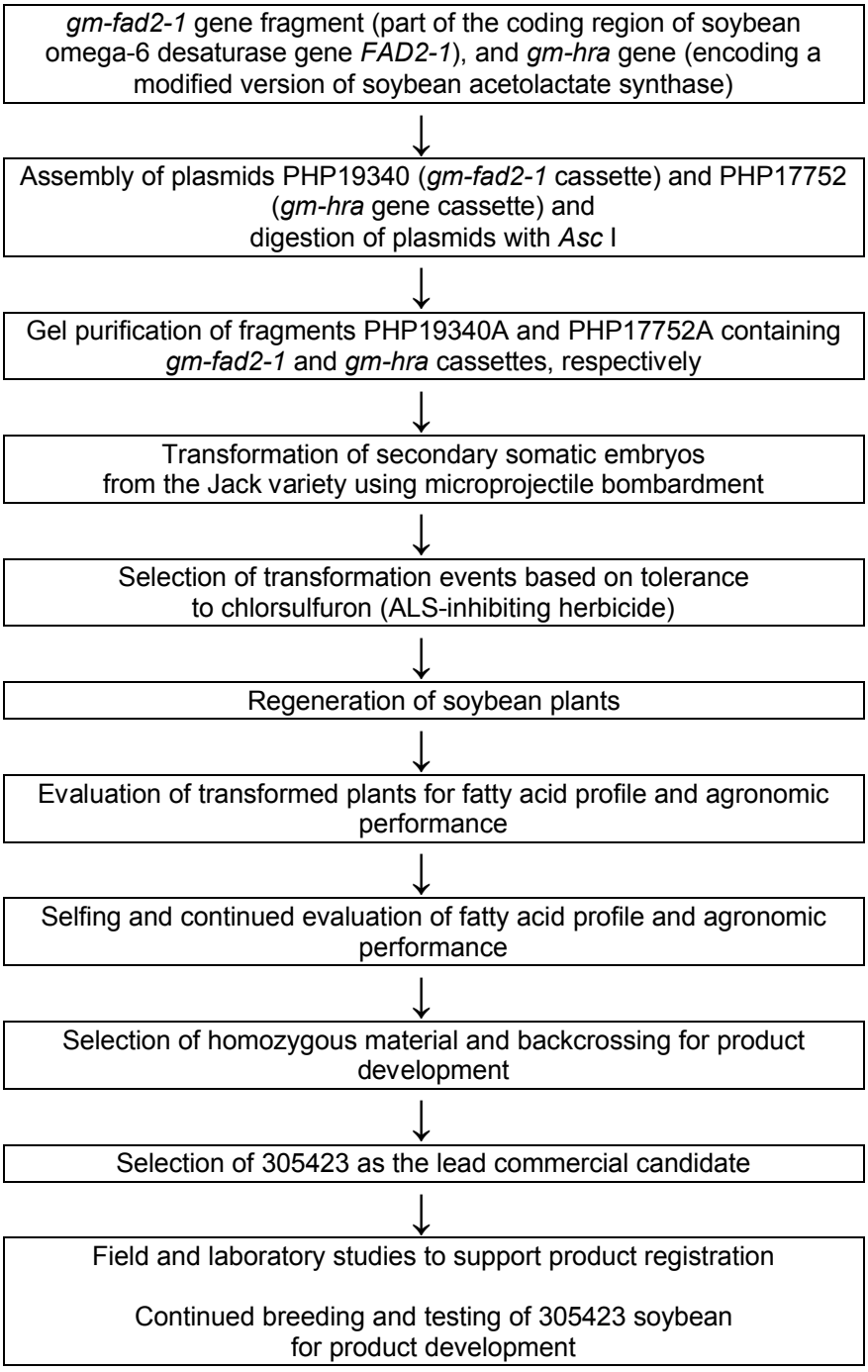
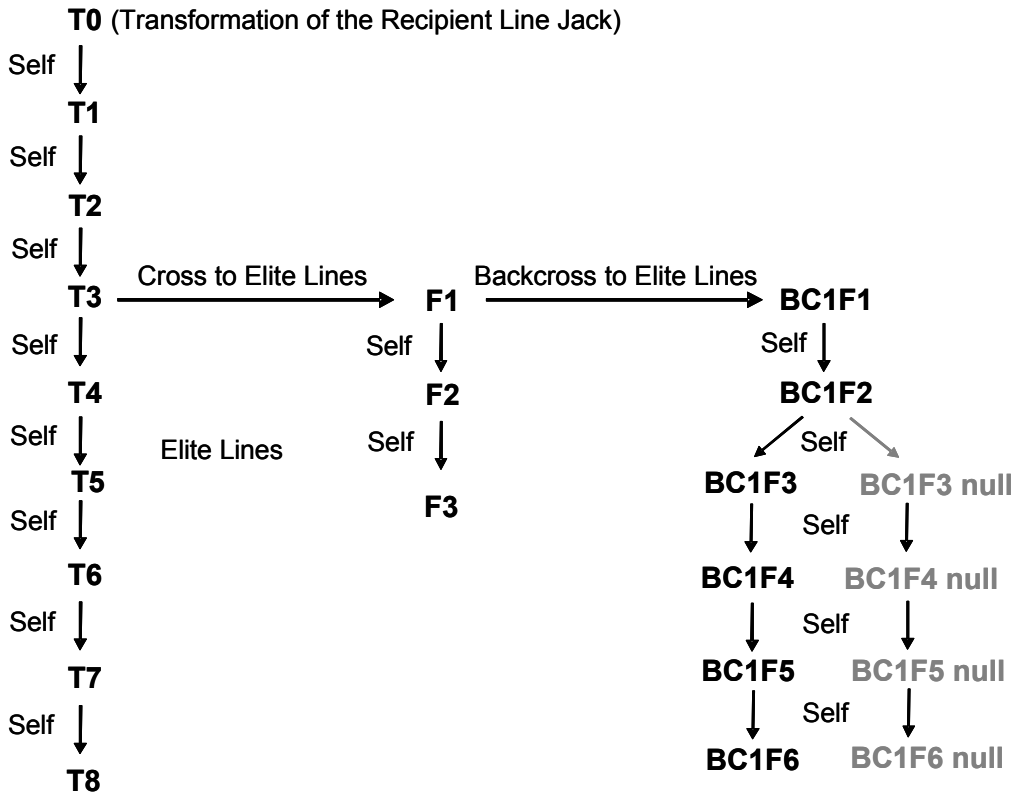


Figure 2. Breeding Diagram for 305423 Soybean and Generations Used for Analyses



Analysis	Relevant Section of petition	305423 soybean	Control/reference soybean
Southern analysis	V-A – V-E	T4, T5, F2	Jack and Elite line
Northern analysis	VI-B, VI-C	T4	Jack
Trait inheritance	V-F	F2	Not applicable
		F3	
		BC1F2	
GM-HRA protein concentration	VII-C	BC1F5	BC1F5 null
Seed germination / dormancy	VIII-A	BC1F6	BC1F6 null
Field agronomic characteristics	VIII-B	T7	Jack
		BC1F5	BC1F5 null and 4 commercial varieties
		T8	Jack
Compositional assessment	IX	BC1F5	BC1F5 null and 4 commercial varieties

IV. Donor Genes and Regulatory Sequences

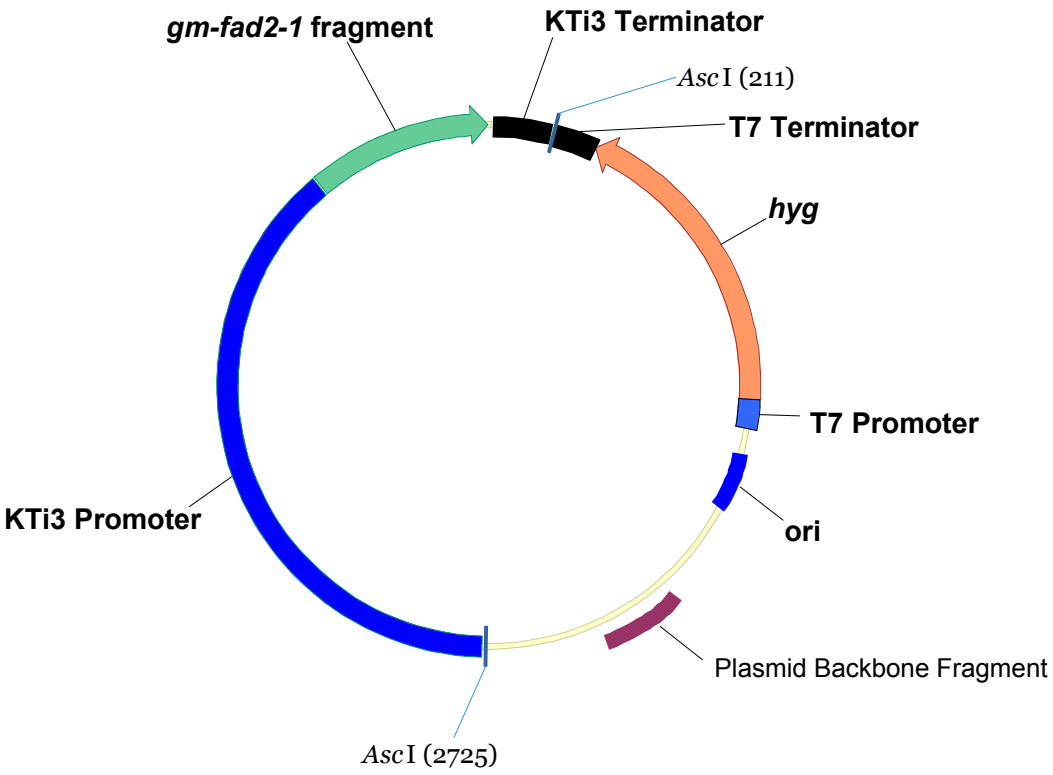
The 305423 soybean was produced by biolistic co-transformation with two linear DNA fragments, PHP19340A and PHP17752A, derived from plasmids PHP19340 and PHP17752, respectively. Plasmid PHP19340 and fragment PHP19340A contain the *gm-fad2-1* cassette (Section IV-A). Plasmid PHP17752 and fragment PHP17752A contain the *gm-hra* gene cassette (Section IV-B). A summary of the elements and their position on source plasmids PHP19340 and PHP17752 is given in Tables 1 and 2, respectively.

IV-A. DNA Fragment PHP19340A Used in Transformation

The map of plasmid PHP19340 is presented in Figure 3 and the genetic elements comprising this plasmid are listed in Table 1. The linear fragment PHP19340A used in co-transformation was obtained by *Asc* I digestion of plasmid PHP19340. The map of fragment PHP19340A is presented in Figure 4, and the genetic elements comprising this fragment are listed in Table 1.

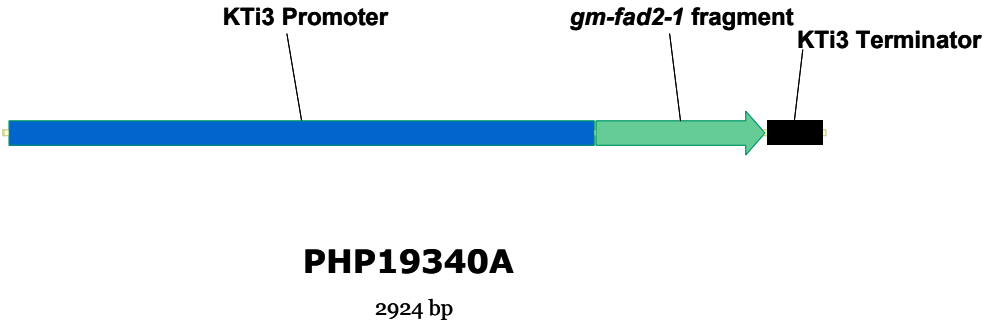
The 2924 bp fragment PHP19340A contains the *gm-fad2-1* cassette with a 597 bp fragment of the soybean microsomal omega-6 desaturase gene 1 (*FAD2-1*) corresponding to about 40% of the middle region of the open reading frame of the *FAD2-1* gene (Heppard *et al.,* 1996). Precisely, the *gm-fad2-1* gene fragment corresponds to base pairs 399 to 995 of the *FAD2-1* gene (GenBank accession number L43920). This region from *FAD2-1* displays 71.5% identity to base pairs 388-984 of the *FAD2-2* gene and 48% identity to base pairs 1143-1748 of the *FAD3* gene (GenBank accession numbers L43921 and L22964 respectively). The presence of the *gm-fad2-1* fragment (in the sense orientation) in the cassette acts to silence expression of soybean endogenous omega-6 desaturases as described in Section VI, resulting in an increased level of oleic acid and decreased levels of linoleic and linolenic acids. Upstream of the *gm-fad2-1* gene fragment is the promoter region from the Kunitz trypsin inhibitor gene 3 (KTi3) (Jofuku and Goldberg, 1989; Jofuku *et al.,* 1989) controlling transcription of the fragment. The KTi3 promoter is highly active in soybean embryos and 1000-fold less active in leaf tissue (Jofuku and Goldberg, 1989). The 3' untranslated region of the KTi3 gene (KTi3 terminator) (Jofuku and Goldberg, 1989; Jofuku *et al.,* 1989) terminates transcription from this cassette.

Figure 3. Map of Plasmid PHP19340



Genes and regulatory elements are indicated. Plasmid size is 5438 base pairs. Fragment PHP19340A (Figure 4) was isolated from this plasmid by an *Asc* I digestion. Restriction enzyme sites for *Asc* I are indicated at base pair positions 211 and 2725.
Plasmid backbone fragment indicates the position of the 495 bp non-functional fragment on plasmid PHP19340 (base pairs 2033-2527) (see Table 1).

Figure 4. Map of Fragment PHP19340A



Schematic representation of linear fragment PHP19340A used in co-transformation to generate 305423 soybean.

Table 1. Description of Genetic Elements in Fragment PHP19340A and Plasmid PHP19340

Genetic elements inserted into 305423 soybean are shown in bold.

Region	Location on plasmid (base pair position)	Location on DNA fragment (base pair position)	Genetic element	Size (base pairs)	Description
PHP19340A fragment	2725 to 5438 1 to 210	1 to 2924	Includes elements listed below	2924	DNA fragment used for transformation
	2743 to 4826	**19 to 2102**	**KTi3 promoter**	**2084**	**Promoter region from the soybean Kunitz trypsin inhibitor gene 3 (Jofuku and Goldberg, 1989; Jofuku *et al.*, 1989)**
	4838 to 5434	**2114 to 2710**	***gm-fad2-1* fragment**	**597**	**Fragment of the soybean microsomal omega-6 desaturase gene *FAD2-1* (Heppard *et al.*, 1996)**
	7 to 202	**2721 to 2916**	**KTi3 terminator**	**196**	**Terminator region from the soybean Kunitz trypsin inhibitor gene 3 (Jofuku and Goldberg, 1989; Jofuku *et al.*, 1989)**
Vector backbone[1]	211 to 2724	NA[2]	Includes elements listed below	2514	DNA from various sources for plasmid construction and replication
	228 to 351	NA	T7 terminator	124	Terminator derived from the Enterobacteria phage T7 genome (GenBank V01146; Dunn and Studier, 1983)
	376 to 1401	NA	*hyg*	1026	Hygromycin resistance gene (hygromycin B phosphotransferase) from *Escherichia coli* (GenBank K01193; Gritz and Davies, 1983))
	1404 to 1487	NA	T7 promoter	84	Promoter derived from the Enterobacteria phage T7 genome (GenBank V01146; Dunn and Studier, 1983)
	1561 to 1930	NA	ori	370	*Hae* II fragment containing bacterial origin of replication (colE1 derived) (Tomizawa *et al.*, 1977)

[1] A small non-functional fragment of 495 bp from the vector backbone is present in 305423 soybean. Based on sequence data this non-functional fragment was determined to originate from base pair positions 2033 to 2527 or 1836 to 2330 on plasmids PHP19340 or PHP17752, respectively (indicated on Figures 3 and 5).
[2] Not applicable

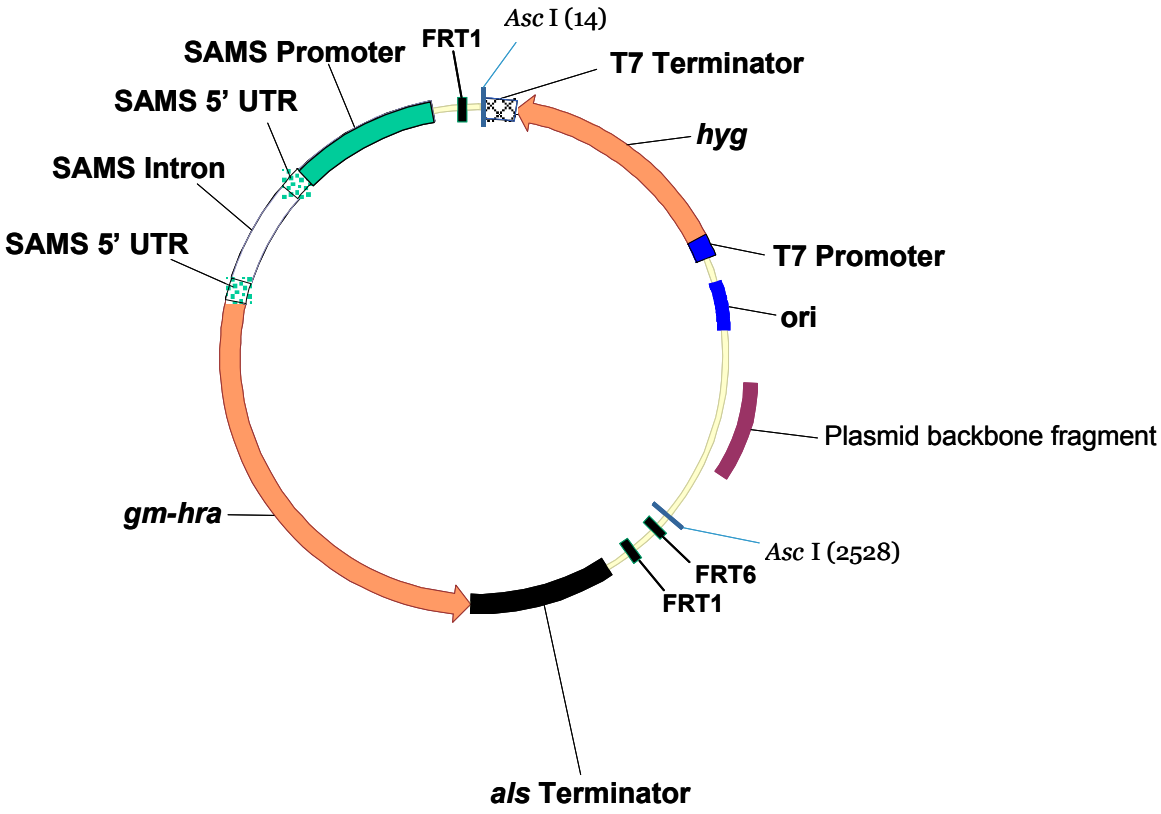
IV-B. DNA Fragment PHP17752A Used in Transformation

The map of plasmid PHP17752 is presented in Figure 5 and the genetic elements comprising this plasmid are listed in Table 2. The linear fragment PHP17752A used in co-transformation was released from plasmid PHP17752 by digestion with *Asc* I restriction enzyme. The map of fragment PHP17752A is presented in Figure 6, and the genetic elements comprising this fragment are listed in Table 2.

The 4512 bp fragment PHP17752A contains a *gm-hra* gene cassette with a modified version of the soybean acetolactate synthase gene (*gm-hra*). The *gm-hra* gene encodes the GM-HRA protein with two amino acid residues modified from the endogenous enzyme, and five additional amino acids at the N-terminal region of the protein derived from translation of the 15 nucleotides from the 5' UTR region of the soybean *als* gene (Falco and Li, 2003). The *gm-hra* gene encodes a form of acetolactate synthase that is tolerant to ALS inhibiting herbicides. The GM-HRA protein is comprised of 656 amino acids and has a molecular weight of approximately 71 kDa (refer to Section VII-B. Figure 22). The *gm-hra* gene is only used as a selectable marker gene in 305423 soybean and does not confer commercial levels of herbicide tolerance in this event.

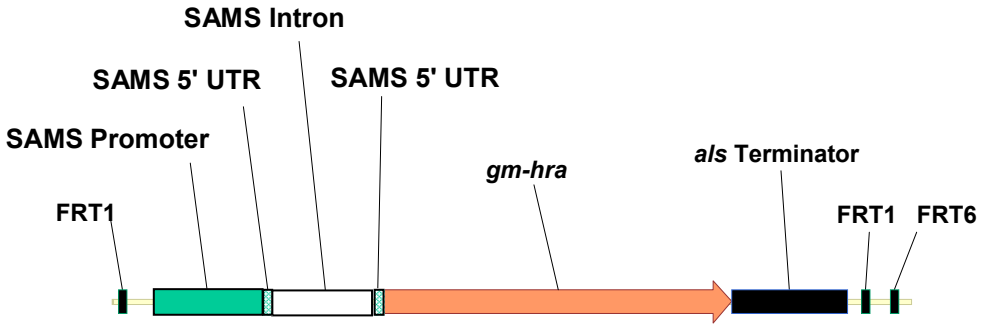
The expression of the *gm-hra* gene is controlled by the 5' regulatory region of the S-adenosyl-L-methionine synthetase (SAMS) gene from soybean (Falco and Li, 2003). This 5' region consists of a constitutive SAMS promoter and an intron that interrupts the SAMS 5' untranslated region (Falco and Li, 2003). The terminator for the *gm-hra* gene is the endogenous soybean acetolactate synthase terminator (*als* terminator) (Falco and Li, 2003). Fragment PHP17752A also contains three Flp recombinase target sequences (two FRT1 and one FRT6 sites); however they were not used in the development of 305423 soybean. Presence of these sites does not cause any recombination. In order to function, these sites need a specific Flp recombinase enzyme that is not present in plants (Cox, 1988).

Figure 5. Map of Plasmid PHP17752



Genes and regulatory elements are indicated. Plasmid size is 7026 base pairs. Fragment PHP17752A (Figure 6) was isolated from this plasmid by an *Asc* I digestion. Restriction enzyme sites for *Asc* I are indicated at base pair positions 14 and 2528.
Plasmid backbone fragment indicates the position of the 495 bp non-functional fragment on plasmid PHP17752 (base pairs 1836-2330) (see Table 2).

Figure 6. Map of Fragment PHP17752A



PHP17752A

4512 bp

Schematic representation of linear fragment PHP17752A used in co-transformation to generate 305423 soybean.

Table 2. Description of Genetic Elements in Fragment PHP17752A and Plasmid PHP17752

Genetic elements inserted into 305423 soybean are shown in bold.

Region	Location on plasmid (base pair position)	Location on DNA fragment (base pair position)	Genetic Element	Size (base pairs)	Description
PHP17752A fragment	2528 to 7026 1 to 13	1 to 4512	Includes elements listed below	4512	DNA fragment used for transformation (in reverse orientation on plasmid map)
	6968 to 7018	**26 to 76**	**FRT1**	**51**	**Flp recombinase recombination site (GenBank accession numbers: J01347; AY737006.1) from *Saccharomyces cerevisiae* (Proteau *et al.*,1986)**
	6177 to 6821	**223 to 867**	**SAMS promoter**	**645**	**Promoter portion of the regulatory region of the soybean S-adenosyl-L-methionine synthetase (SAMS) gene (Falco and Li, 2003)**
	6118 to 6176	**868 to 926**	**SAMS 5' UTR**	**59**	**5' untranslated region of the soybean SAMS gene (Falco and Li, 2003)**
	5527 to 6117	**927 to 1517**	**SAMS intron**	**591**	**Intron within the 5' UTR of the soybean SAMS gene (Falco and Li, 2003)**
	5511 to 5526	**1518 to 1533**	**SAMS 5' UTR**	**16**	**5' untranslated region of the soybean SAMS gene (Falco and Li, 2003)**
	3540 to 5510	**1534 to 3504**	***gm-hra* gene**	**1971**	**Modified version of the acetolactate synthase (*als*) gene from soybean with 15 additional nucleotides on the 5' end (1534 to 1548) derived from the *als* 5' UTR and two nucleotide changes within the coding sequence (Falco and Li, 2003)**
	2888 to 3539	**3505 to 4156**	***als* terminator**	**652**	**Terminator from the soybean acetolactate synthase gene (Falco and Li, 2003)**
	2762 to 2812	**4232 to 4282**	**FRT1**	**51**	**Flp recombinase recombination site (GenBank accession numbers: J01347; AY737006.1) from *Saccharomyces cerevisiae* (Proteau *et al.*,1986)**
	2597 to 2647	**4397 to 4447**	**FRT6**	**51**	**Modified Flp recombinase recombination site (94% homology to GenBank accession numbers: J01347; AY737006.1) from *Saccharomyces cerevisiae* (Baszczynski *et al.*, 2001)**

Vector backbone[1]	14 to 2527	NA[2]	Includes elements listed below	2514	DNA from various sources for plasmid construction and replication
	31 to 154	NA	T7 terminator	124	Terminator derived from the Enterobacteria phage T7 genome (GenBank V01146; Dunn and Studier, 1983)
	179 to 1204	NA	*hyg*	1026	Hygromycin resistance gene (hygromycin B phosphotransferase) from *Escherichia coli* (GenBank K01193; Gritz and Davies, 1983)*)*
	1207 to 1290	NA	T7 promoter	84	Promoter derived from the Enterobacteria phage T7 genome (GenBank V01146; Dunn and Studier, 1983)
	1364 to 1733	NA	ori	370	*Hae* II fragment containing bacterial origin of replication (colE1 derived) (Tomizawa *et al.*, 1977)

[1] A small non-functional fragment of 495 bp from the vector backbone is present in 305423 soybean. Based on sequence data this non-functional fragment was determined to originate from base pair positions 2033 to 2527 or 1836 to 2330 on plasmids PHP19340 or PHP17752, respectively (indicated on Figures 3 and 5).
[2] Not applicable

V. Genetic Characterization of 305423 Soybean

V-A. Molecular Analysis Overview

Molecular characterization of 305423 soybean was conducted by both Southern blot and sequence analyses of the inserted DNA. The 305423 soybean was generated via microprojectile co-bombardment with fragments PHP19340A (Figure 4) and PHP17752A (Figure 6) that were isolated from plasmids PHP19340 and PHP17752, respectively (Figures 3 and 5). Fragment PHP19340A contains the *gm-fad2-1* cassette that is comprised of the KTi3 promoter, the *gm-fad2-1* fragment, and the KTi3 terminator. Fragment PHP17752A contains the *gm-hra* cassette that is comprised of the SAMS regulatory region, *gm-hra* gene, and *als* terminator. Individual plants of the T4 generation were analyzed by Southern blot to determine the copy number of each of the genetic elements and to examine the integrity of the PHP19340A and PHP17752A fragments inserted into the 305423 soybean genome (Section V-B; Appendix 2-1); results were further confirmed by sequence data (Section V-B; Appendix 2-3). It was determined that multiple intact and truncated copies of PHP19340A have been inserted into 305423 soybean that contain, in total, eight copies of the KTi3 promoter, seven copies of the *gm-fad2-1* fragment, and five copies of the KTi3 terminator. A single, intact copy of the *gm-hra* cassette of fragment PHP17752A has been inserted into 305423 soybean that contains the SAMS regulatory region, the *gm-hra* gene and the *als* terminator.

Both the T4 and T5 generations of 305423 soybean were analyzed for presence of the plasmid backbone sequence derived from PHP19340 or PHP17752 (Section V-C; Appendix 2-2). Southern blot analysis confirmed the absence of all functional elements from the plasmid backbones (*i.e.* the hygromycin resistance gene (*hyg*) and the plasmid origin of replication (ori)) in 305423 soybean. The analysis also determined that a small non-functional fragment of the plasmid backbone DNA was present in 305423 soybean. Sequence data confirmed that this region was identical to a non-functional 495 bp section of the plasmid backbone of either PHP19340 or PHP17752 and did not contain the plasmid origin of replication or the hygromycin resistance gene.

A physical map of the inserted DNA in 305423 soybean was generated based on sequence data and additional Southern blot analysis that confirmed the insertions (Section V-D; Appendix 2-3). It was determined that the inserted DNA of 305423 soybean is comprised of four insertions.

Southern blot analysis demonstrated that the DNA inserted into 305423 soybean remained stable during soybean breeding. All tested individual plants from two self-crossed generations, T4 and T5 (27 and 30 plants, respectively), showed identical hybridization patterns, thus verifying stability of inheritance of the inserted DNA. In addition, 100 individual plants from a segregating F2 population were analyzed by Southern blot analysis and demonstrated stability of inheritance and linkage of the inserted DNA at a single genetic locus. These analyses confirmed the stability of the inserted DNA in 305423 soybean across multiple breeding generations (Section V-E; Appendix 2-4).

The genetic characterization of 305423 soybean was further confirmed by verifying Mendelian trait inheritance. Segregation data for three generations confirmed Mendelian inheritance of the inserted DNA in 305423 soybean and the high oleic phenotype (Section V-F).

Sections V-B through V-F summarize the conclusions from the characterization of the insertion in 305423 soybean that were obtained through comprehensive Southern blot analysis and sequence data. Please refer to Figure 2 for a breeding diagram of the 305423 soybean generations used in the molecular analysis. Details on the methods used for Southern blot hybridizations are further described in Appendix 1-1, and results of the Southern blot analysis are presented in Appendix 2.

V-B. Transgene Copy Number and Insertion Integrity

The integration pattern of the inserted DNA in 305423 soybean was first investigated by Southern blot analysis using selected restriction enzyme digestions and probes homologous to each genetic element comprising the *gm-fad2-1* and *gm-hra* cassettes. These were done to determine copy number and integrity of the inserted PHP19340A and PHP17752A fragments (Figures 4 and 6). Probes homologous to the KTi3 promoter, the *gm-fad2-1* fragment, and the KTi3 terminator were used to analyze the PHP19340A integration pattern, and probes homologous to the SAMS regulatory region, the *gm-hra* gene, and the *als* terminator were used to analyze the PHP17752A integration pattern. Refer to Appendix 2-1 for a complete description of the Southern blot analysis.

Based on the Southern blot analysis, it was determined that multiple copies, both intact and truncated, of PHP19340A have been inserted into the genome of 305423 soybean comprising, in total, eight copies of the KTi3 promoter, seven copies of the *gm-fad2-1* fragment, and five copies of the KTi3 terminator. One copy of the PHP19340A fragment is intact and contains a complete KTi3 promoter, *gm-fad2-1* fragment, and KTi3 terminator. Four copies of the PHP19340A fragment have some truncation but contain portions of all three cassette elements. Two copies of the PHP19340A fragment retain only the KTi3 promoter with the *gm-fad2-1* fragment. Finally, one copy of the KTi3 promoter is associated with a small non-functional fragment of backbone DNA (Sections V-C and V-D). For the PHP17752A fragment, it was determined that a single, intact *gm-hra* cassette has been inserted into the genome of 305423 soybean.

Conclusions from the Southern blot analysis were confirmed by sequencing of the DNA inserted in 305423 soybean. Based on these data, it was determined that 305423 soybean has incorporated four insertions (hereafter referred to as Insertions 1 through 4), and a physical map of the inserted elements was generated (discussed in Section V-D). It was confirmed that all four insertions are genetically linked and segregate as a single locus (refer to Section V-E).

V-C. Plasmid Backbone DNA Analysis

Southern blot analysis with the probes covering the complete backbone DNA of plasmids PHP19340 and PHP17752 (Figures 3 and 5) was used to examine the presence of regions outside the two transformation fragments, PHP19340A and PHP17752A, in 305423 soybean. A complete description of the results of Southern analysis can be found in Appendix 2-2.

Southern blot analysis confirmed the absence of all functional elements from the plasmid backbone in 305423 soybean. The analysis confirmed the absence of both the hygromycin resistance gene (*hyg*) and the plasmid origin of replication (ori) from PHP19340 and PHP17752 (for regions see Figures 3 and 5, Tables 1 and 2).

The analysis also determined that a small non-functional fragment from the plasmid backbone DNA was present in 305423 soybean. This fragment was found to be associated with a truncated copy of the KTi3 promoter as a part of Insertion 3 in 305423 soybean (refer to Section V-D). Sequence data confirmed that this fragment was identical to a 495 bp non-functional region of the plasmid backbone of either PHP19340 or PHP17752.

V-D. Physical Map of the Inserted DNA

As discussed in Section V-B, Southern blot analysis revealed that intact and truncated copies of PHP19340A have been inserted into 305423 soybean comprising eight copies of the KTi3 promoter, seven copies of the *gm-fad2-1* fragment, and five copies of the KTi3 terminator. In addition, a single copy of the *gm-hra* cassette of PHP17752A has been integrated. It was also determined that a non-functional fragment of the plasmid backbone DNA was inserted in 305423 soybean (Section V-C).

The complete sequence of the 305423 soybean inserted DNA was obtained. Sequence data confirmed that the inserted DNA in 305423 soybean consists of four insertions. A schematic overview map of these insertions in 305423 soybean is presented in Figures 7 through 10.

- Insertion 1 contains one intact PHP19340A fragment, one intact PHP17752A fragment, and three truncated PHP19340A fragments. The order of these fragments, from 5' end to 3' end (left to right, as diagrammed in Figure 7), is as follows: one PHP19340A fragment truncated at the KTi3 terminator, one intact copy of PHP19340A, one intact copy of PHP17752A, one truncated PHP19340A fragment with an intact KTi3 promoter and a truncated *gm-fad2-1* fragment, and one truncated PHP19340A fragment with a truncated KTi3 promoter and truncated *gm-fad2-1* fragment.

- Insertion 2 contains one truncated PHP19340A fragment that includes all three elements but is truncated at the KTi3 promoter (Figure 8).

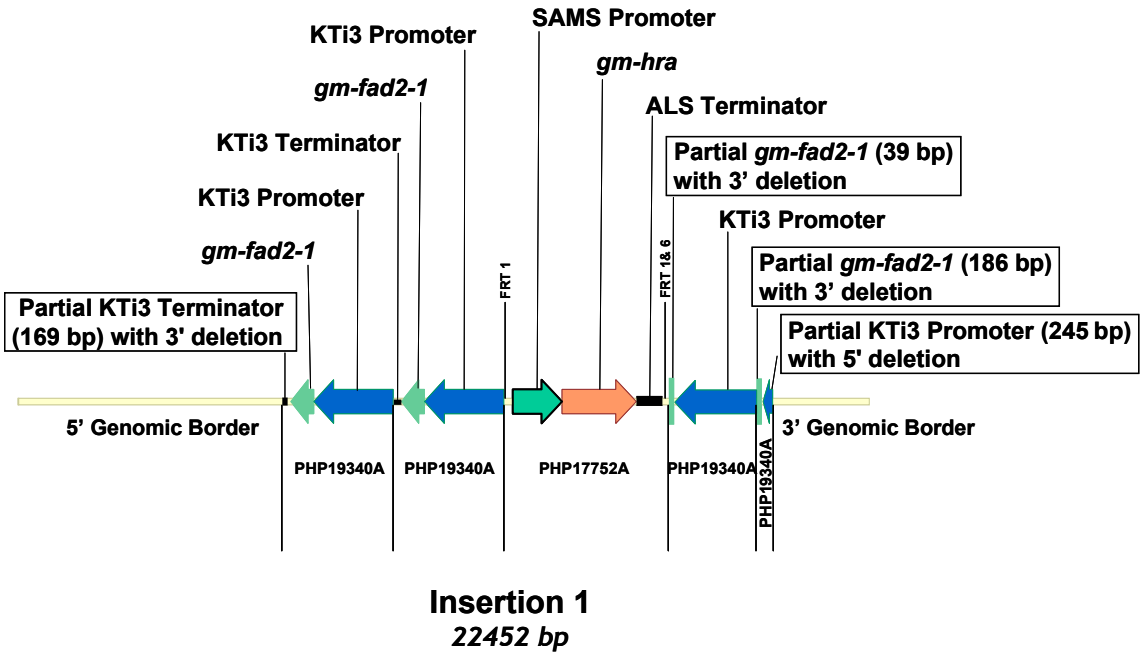
- Insertion 3 consists of one truncated copy of the KTi3 promoter with a non-functional 495 bp fragment of the plasmid backbone (Figure 9).

- Insertion 4 contains two inverted truncated copies of the PHP19340A fragment connected by the KTi3 terminators. Both copies are comprised of all three elements of the cassette but are truncated at the KTi3 promoters (Figure 10).

Additional Southern blot analysis of 305423 soybean using selected restriction enzyme digestions was conducted and is described in Appendix 2-3. These results supported the sequence analyses of 305423 soybean.

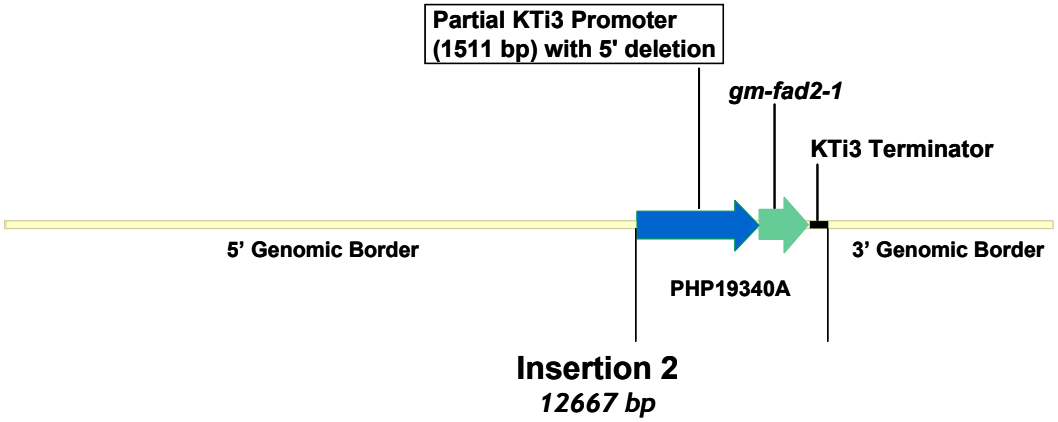
All insertions in 305423 soybean are present in a segregating out-crossed population based on Southern blot analysis (refer to Section V-E), indicating that the insertions are genetically linked and segregate in a Mendelian fashion (Section V-F).

Figure 7. Schematic Map of Insertion 1 in 305423 Soybean



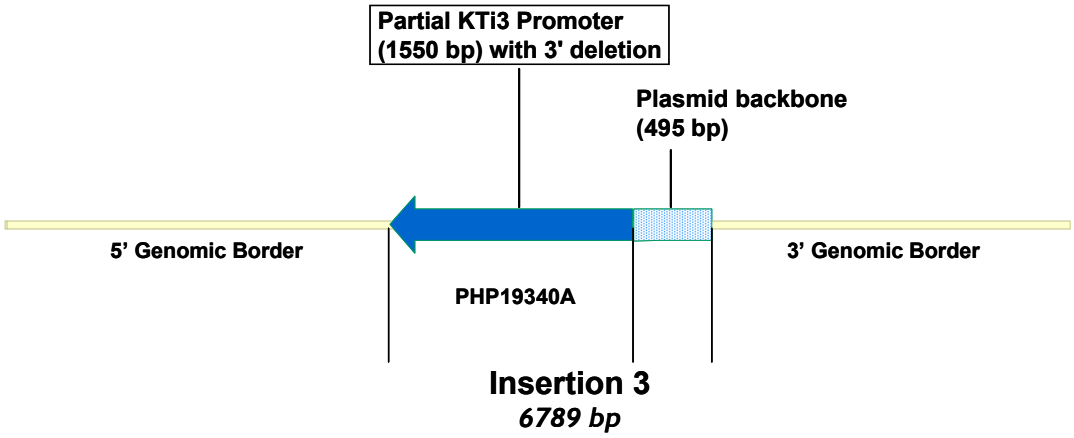
An overview of Insertion 1 in 305423 soybean. From the 5' end to 3' end (left to right), this insertion contains one PHP19340A fragment truncated at the KTi3 terminator, one intact copy of PHP19340A, one intact copy of PHP17752A, and two truncated PHP19340A fragments with only the KTi3 promoter and the *gm-fad2-1* fragment. Arrows indicate the orientation of each inserted element (arrows pointing to the right match orientation provided in the fragment maps in Figure 4 and Figure 6).

Figure 8. Schematic Map of Insertion 2 in 305423 Soybean



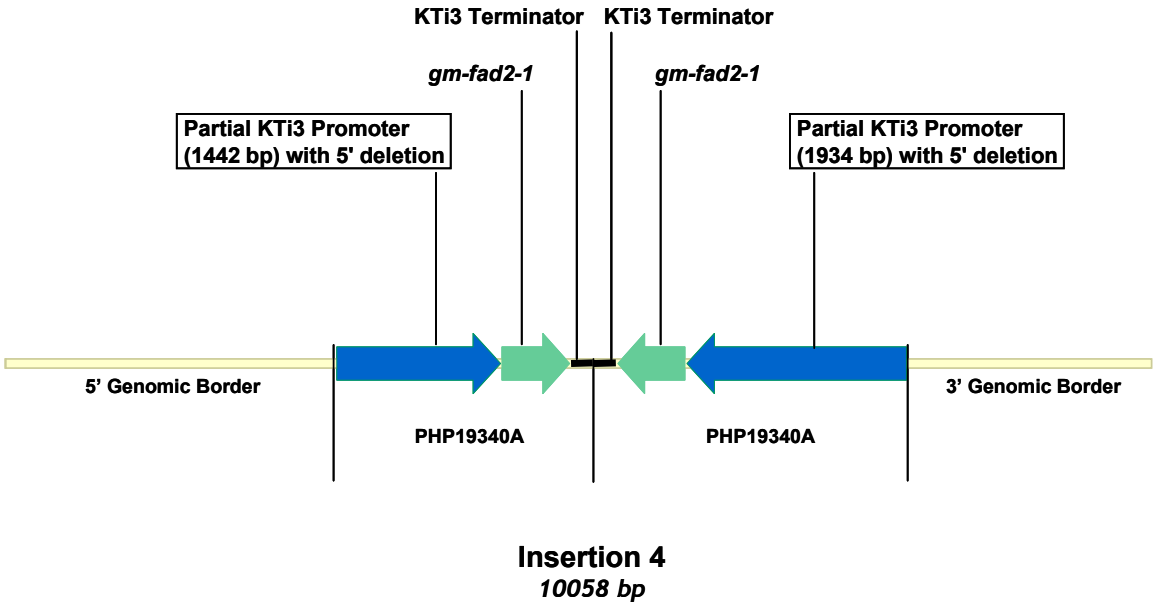
Overview of Insertion 2 in 305423 soybean. Insertion 2 contains one PHP19340A fragment truncated at the KTi3 promoter. Arrows indicate the orientation of each inserted element (arrows pointing to the right match orientation provided in the fragment maps in Figure 4 and Figure 6).

Figure 9. Schematic Map of Insertion 3 in 305423 Soybean



Overview of Insertion 3 in 305423 soybean. Insertion 3 contains a truncated copy of the KTi3 promoter with a non-functional 495 bp fragment of the plasmid backbone. Arrows indicate the orientation of each inserted element (arrows pointing to the right match orientation provided in the fragment maps in Figure 4 and Figure 6).

Figure 10. Schematic Map of Insertion 4 in 305423 Soybean



Overview of Insertion 4 in 305423 soybean. Insertion 4 contains, from 5' to 3' end (left to right), one PHP19340A fragment truncated at the KTi3 promoter and an inverted copy of the PHP19340A fragment also truncated at the KTi3 promoter. Arrows indicate the orientation of each inserted element (arrows pointing to the right match orientation provided in the fragment maps in Figure 4 and Figure 6).

Using the sequence data encompassing the 5' and 3' genomic border sequences, each of the four insertions in 305423 soybean was screened for the presence of open reading frames (ORFs) containing both start and stop codons that spanned any novel junctions. When identified, these ORFs were screened to identify any significant similarity to known or putative allergens and to ascertain any identities to potentially toxic proteins. Two junction spanning ORFs were identified from the search and were compared to a database containing known protein toxins (NCBI Protein Dataset, Release 156.0, http://www.ncbi.nlm.nih.gov) and to a database of allergens derived from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 6.0, January 2006), which contains the amino acid sequences of known and putative allergenic proteins. The *in silico* analysis of the two ORFs showed the lack of biologically significant identities to known protein toxins or allergens, indicating that there are no safety concerns with these ORFs.

V-E. Stability of the Insertion Across Generations

Southern blot analysis was conducted on a total of 157 individual plants from the three generations of 305423 soybean (T4, T5, and a segregating F2 generation; Figure 2) to confirm that the DNA inserted into 305423 soybean remained stable during soybean breeding. DNA probes homologous to the *gm-fad2-1* fragment and the *gm-hra* gene were used to examine the event-specific hybridization pattern. A detailed description of the results of the Southern analysis is provided in Appendix 2-4.

In summary, the Southern blot analysis of the T4, T5, and F2 generations showed consistent hybridization results with the *gm-fad2-1* and *gm-hra* probes and confirmed the stability of inheritance of the inserted DNA during soybean breeding. A single recombination in the F2 population of 100 individuals that removed the *gm-hra* cassette in Insertion 1 was observed. However, analysis of an additional 1000 segregating 305423 soybean F2 individuals failed to show any recombinants as determined by PCR-based assays specific for the event and for the *gm-hra* gene. The occurrence of a single recombinant individual deviating from the expected hybridization pattern for 305423 soybean was determined to be at a very low frequency and is not a concern to the stability of 305423 soybean.

These analyses show that the inserted DNA in 305423 soybean is stable across multiple breeding generations.

V-F. Inheritance of the Traits in 305423 Soybean

Chi-square analysis of trait inheritance data from three different generations (F2, F3 and BC1F2) was performed to determine the Mendelian heritability and stability of the *gm-fad2-1* gene fragment and *gm-hra* gene in various generations of 305423 soybean. The breeding history of the three generations evaluated for Mendelian inheritance is described in Figure 2. For each of the generations tested, the plants were expected to segregate 1:2:1 (homozygous positive plants:hemizygous positive plants:homozygous negative [null] plants). In the studies presented below (Table 3), homozygous positive plants were not differentiated from hemizygous positive plants, resulting in a 3:1 positive:negative segregation pattern.

Various methods were used to score the plants as positive or negative:

- a gas chromatography (GC) assay for the high oleic phenotype. GC data is an indication of the presence of the *gm-fad2-1* gene fragment. In this assay, only seed containing the *gm-fad2-1* gene fragment will show an elevated high oleic profile (*i.e.* ~ 80% of total fatty acids) while the other seed will show oleic acid levels around 20% of total fatty acids (F2, F3 and BC1F2 generations), or
- a GC assay to identify seed with the high oleic phenotype followed by confirmation of plants grown from the seed by Southern analysis with the *gm-fad2-1* probe (F2 generation), or
- an event specific PCR assay for 305423 soybean and a gene specific PCR assay for the *gm-hra* gene. Both assays identify the homozygous negative (null) plants versus plants that are positive (homozygous or hemizygous) for the *gm-fad2-1* gene fragment and *gm-hra* gene (BC1F2 generation).

The above methods were used to confirm the presence of the *gm-fad2-1* gene fragment and the *gm-hra* gene. Because the *gm-fad2-1* gene fragment and the *gm-hra* gene were co-transformed and are expected to have identical segregation ratios in the progeny of 305423 soybean, the *gm-fad2-1* results are applicable to the inheritance of *gm-hra* and to the inserted DNA.

Results from the chi-square analysis are summarized in Table 3. Details of the statistical methodology can be found in Appendix 3. All P-values were greater than 0.05, indicating no statistically significant differences between the observed and expected frequencies of the segregation ratios in three generations of 305423 soybean. In the BC1F2 generation, where both traits were analyzed in the same plants, identical segregation data experimentally confirmed co-segregation of the *gm-fad2-1* gene fragment and *gm-hra* gene.

The results of this analysis are consistent with the finding of a single, genetically-linked locus of insertion that segregates in 305423 soybean progeny according to Mendel's laws of genetics. The stability of inheritance of the inserted DNA has been demonstrated in three generations of self- and cross-pollinated 305423 soybeans.

Table 3. Comparison of Observed and Expected 3:1 Segregation Ratios for 305423 Soybean

Generation	Method	Observed		Expected		Chi-square test
		Positives +/+ or +/-	Negatives -/-	Positives +/+ or +/-	Negatives -/-	P-value
F2						
Elite 1 background	GC [1]	73	27	75	25	0.729
Elite 1 background	GC followed by Southern analyses	76	24	75	25	0.908
F3						
Elite 1 background	GC	34	14	36	12	0.617
Elite 2 background		59	22	60.75	20.25	0.748
BC1F2						
Elite 1 background	Event and *gm-hra* gene specific PCR	111	33	108	36	0.630
Elite 2 background		74	24	73.5	24.5	1.000
Elite 1 background	GC	160	60	165	55	0.484
Elite 2 background		155	63	163.5	54.5	0.211

[1] Gas chromatography

V-G. Summary and Conclusions

Based on comprehensive Southern blot analysis and supporting sequence data, it was determined that multiple intact and truncated copies of fragment PHP19340A have been inserted into 305423 soybean comprising, in total, eight copies of the KTi3 promoter, seven copies of the *gm-fad2-1* fragment, and five copies of the KTi3 terminator. A single, intact copy of the *gm-hra* cassette of fragment PHP17752A has been inserted into 305423 soybean.

Complete backbone analysis confirmed the absence of all functional elements from the plasmid backbone (*i.e.* the hygromycin resistance gene and the plasmid origin of replication) in 305423 soybean. A small non-functional fragment of the plasmid backbone DNA was present in 305423 soybean but as concluded from the ORF analysis, does not represent any safety concerns.

A physical map of the insertion region in 305423 soybean was generated based on sequence data and additional Southern blot analysis. The following four insertions were determined to comprise the inserted DNA in 305423 soybean:

- Insertion 1: one intact PHP19340A, one intact PHP17752A, and three truncated PHP19340A fragments (one with all three elements and two with only the KTi3 promoter and the *gm-fad2-1* fragment);
- Insertion 2: one truncated PHP19340A fragment (with all three elements);
- Insertion 3: one truncated copy of the KTi3 promoter with a non-functional 495 bp fragment of the plasmid backbone;
- Insertion 4: two inverted truncated copies of the PHP19340A fragment (with all three elements) connected by the KTi3 terminators.

Sequence of the 5' and 3' genomic DNA borders of the four insertions was analyzed for ORFs spanning any novel junctions and no safety concerns were identified.

The inserted DNA in 305423 soybean remained stable during soybean breeding as confirmed by Southern blot analysis conducted on three different generations. Inheritance studies demonstrated that the inserted DNA in 305423 soybean segregated in normal Mendelian fashion. None of the P-values obtained in the studies indicated a statistically significant difference between observed and expected segregation ratios for the inserted DNA or phenotype over three different plant generations. The results are consistent with the molecular characterization data, which indicates stable integration of the inserted DNA in the genome of 305423 soybean.

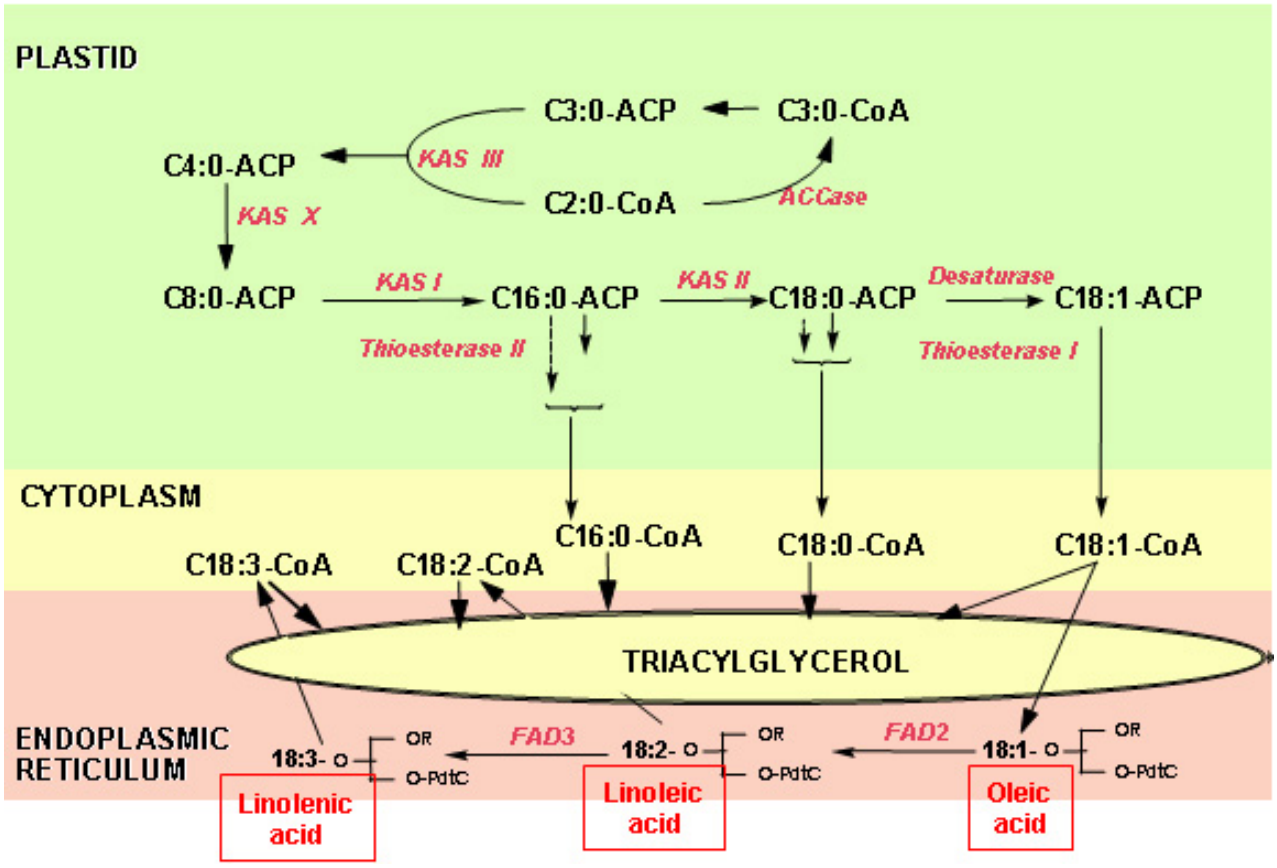
VI. Silencing of the Endogenous *FAD2-1* and KTi3 Genes

This section describes the molecular basis for the high oleic phenotype of the 305423 soybean achieved through silencing of the endogenous *FAD2-1* gene encoding omega-6 desaturase that is involved in the fatty acid biosynthesis (Section VI-A), and provides experimental evidence of the *FAD2-1* gene silencing in 305423 soybean (Section VI-B). Additionally, the expression level of the endogenous Kunitz trypsin inhibitor 3 (KTi3) gene was evaluated, since the promoter of this gene was used to drive the transcription of the introduced *gm-fad2-1* gene fragment and this was expected to cause a silencing effect on the endogenous KTi3 gene expression (Section VI-C).

VI-A. Molecular Basis of the High Oleic Phenotype

As outlined in Section I, the high oleic phenotype of 305423 soybean refers to the modified fatty acid profile of soybean seeds characterized by increased levels of monounsaturated (oleic) acid, and decreased levels of polyunsaturated (linoleic and linolenic) acids. Such a phenotype was achieved by modifying expression of the *FAD2-1* gene involved in fatty acid biosynthesis in soybean seeds (illustrated in Figure 11).

Figure 11. Fatty Acid Biosynthesis in Soybean.



Adapted from Kinney, 1994.

The synthesis of polyunsaturated fatty acids in developing oilseeds is catalyzed by two membrane-associated fatty acid desaturases (FAD) that sequentially add a second and third double bond to oleic acid, a monounsaturated fatty acid (Kinney, 1994). The second double bond is added at the δ-12 (ω-6) position by ω-6 desaturase, encoded in soybean by the *FAD2* gene (Okuley *et al*., 1994; Heppard *et al*., 1996). The third double bond is added at the ω-3 (δ-15) position by ω-3 desaturase, encoded in soybean by the *FAD3* gene (Yadav *et al*., 1993).

In soybean, there are two *FAD2* genes: *FAD2-1* and *FAD2-2* (Heppard *et al*., 1996). Expression of the *FAD2-1* gene is seed-specific; it increases during the period of oil deposition, starting around 19 days after flowering, and its gene product is responsible for the synthesis of the polyunsaturated fatty acids found in soybean oil. The *FAD2-2* gene is constitutively expressed in seed, leaf, root and stem. It is a housekeeping ω-6 desaturase gene responsible for synthesis of polyunsaturated fatty acids for cell membranes (Heppard *et al*., 1996).

One of the approaches to decrease the content of polyunsaturated fatty acids and elevate the level of monounsaturated (oleic) acid in soybean seeds is to down-regulate the expression of the endogenous *FAD2-1* gene. Decrease in the level of the desaturase encoded by *FAD2-1* inhibits the conversion of oleic acid to linoleic acid, thus elevating the oleic acid level in soybean seed. Such down-regulation strategy has been successfully demonstrated for the *FAD2* genes (Buhr *et al*., 2002; Stoutjesdijk *et al*., 2002). Silencing of the endogenous *FAD2-1* gene can be achieved through the introduction of a gene fragment driven by a seed-preferred promoter. A 597 bp *gm-fad2-1* gene fragment (in the sense orientation) corresponding to about 40% of the middle of the open reading frame (ORF) of the *FAD2-1* gene and driven by seed-preferred KTi3 promoter was used to silence the endogenous *FAD2-1* gene. Multiple copies of the *gm-fad2-1* fragment appear to be necessary for effective co-suppression of the endogenous gene (El-Shemy *et al*., 2004; Han *et al*., 2004; Mishra and Handa, 2005) (refer to Section V).

VI-B. Confirmation of the *FAD2-1* Gene Silencing in 305423 Soybean

To demonstrate effective silencing of the endogenous *FAD2-1* gene in 305423 soybean resulting in the high oleic phenotype, Northern blot analysis was conducted. Along with assessing the expression level of the *FAD2-1* gene, two other endogenous *FAD* genes, *FAD2-2* and *FAD3*, were also evaluated. The *FAD2-2* transcript analysis was performed to asses the effect of the introduction of the *gm-fad2-1* gene fragment on a closely related gene family member; introduced *gm-fad2-1* gene fragment shares 71.5% of homology with *FAD2-2* coding region. Transcription analysis on the *FAD3* gene was used to determine whether a gene located downstream in the same fatty acid biosynthesis pathway and sharing less homology to the introduced *gm-fad2-1* gene fragment (48% within the corresponding sequence stretch) was affected by the introduction of the *gm-fad2-1* gene fragment.

Gene expression was analyzed in leaf and developing seed of ten 305423 soybean plants of the T4 generation and five control Jack plants (Figure 2). Probes used for Northern analysis and their hybridization targets are listed in Table 4. Detailed description of the method is provided in Appendix 1-2.

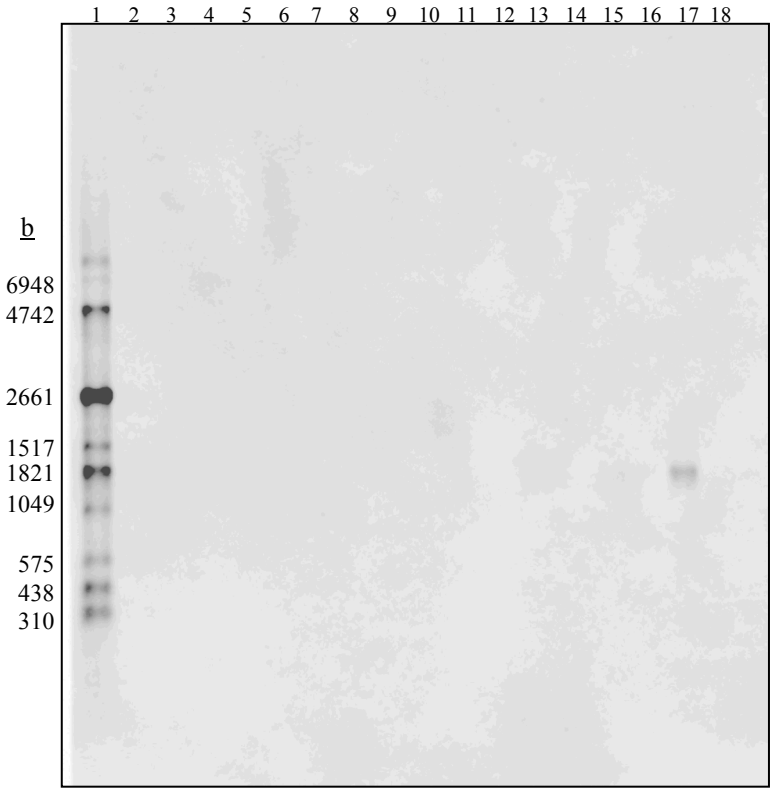
Table 4. Probes Used for Expression Analysis of Endogenous Soybean Genes

Probe name	Hybridization target	*In vivo* transcript size (kilobase)
***fad2-1* 3'UTR**	*FAD2-1* transcript[1]	1.5
***fad2-2* 3'UTR**	*FAD2-2* transcript	1.6
fad3	*FAD3* transcript	1.5
dapA	*dapA* transcript[2]	1.5

[1] Probe does not hybridize to the transcript from the *gm-fad2-1* gene fragment, as the introduced fragment corresponds to the coding region of the *FAD2-1* gene.
[2] Encodes dihydrodipicolinate synthase constitutively expressed in most soybean tissues; control for RNA quality and method sensitivity.

Results of Northern blot analysis are presented in Figures 12 through 15 for leaf tissue, and Figures 16 through 19 for developing seed. As expected, no endogenous *FAD2-1* transcript was detected in leaf tissue of both 305423 and control soybean (Figure 12) since expression of this soybean gene is restricted to seeds (Heppard *et al*., 1996). Transcript levels of constitutively expressed *FAD2-2* and *FAD3* genes (Figures 13 and 14, respectively) in leaves of 305423 and control soybean plants were similar as expected. Hybridization of the *dapA* probe to endogenous leaf dihydrodipicolinate synthase mRNA confirmed the integrity of mRNA used for Northern analysis and method sensitivity sufficient to detect low-level transcripts (Figure 15).

Figure 12. Expression of the *FAD2-1* Gene in Leaf Tissue of 305423 and Control Soybean

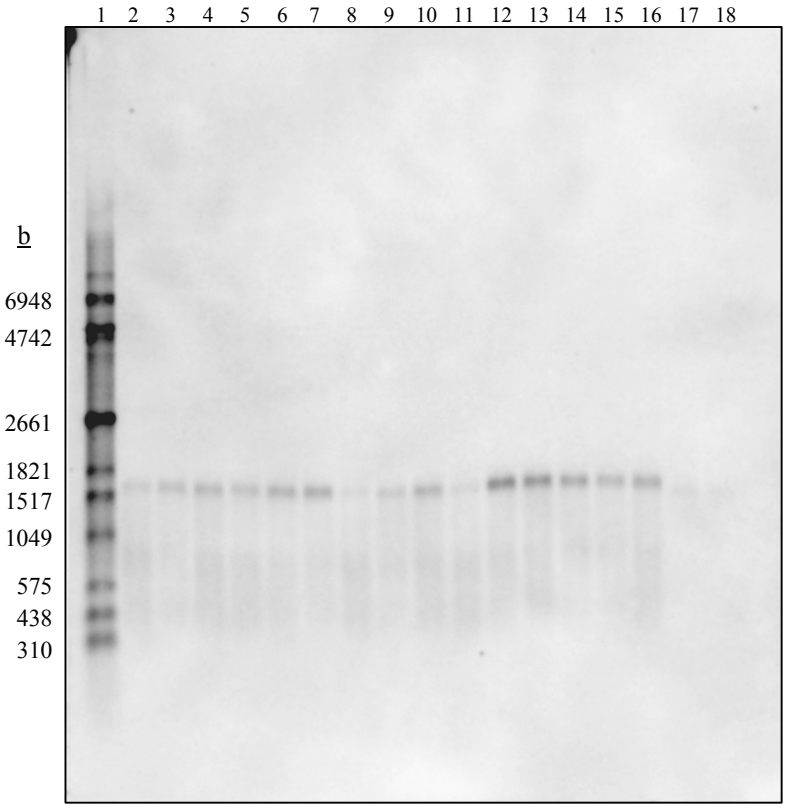


Messenger RNA samples isolated from T4 leaf tissue of 305423 and control (Jack) soybean lines were loaded in each well (200 ng) and hybridized to *fad2-1* 3'UTR probe. The positive sense *in vitro* transcript control (1492 b) is located in lanes 17 and 18. Fragment sizes in bases (b) of the DIG-labeled RNA molecular weight Marker I are indicated adjacent to the blot image.

Lane	Sample
1	RNA molecular weight markers DIG RNA I
2	305423-Leaf/plant 1
3	305423-Leaf/plant 2
4	305423-Leaf/plant 3
5	305423-Leaf/plant 4
6	305423-Leaf/plant 5
7	305423-Leaf/plant 6
8	305423-Leaf/plant 7
9	305423-Leaf/plant 8

Lane	Sample
10	305423-Leaf/plant 9
11	305423-Leaf/plant 10
12	Control-Leaf/plant 1
13	Control-Leaf/plant 2
14	Control-Leaf/plant 3
15	Control-Leaf/plant 4
16	Control-Leaf/plant 5
17	*fad2-1* sense transcript (*in vitro*) 25 pg
18	*fad2-1* sense transcript (*in vitro*) 5 pg

Figure 13. Expression of the *FAD2-2* Gene in Leaf Tissue of 305423 and Control Soybean

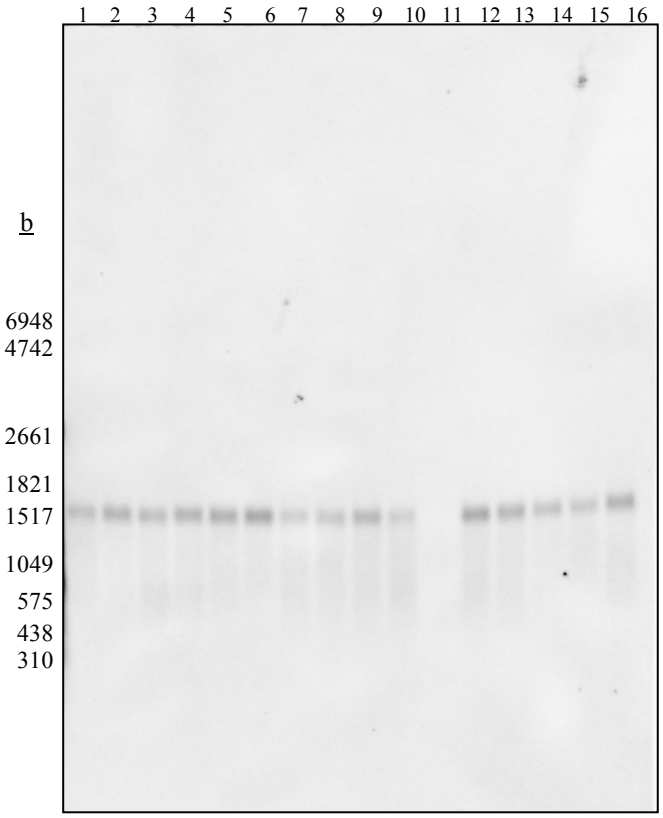


Messenger RNA samples isolated from T4 leaf tissue of 305423 and control (Jack) soybean lines were loaded in each well (200 ng) and hybridized to *fad2-2* 3'UTR probe. The positive sense *in vitro* transcript control (1577 b) is located in lanes 17 and 18. Fragment sizes in bases (b) of the DIG-labeled RNA molecular weight Marker I are indicated adjacent to the blot image.

Lane	Sample
1	RNA molecular weight markers DIG RNA I
2	305423-Leaf/plant 1
3	305423-Leaf/plant 2
4	305423-Leaf/plant 3
5	305423-Leaf/plant 4
6	305423-Leaf/plant 5
7	305423-Leaf/plant 6
8	305423-Leaf/plant 7
9	305423-Leaf/plant 8

Lane	Sample
10	305423-Leaf/plant 9
11	305423-Leaf/plant 10
12	Control-Leaf/plant 1
13	Control-Leaf/plant 2
14	Control-Leaf/plant 3
15	Control-Leaf/plant 4
16	Control-Leaf/plant 5
17	*fad2-2* sense transcript (*in vitro*) 10 pg
18	*fad2-2* sense transcript (*in vitro*) 5 pg

Figure 14. Expression of the *FAD3* Gene in Leaf Tissue of 305423 and Control Soybean

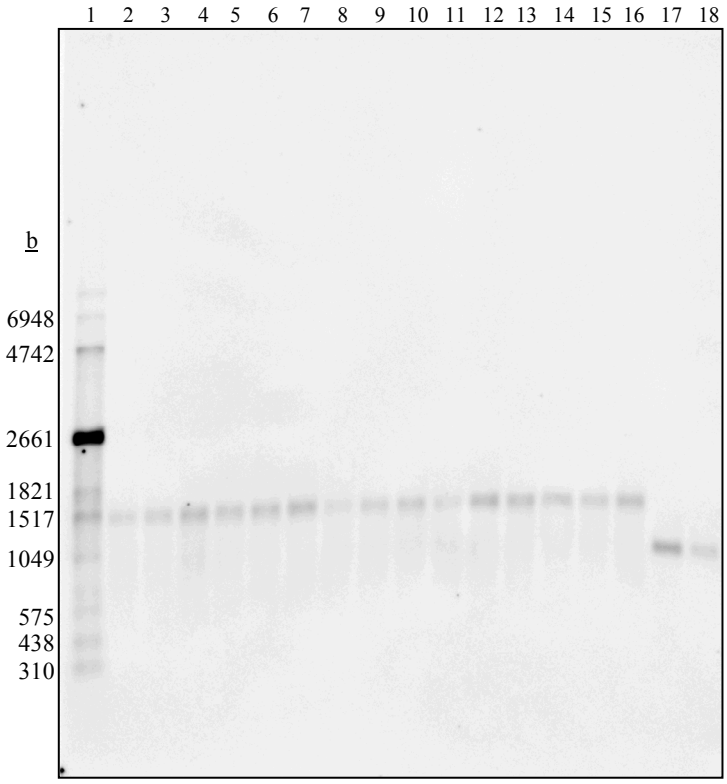


Messenger RNA samples isolated from T4 leaf tissue of 305423 and control (Jack) soybean lines were loaded in each well (200 ng) and hybridized to *fad3* probe. This blot does not contain a separate positive sense probe control however, hybridization to unmodified Jack soybean RNA samples serves as a positive control of hybridization. Fragment sizes in bases (b) of the DIG-labeled RNA molecular weight Marker I are indicated adjacent to the blot image. In this blot the DIG RNA marker lane has been excised because the bands were too intensive and interfered with the clear depiction of transcript signals. The positioning of the markers was done using a shorter exposure of the blot to X-ray film and with the help of unlabeled markers

Lane	Sample
1	305423-Leaf/plant 1
2	305423-Leaf/plant 2
3	305423-Leaf/plant 3
4	305423-Leaf/plant 4
5	305423-Leaf/plant 5
6	305423-Leaf/plant 6
7	305423-Leaf/plant 7
8	305423-Leaf/plant 8

Lane	Sample
9	305423-Leaf/plant 9
10	305423-Leaf/plant 10
11	Blank
12	Control-Leaf/plant 1
13	Control-Leaf/plant 2
14	Control-Leaf/plant 3
15	Control-Leaf/plant 4
16	Control-Leaf/plant 5

Figure 15. Expression of the *dapA* Gene in Leaf Tissue of 305423 and Control Soybean



Messenger RNA samples isolated from T4 leaf tissue of 305423 and control (Jack) soybean lines were loaded in each well (400 ng) and hybridized to *dapA* probe. The positive sense *in vitro* transcript control (998 b) is located in lanes 17 and 18. Fragment sizes in bases (b) of the DIG-labeled RNA molecular weight Marker I are indicated adjacent to the blot image.

Lane	Sample
1	RNA molecular weight markers DIG RNA I
2	305423-Leaf/plant 1
3	305423-Leaf/plant 2
4	305423-Leaf/plant 3
5	305423-Leaf/plant 4
6	305423-Leaf/plant 5
7	305423-Leaf/plant 6
8	305423-Leaf/plant 7
9	305423-Leaf/plant 8

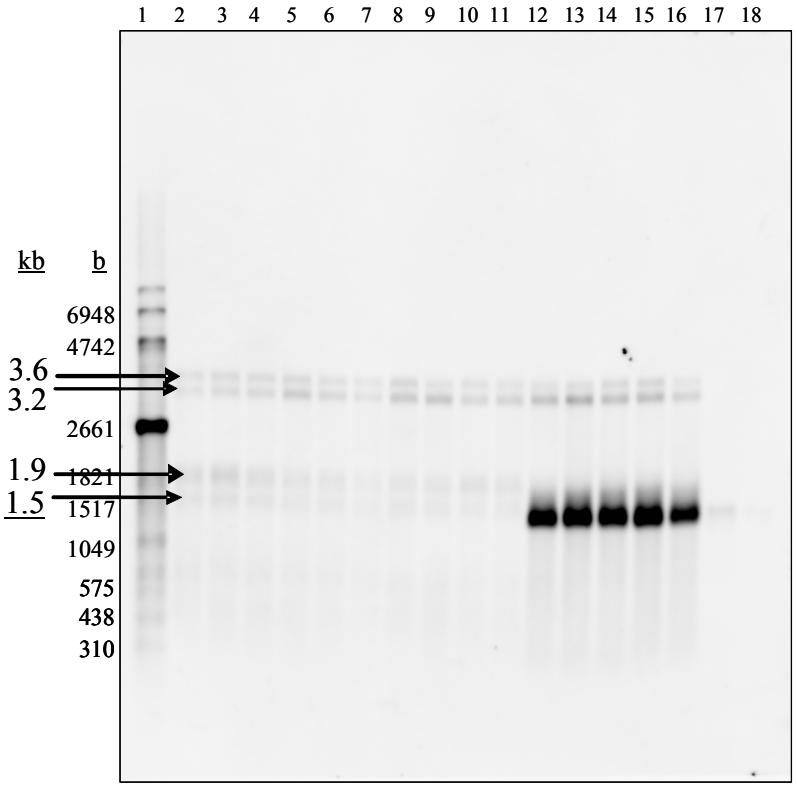
Lane	Sample
10	305423-Leaf/plant 9
11	305423-Leaf/plant 10
12	Control-Leaf/plant 1
13	Control-Leaf/plant 2
14	Control-Leaf/plant 3
15	Control-Leaf/plant 4
16	Control-Leaf/plant 5
17	*dap*A sense transcript (*in vitro*) 5 pg
18	*dap*A sense transcript (*in vitro*) 2 pg

Expression of the *FAD2-1, FAD2-2,* and *FAD3* genes in developing soybean seed was examined at two developmental time points, 20 and 30 days after flowering (daf). No differences in gene expression patterns were observed between the two time points, therefore only the 30 daf seed data is presented in Figures 16 through 19.

Hybridization with the *fad2-1* 3'UTR probe showed that the expected 1.5 kb band corresponding to the correctly processed endogenous *FAD2-1* transcript was present in both 305423 and control soybean seed, confirming that expression of the *FAD2-1* gene is seed-specific (Heppard *et al*., 1996). The level of the 1.5 kb *FAD2-1* transcript was significantly reduced in 305423 soybean seed compared to the control soybean, thus confirming the effective silencing of the endogenous *FAD2-1* gene in 305423 soybean developing seed (Figure 16).

Besides the 1.5 kb band detected in seed with the *FAD2-1*-specific probe, three additional bands were also observed. Two bands, approximately of 3.2 kb and 3.6 kb, were present at equal intensity in both 305423 and control soybean seed, and likely correspond to the alternatively processed endogenous *FAD2-1* transcripts. The population of the soybean *FAD2-1* transcripts is complex, as evident from the National Center for Biotechnology Information (NCBI) Expressed Sequence Tags (EST) database (www.ncbi.nih.gov) and from publications (Fillatti, 2005; Tang *et al*., 2005). Various EST clones corresponding to the *FAD2-1* transcript are documented, with some of these being initiated from an early transcription start site or containing an unspliced intron (GenBank accession numbers AJ271842, AY954300, AW397948, AW567956, CK769122). The 3.2 kb transcript is likely generated from the early transcription start site, and the 3.6 kb transcript in addition contains an unspliced 0.4 kb intron. These 3.2 kb and 3.6 kb messages could also be generated by read-through transcription. An approximately 1.9 kb *FAD2-1* transcript was unique to 305423 soybean seeds. A similar transcript was also observed in another high oleic soybean line generated by co-suppression of the endogenous *FAD2-1* gene (Buhr *et al*., 2002). It is very likely that this is an intermediate or aberrant RNA involved in gene silencing (Han and Grierson, 2002), and is further evidence of gene silencing caused by the introduced *gm-fad2-1* gene fragment.

Figure 16. Expression of the *FAD2-1* Gene in Developing Seed of 305423 and Control Soybean



Messenger RNA samples isolated from 30 daf seed tissue of 305423 (T4 generation) and control (Jack) soybean lines were loaded in each well (200 ng) and hybridized to *fad2-1* 3'UTR probe. The positive sense *in vitro* transcript control (1492 b) is located in lanes 17 and 18. Fragment sizes in bases (b) of the DIG-labeled RNA molecular weight Marker I are indicated adjacent to the blot image. Arrows point to the hybridized fragments with sizes given in kilobases (kb).

Lane	Sample
1	RNA molecular weight markers DIG RNA I
2	305423-Seed/plant 1
3	305423-Seed/plant 2
4	305423-Seed/plant 3
5	305423-Seed/plant 4
6	305423-Seed/plant 5
7	305423-Seed/plant 6
8	305423-Seed/plant 7
9	305423-Seed/plant 8

Lane	Sample
10	305423-Seed/plant 9
11	305423-Seed/plant 10
12	Control-Seed/plant 1
13	Control-Seed/plant 2
14	Control-Seed/plant 3
15	Control-Seed/plant 4
16	Control-Seed/plant 5
17	*fad2-1* sense transcript (*in vitro*) 25 pg
18	*fad2-1* sense transcript (*in vitro*) 5 pg

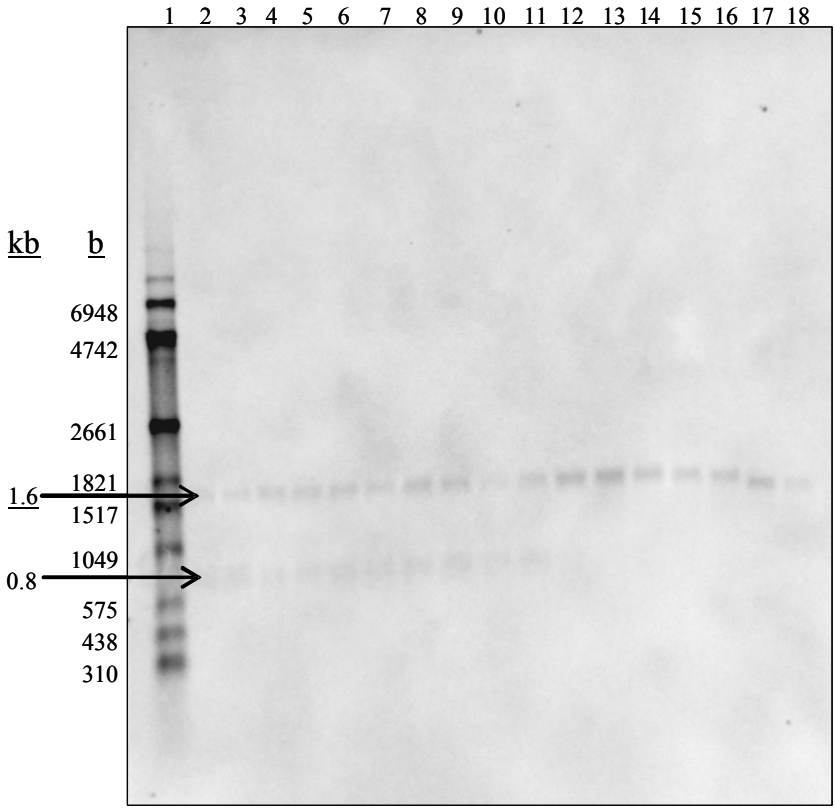
Two distinct transcripts of the *FAD2-2* gene, approximately of 1.6 kb and 0.8 kb, were detected in 305423 soybean seed upon hybridization with the *fad2-2* 3'UTR probe (Figure 17). The correctly processed transcript of 1.6 kb was found in both 305423 and control soybean seed. The transcript level was slightly lower in 305423 soybean than in control seed, suggesting that *FAD2-2* gene expression might also be slightly reduced due to introduction of the *gm-fad2-1* gene fragment. This is an anticipated result, since homology of introduced *gm-fad2-1* gene fragment to *FAD2-2* coding region is 71.5%. The minor 0.8 kb transcript was specific to 305423 soybean seed, and appears to represent an intermediate or aberrant RNA involved in partial *FAD2-2* gene silencing (Han and Grierson, 2002).

Similar to leaf tissue, the level of the 1.5 kb *FAD3* transcript detected with the *fad3* probe remained unchanged in 305423 soybean seed as compared to the control plants (Figure 18). This result confirms that introduction of the *gm-fad2-1* gene fragment does not affect *FAD3* gene expression, which was also anticipated, as the shared homology between the introduced *gm-fad2-1* gene fragment and the *FAD3* coding sequence is 48%.

Hybridization of the *dapA* probe to endogenous seed dihydrodipicolinate synthase mRNA confirmed integrity of mRNA used for Northern analysis and method sensitivity sufficient to detect low-level transcripts (Figure 19).

The results of gene expression analysis confirm that expression of the endogenous *FAD2-1* gene is effectively silenced in developing seed of the high oleic 305423 soybean. As expected, no expression of the endogenous *FAD2-1* gene was found in leaf tissue since expression of this soybean gene is restricted to seeds. Introduction of the *gm-fad2-1* gene fragment likely also exhibits a limited suppression effect on the closely related *FAD2-2* gene transcript level in developing seeds. As expected, introduction of the *gm-fad2-1* gene fragment does not affect the transcript level of the *FAD3* gene encoding the downstream omega-3 desaturase in the fatty acid biosynthetic pathway.

Figure 17. Expression of the *FAD2-2* Gene in Developing Seed of 305423 and Control Soybean

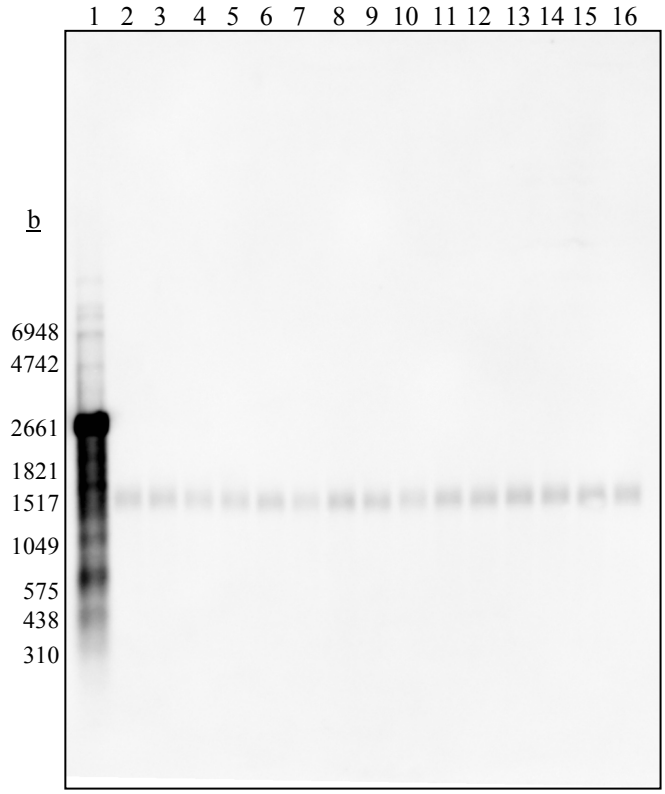


Messenger RNA samples isolated from 30 daf seed tissue of 305423 (T4 generation) and control (Jack) soybean lines were loaded in each well (400 ng) and hybridized to *fad2-2* 3'UTR probe. The positive sense *in vitro* transcript control (1577 b) is located in lanes 17 and 18. Fragment sizes in bases (b) of the DIG-labeled RNA molecular weight Marker I are indicated adjacent to the blot image. Arrows point to the hybridized fragments with sizes given in kilobases (kb).

Lane	Sample
1	RNA molecular weight markers DIG RNA I
2	305423-Seed/plant 1
3	305423-Seed/plant 2
4	305423-Seed/plant 3
5	305423-Seed/plant 4
6	305423-Seed/plant 5
7	305423-Seed/plant 6
8	305423-Seed/plant 7
9	305423-Seed/plant 8

Lane	Sample
10	305423-Seed/plant 9
11	305423-Seed/plant 10
12	Control-Seed/plant 1
13	Control-Seed/plant 2
14	Control-Seed/plant 3
15	Control-Seed/plant 4
16	Control-Seed/plant 5
17	*fad2-2* sense transcript (*in vitro*) 10 pg
18	*fad2-2* sense transcript (*in vitro*) 5 pg

Figure 18. Expression of the *FAD3* Gene in Developing Seed of 305423 and Control Soybean

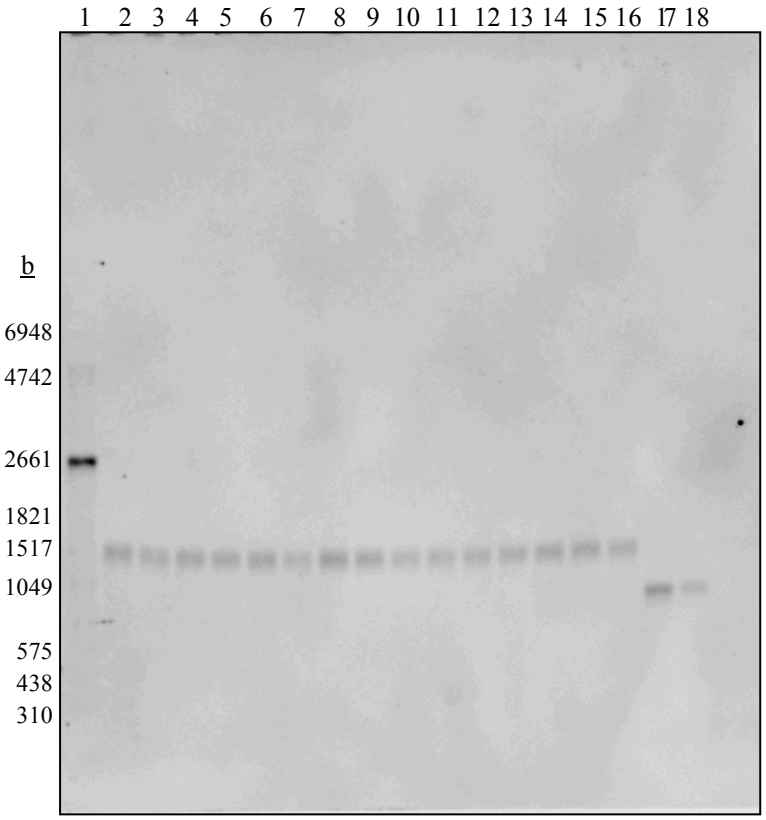


Messenger RNA samples isolated from 30 daf seed tissue of 305423 (T4 generation) and control (Jack) soybean lines were loaded in each well (200 ng) and hybridized to *fad3* probe. This blot does not contain a separate positive sense probe control however, hybridization to unmodified Jack soybean RNA samples serves as a positive control of hybridization. Fragment sizes in bases (b) of the DIG-labeled RNA molecular weight Marker I are indicated adjacent to the blot image.

Lane	Sample
1	RNA molecular weight markers DIG RNA I
2	305423-Seed/plant 1
3	305423-Seed/plant 2
4	305423-Seed/plant 3
5	305423-Seed/plant 4
6	305423-Seed/plant 5
7	305423-Seed/plant 6
8	305423-Seed/plant 7

Lane	Sample
9	305423-Seed/plant 8
10	305423-Seed/plant 9
11	305423-Seed/plant 10
12	Control-Seed/plant 1
13	Control-Seed/plant 2
14	Control-Seed/plant 3
15	Control-Seed/plant 4
16	Control-Seed/plant 5

Figure 19. Expression of the *dapA* Gene in Developing Seed of 305423 and Control Soybean



Messenger RNA samples isolated from 30 daf seed tissue of 305423 (T4 generation) and control (Jack) soybean lines were loaded in each well (400 ng) and hybridized to *dapA* probe. The positive sense *in vitro* transcript control (998 b) is located in lanes 17 and 18. Fragment sizes in bases (b) of the DIG-labeled RNA molecular weight Marker I are indicated adjacent to the blot image.

Lane	Sample
1	RNA molecular weight markers DIG RNA I
2	305423-Seed/plant 1
3	305423-Seed/plant 2
4	305423-Seed/plant 3
5	305423-Seed/plant 4
6	305423-Seed/plant 5
7	305423-Seed/plant 6
8	305423-Seed/plant 7
9	305423-Seed/plant 8

Lane	Sample
10	305423-Seed/plant 9
11	305423-Seed/plant 10
12	Control-Seed/plant 1
13	Control-Seed/plant 2
14	Control-Seed/plant 3
15	Control-Seed/plant 4
16	Control-Seed/plant 5
17	*dap*A sense transcript (*in vitro*) 5 pg
18	*dap*A sense transcript (*in vitro*) 2 pg

VI-C. Silencing of the Endogenous KTi3 Gene in 305423 Soybean

Transcription of the *gm-fad2-1* gene fragment in 305423 soybean is driven by the promoter for the Kunitz trypsin inhibitor gene 3 (KTi3) (Jofuku and Goldberg, 1989). It has been reported in the literature that the insertion of a promoter fragment can effectively silence its endogenous gene expression (Morino *et al.,* 2004; Cigan *et al.*, 2005; Eike *et al*., 2005; Yang *et al*., 2005). Endogenous KTi3 gene expression was therefore examined to find if this effect has taken place in 305423 soybean.

A PCR-amplified fragment corresponding to the 491 bp (from base pair 20 to base pair 511 of GenBank accession number X64447 sequence) of the KTi3 internal coding region was used as a probe (*kti3* coding probe) to detect KTi3 transcript in 305423 and control soybean seeds at 20 days after flowering (daf) by Northern hybridization. Details on the method can be found in Appendix 1-2. Ten 305423 soybean plants (T4 generation), and five control Jack plants were used for analysis (Figure 2). The 20 daf seed stage was chosen because KTi3 is expressed at high levels in both 20 and 30 daf seeds (Jofuku *et al*., 1989).

Results of the Northern blot hybridization with the *kti3* coding probe are presented in Figure 20. A band of ~0.9 kb was detected in all the plants, which correlates well with the expected size of the KTi3 transcript. The hybridization signal was intense in control soybean plants but very faint in 305423 soybean. This result indicates that the KTi3 gene was silenced by the introduction of the KTi3 promoter in 305423 soybean.

It should be noted that the KTi3 coding region shares about 80% homology with the KTi1 and the KTi2 genes of similar transcript size (Jofuku and Goldberg, 1989; Jofuku *et al.*, 1989), therefore the *kti3* coding probe likely cross-hybridizes with the transcripts from KTi1 and KTi2 genes. However, the KTi3 gene encodes the predominant Kunitz trypsin inhibitor in soybean seeds, whereas expression of the constitutive KTi1/KTi2 genes in seeds is much lower and they are primarily expressed in leaves, roots and stems (Jofuku and Goldberg, 1989). It is therefore unlikely that the presence of the KTi3 promoter from the 305423 soybean insertion would affect expression of the KTi1 and KTi2 genes in seeds.

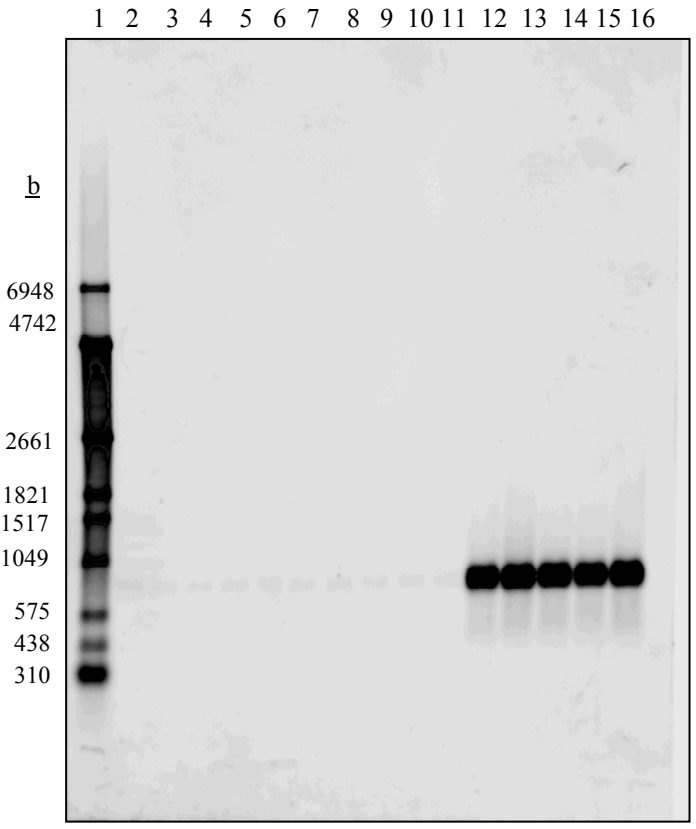
The result of expression analysis of endogenous KTi3 gene is in agreement with the observation from the compositional assessment of 305423 soybean (discussed further in Section IX) showing that the amount of trypsin inhibitor was statistically significantly decreased in 305423 soybean as compared to the control isoline.

VI-D. Summary and Conclusions

The high oleic phenotype of 305423 soybean refers to the fatty acid composition of soybean seed, and specifically, to the increased level of the oleic acid and decreased levels of the linoleic and linolenic acids. The high oleic phenotype in 305423 soybean was achieved through the introduction of a molecular construct containing the *gm-fad2-1* gene fragment. The *gm-fad2-1* fragment sequence is identical to a portion of the coding region of the endogenous soybean *FAD2-1* gene encoding omega-6 desaturase that causes the conversion of oleic acid to linoleic acid. Transcription of the *gm-fad2-1* gene fragment under the control of a seed-preferred KTi3 promoter was expected to cause a silencing effect on expression of endogenous *FAD2-1* gene in 305423 soybean seed, thus resulting in accumulation of oleic acid. Northern blot analysis of the *FAD2-1* gene expression confirmed effective silencing of this gene in 305423 soybean. Expression of two other *FAD* gene family members, *FAD2-2* and *FAD3*, involved in subsequent steps of the fatty acid biosynthesis in soybean seed was also evaluated, and indicated partial gene silencing of the *FAD2-2* gene (sharing 71.5% homology with the *gm-fad2-1* gene fragment along the corresponding sequence stretch), and no effect on the expression of the *FAD3* gene (sharing 48% homology with the *gm-fad2-1* gene fragment along the corresponding sequence stretch). Additionally, silencing of the endogenous KTi3 gene encoding the Kunitz trypsin inhibitor was seen in 305423 soybean seed;

this was not unexpected since the KTi3 gene promoter was used to drive transcription of the *gm-fad2-1* gene fragment and, as reported in the literature, this can result in silencing of the associated endogenous gene.

Figure 20. Expression of the KTi3 Gene in Developing Seed of 305423 and Control Soybean



Messenger RNA samples isolated from 20 daf seed tissue of 305423 (T4 generation) and control (Jack) soybean lines were loaded in each well (400 ng) and hybridized to *kti3* coding probe. This blot does not contain a separate positive sense probe control however, hybridization to unmodified Jack soybean RNA samples serves as a positive control of hybridization. Fragment sizes in bases (b) of the DIG-labeled RNA molecular weight Marker I are indicated adjacent to the blot image.

Lane	Sample
1	RNA molecular weight markers DIG RNA I
2	305423-Seed/plant 1
3	305423-Seed/plant 2
4	305423-Seed/plant 3
5	305423-Seed/plant 4
6	305423-Seed/plant 5
7	305423-Seed/plant 6
8	305423-Seed/plant 7

Lane	Sample
9	305423-Seed/plant 8
10	305423-Seed/plant 9
11	305423-Seed/plant 10
12	Control-Seed/plant 1
13	Control-Seed/plant 2
14	Control-Seed/plant 3
15	Control-Seed/plant 4
16	Control-Seed/plant 5

VII. Characterization of the Introduced GM-HRA Protein

The GM-HRA protein is a modified version of the endogenous acetolactate synthase (ALS) protein from soybean. When expressed in plants, GM-HRA confers tolerance to ALS-inhibiting herbicides. The herbicide tolerant *gm-hra* gene was made by isolating the herbicide sensitive soybean *gm-als* gene and introducing two specific amino acid changes in the mature protein. The *gm-hra* gene is used as a selectable marker gene in 305423 soybean.

The 305423 soybean was not selected for commercial sulfonylurea or other ALS-inhibitor herbicide tolerance during the breeding and product development process. All conventional soybean lines, as well as 305423 soybean, have an inherent level of tolerance to certain sulfonylurea herbicides. Expression of the GM-HRA protein can increase this inherent tolerance level and additionally broaden the tolerance to a larger class of sulfonylurea herbicides however; Pioneer does not currently plan to market 305423 soybean as having a weed control advantage over conventional soybeans. As with conventional soybeans, 305423 soybean should only be sprayed using sulfonylurea herbicides labeled for use on conventional soybeans.

VII-A. Biochemistry and Mode of Action of the GM-HRA Protein

The endogenous ALS, also known as acetohydroxyacid synthase (AHAS)[1], is a key enzyme that catalyzes the first common step in the biosynthesis of the essential branched-chain amino acids isoleucine, leucine, and valine (LaRossa and Schloss, 1984; LaRossa and Falco, 1984; Duggleby and Pang, 2000; Coruzzi and Last, 2000). Two reactions are catalyzed by ALS enzymes: the conversion of two molecules of pyruvate to form acetolactate leading to the synthesis of leucine and valine, and the condensation of pyruvate with 2-ketobutyrate to form 2-acetohydroxybutyrate in the pathway to isoleucine (Figure 21).

ALS enzymes are widely distributed in nature; ALS genes have been isolated from bacteria, fungi, algae and plants (Friden *et al.,* 1985; Falco *et al.,* 1985; Reith and Munholland, 1995; Mazur *et al.,* 1987). ALS enzymes generally consist of a larger catalytic subunit that is active alone and a smaller regulatory subunit. The large catalytic subunit of ALS in soybean was used to derive GM-HRA. The large catalytic subunit of ALS typically functions as a homotetramer or homodimer with the active site located at a dimer interface. Recent studies on the crystal structures of ALS from yeast and *Arabidopsis thaliana* in the presence of ALS-inhibiting herbicides have provided evidence that the inhibitors are located at the entrance to and extending into a funnel-type channel leading to the active site, blocking access to the substrate (McCourt *et al*., 2006; Pang *et al*., 2002, 2003, 2004). These compounds inhibit plant growth by inactivating ALS, a key enzyme in the essential amino acid biosynthetic pathway; the negative effects on plant growth may be attributed to the physiological consequences of the enzyme inhibition (Duggleby and Pang, 2000). ALS has been shown to be inhibited by five chemical classes of herbicides.

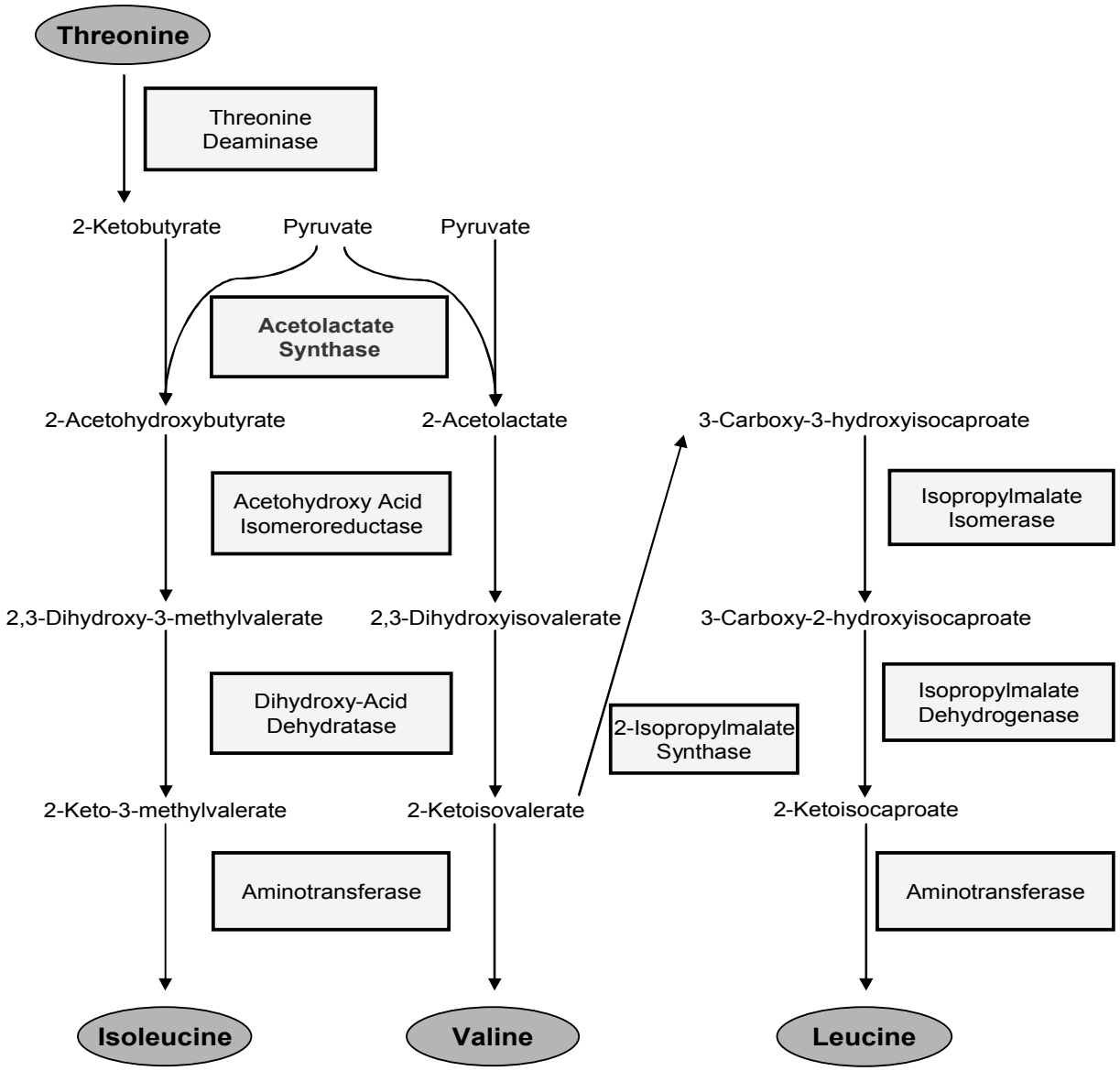
In particular, ALS was identified as being the target site for sulfonylurea herbicides (Levitt, 1978); this triggered the first enzymes with herbicide tolerant activity to be identified in bacteria (LaRossa and Schloss, 1984), yeast (*Sacchromyces cerevisiae*) (Falco and Dumas, 1985), and plants (Chaleff and Mauvais, 1984). The respective genes were then isolated from various species and amino acid sequence changes accountable for the tolerant phenotype were identified (Hartnett *et al*., 1990, 1991; Falco *et al*., 1989; Duggleby and Pang, 2000). Various tolerance sites were identified, and the substituted amino acid at those sites, result in ALS enzymes with various tolerance levels or specific tolerance to the different chemical classes of inhibitors. Natural

[1] Although both 'ALS' and 'AHAS' are acceptable nomenclature for acetohydroxyacid synthase, recent literature more commonly refers to the enzyme in plants as 'AHAS' (EC 2.2.1.6, formerly EC 4.1.3.18). However, 'ALS' will be used throughout this submission for consistency with the early literature.

herbicide tolerance mutations are also found, and most commonly occur at four particular amino acid locations (Duggleby and Pang, 2000). Soybean, maize, wheat, rice, canola and sunflower commercial crops tolerant to ALS-inhibiting herbicides have been developed through mutagenesis or selection processes (Tan *et al.*, 2006). In plants, *als* genes are nuclear encoded and the enzymes contain a chloroplast directed N-terminal transit peptide, estimated to be between 80 to 100 amino acids in length. The transit peptide serves to import the protein into the chloroplast, where the majority of branched chain amino acid biosynthesis occurs.

The *gm-hra* gene encoding the GM-HRA protein was generated by site-specific mutagenesis of the endogenous soybean *als I* gene. These mutations cause substitution of the two amino acids in the protein sequence (Falco and Li, 2003). Mutations are analogous to those in the herbicide tolerant tobacco mutant (Creason and Chaleff, 1988; Lee *et al.,* 1988), and also represent commonly found natural tolerance mutations. As a result of such modification, the GM-HRA protein encoded by the *gm-hra* gene confers plant tolerance to ALS-inhibiting herbicides. This feature was used as a selectable marker in the process of development of 305423 soybean (Section III-A).

Figure 21. Branched Chain Amino Acid Biosynthesis in Plants



Adapted from Coruzzi and Last, 2000.

VII-B. Characterization of the GM-HRA Protein Produced in 305423 Soybean

The GM-HRA protein is 656 amino acids in length with a predicted molecular weight of 71 kDa for the full-length protein (Figure 22). The start of the mature protein is located at residue S53 (double underlined) resulting in a protein of 604 amino acids with a predicted molecular weight of 65 kDa. The two boxed amino acid residues, A183 and L560, have been changed from P178 and W555, respectively, of the GM-ALS I sequence and are responsible for GM-HRA tolerance to ALS-inhibiting herbicides. The first five amino acids in bold are derived from translation of the 15 nucleotides from the *als* 5' UTR.

Figure 22. Deduced Amino Acid Sequence of the GM-HRA Protein Introduced into 305423 Soybean

```
  1    MPHNTMAATA SRTTRFSSSS SHPTFPKRIT RSTLPLSHQT LTKPNHALKI
 51    KCSISKPPTA APFTKEAPTT EPFVSRFASG EPRKGADILV EALERQGVTT
101    VFAYPGGASM EIHQALTRSA AIRNVLPRHE QGGVFAAEGY ARSSGLPGVC
151    IATSGPGATN LVSGLADALM DSVPVVAITG QVARRMIGTD AFQETPIVEV
201    SRSITKHNYL ILDVDDIPRV VAEAFFVATS GRPGPVLIDI PKDVQQQLAV
251    PNWDEPVNLP GYLARLPRPP AEAQLEHIVR LIMEAQKPVL YVGGGSLNSS
301    AELRRFVELT GIPVASTLMG LGTFPIGDEY SLQMLGMHGT VYANYAVDNS
351    DLLLAFGVRF DDRVTGKLEA FASRAKIVHI DIDSAEIGKN KQAHVSVCAD
401    LKLALKGINM ILEEKGVEGK FDLGGWREEI NVQKHKFPLG YKTFQDAISP
451    QHAIEVLDEL TNGDAIVSTG VGQHQMWAAQ FYKYKRPRQW LTSGGLGAMG
501    FGLPAAIGAA VANPGAVVVD IDGDGSFIMN VQELATIRVE NLPVKILLLN
551    NQHLGMVVQL EDRFYKSNRA HTYLGDPSSE SEIFPNMLKF ADACGIPAAR
601    VTKKEELRAA IQRMLDTPGP YLLDVIVPHQ EHVLPMIPSN GSFKDVITEG
651    DGRTRY
```

Double underlining indicates the start of the mature protein at residue S53. The two boxed residues, A183 and L560, are differences from the endogenous soybean acetolactate synthase protein (GM-ALS I). The first five amino acids in bold are derived from translation of the 15 nucleotides from the *als* 5' UTR.

The GM-HRA protein was partially purified from 305423 soybean leaf tissue by immunoaffinity chromatography. To obtain the protein amounts sufficient to conduct toxicological and biochemical studies, the GM-HRA protein was overexpressed in *E. coli* strain BL 21(DE3) using the recombinant DNA technology. Various characterization tests were conducted to confirm protein identity and equivalency of the microbially expressed and *in planta* produced GM-HRA proteins. A detailed description of methods used in these protein characterization studies and the resulting data are included in Appendix 5.

Characterization of the physicochemical properties of the microbially expressed and plant-derived GM-HRA proteins was accomplished through the use of the following techniques:

1) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the purity and confirm molecular weight of the proteins;
2) Western blot analysis to confirm the molecular weight and immunoreactivity of the proteins;

3) Glycoprotein staining of the proteins to determine the post-translational modification (glycosylation);
4) Mass determination of the tryptic peptides by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) to confirm the identity of the proteins;
5) N-terminal amino acid sequence analysis to further confirm the identity of the proteins.

Utilizing the above characterization methods, the identity of both the microbially expressed and the 305423 soybean plant-derived GM-HRA proteins were verified. Also, the above characterization tests confirmed the equivalency of the GM-HRA protein expressed in *E. coli* to the GM-HRA protein expressed *in planta* in 305423 soybean. Microbial GM-HRA protein was used subsequently for *in vitro* and *in vivo* safety assessment studies summarized in Section VII-D.

VII-C. Levels of Transgenic Protein in 305423 Soybean

Expression of the *gm-hra* gene in 305423 soybean is controlled by the soybean S-adenosyl-L-methionine synthetase (SAMS) promoter conferring constitutive expression of the transgene in soybean tissues. The GM-HRA protein levels were measured in replicated samples of leaf, root, forage, and grain tissues of 305423 soybean, generation BC1F5, and control null segregant plants of generation BC1F5 (Figure 2). Tissues were collected from six separate field sites in North America in 2005. Three replicated samples per tissue per site were collected for 305423 soybean, and one sample per tissue per site was collected for control soybean.

Leaf, root and forage tissues were collected at the R3 growth stage; this is the “beginning pod” stage and is representative of the feed commodity stage i.e. the stage that forage would be harvested at if it were to be fed to animals. The grain was collected at the R8 growth stage; this reflects the stage of full maturity when soybean grain would be harvested (refer to Gaska, 2006 for the description of soybean growth stages). Leaf and root tissues were analyzed to obtain protein expression data on the above- and below-ground portions of the plant, respectively. Forage tissue was analyzed because soybeans are occasionally used as alternative forage when alfalfa or clover are in short supply due to winter-killing or drought conditions. Expression in grain tissue was measured because fractions derived from soybean grain are the most commonly consumed portion of the plant for both humans and animals. Tissue samples were processed and the GM-HRA protein levels were determined using a quantitative enzyme-linked immunosorbent assay (ELISA) method as described in Appendix 6.

Results of the GM-HRA protein expression analysis are presented in Table 5. The mean GM-HRA protein concentrations across six sites in 305423 soybean leaf, root, forage and grain were 4.0, 0.18, 5.7, and 2.5 ng/mg tissue dry weight, respectively. No GM-HRA protein was detected in any of the non-transgenic control samples analyzed.

The results confirm that the GM-HRA protein is expressed in leaf, root, forage and grain of 305423 soybean.

Table 5. Levels of the GM-HRA Protein in 305423 Soybean

Growth stage/ tissue	ng/mg tissue dry weight		Standard deviation
	Mean	Range[1; 2]	
	305423 soybean		
R3 / Leaf	4.0	1.2 – 6.3	1.8
R3 / Root	0.18	0 – 0.63	0.22
R3 / Forage	5.7	0.78 - 51	12
R8 / Grain	2.5	0 – 4.9	1.1
	Control soybean		
R3 / Leaf	0	0	0
R3 / Root	0	0	0
R3 / Forage	0	0	0
R8 / Grain	0	0	0

[1] Range denotes the lowest and highest individual values across locations.
[2] For values below the sample LLOQ (lower limit of quantitation), a value of zero was assigned for calculation purposes.

VII-D. Summary of the Food and Feed Safety Assessment of the GM-HRA Protein

A detailed assessment of human and animal safety of the GM-HRA protein has been provided to FDA as part of the consultation on food and feed safety for 305423 soybean. The conclusions of the safety assessment of the GM-HRA protein are summarized below. The allergenic potential of the GM-HRA protein was assessed using a step-wise, weight of evidence approach (Codex Alimentarius Commission, 2003) through the assessment of the *gm-hra* gene source and history of use or exposure, bioinformatic comparison of the amino acid sequence of the GM-HRA protein with known protein allergen sequences, evaluation of the stability of the GM-HRA protein using *in vitro* gastric and intestinal digestion models and determination of the GM-HRA protein glycosylation status. The potential toxicity of the GM-HRA protein was assessed by bioinformatic comparison of the amino acid sequence of the GM-HRA protein with known protein toxins as well as by carrying out a study of the acute oral toxicity of GM-HRA in mice.

1) The GM-HRA protein is an acetolactate synthase (ALS) enzyme found in bacteria, fungi, algae and plants (Friden *et al*.; 1985, Falco *et al*.; 1985; Reith and Munholland, 1995; Mazur *et al*., 1987).
2) The donor organism of the GM-HRA protein is soybean (*Glycine max*). The GM-HRA protein is only two amino acids different from the endogenous soybean ALS protein from which it was derived, with an additional five amino acids on the N-terminus derived from translation of the 15 nucleotides from the *als* 5' UTR.
3) The amino acid sequence of the GM-HRA protein was compared to a database of allergens derived from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 6.0, January 2006), which contains the amino acid sequences of known and putative allergenic proteins. Bioinformatics analyses revealed no biologically relevant sequence identities between known or putative protein allergens and the GM-HRA protein sequence. Furthermore, no short (eight or more contiguous identical amino acids) polypeptide matches were shared between the GM-HRA protein and protein allergens. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the GM-HRA protein and known allergens.
4) The GM-HRA protein is rapidly hydrolyzed in both simulated gastric and intestinal fluids (less than 30 seconds in simulated gastric fluid containing pepsin at pH 1.2 as

demonstrated by SDS-PAGE analysis, and less than 30 seconds in simulated intestinal fluid containing pancreatin at pH 7.5 as demonstrated by SDS-PAGE analysis or less than 1 minute as demonstrated by western blot analysis).

5) The GM-HRA protein is not glycosylated as demonstrated by glycoprotein staining.
6) No biologically relevant amino acid sequence identities were observed between known protein toxins (NCBI Protein Dataset, Release 156.0, http://www.ncbi.nlm.nih.gov) and the GM-HRA protein sequence.
7) There was no evidence of acute toxicity in mice at a dose of 2000 mg purified protein preparation per kg of body weight (equivalent to approximately 582 mg of full-length GM-HRA protein per kg of body weight). Based on expression levels of GM-HRA protein in 305423 soybean grain measured in several studies, a child weighing 10 kg would have to consume 2,120 kg/day of 305423 soybean grain to match the dose used in the mouse acute toxicity test. An adult weighing 60 kg would have to consume 12,720 kg/day of 305423 soybean grain. Based on these simplistic worse-case calculations, it is clear there is a wide margin of safety for GM-HRA, especially if other factors such as identity preservation or soybean grain processing are taken into account.

Results of the safety assessment indicate that the GM-HRA protein is unlikely to cause an allergic reaction in humans or be a toxin in humans or animals, and therefore is safe for human and animal consumption.

VIII. Agronomic Performance and Ecological Observations

An agronomic and phenotypic evaluation of 305423 soybean was conducted to assess its familiarity and agronomic equivalence compared to conventional soybean. 305423 soybean was also observed for response to insect and disease stressors. These evaluations form the basis for the determination that 305423 soybean is no more likely to pose a plant pest risk than conventional soybeans.

The agronomic and phenotypic evaluation was based on both laboratory experiments and replicated, multi-site field trials conducted by agronomists and scientists who are considered experts in the production and evaluation of soybean. In each of these assessments, 305423 soybean was compared to either a Jack control or a null segregant line that did not carry the *gm-fad2-1* gene fragment or *gm-hra* gene.

To evaluate the agronomic and phenotypic characteristics of 305423 soybean, data were collected to address specific characteristics that influence reproductive and survival biology.

VIII-A. Germination and Dormancy Evaluations

In order to test germination and potential dormancy, seeds from the BC1F6 generation (Figure 2) of 305423 soybean were tested for germination under both cold and warm growing conditions using standard laboratory tests. The control was null segregant soybean of the BC1F6 generation.

The cold germination test consisted of four replicates containing 100 seeds each of 305423 and control soybean. The seeds were sown into a saturated soil/sand mixture and placed in a 10°C chamber for four days. After four days, the seed was moved to a 25°C chamber for an additional three days. At the end of the seven days, the number of germinated seeds was recorded.

The warm germination test consisted of eight replicates containing 50 seeds each of 305423 soybean and the control. The seeds were placed between sheets of germination towels and placed in a 25°C chamber at approximately 90% relative humidity for five days. After five days, the number of germinated seeds was recorded.

Results of the seed germination testing are presented in Table 6. Both 305423 and control soybean showed a high rate of germination (99% or greater) in both the warm and cold germination tests. Out of the 800 total seeds tested from each of 305423 and control soybean, no seeds remained hard after the germination tests confirming none of them were dormant.

These results show that the germination and dormancy characteristics of 305423 soybean were not altered when compared to nontransgenic soybean. Based on this information, 305423 soybean is unlikely to exhibit an increased potential for weediness as compared to conventional soybeans.

Table 6. Germination and Dormancy Evaluation of 305423 Soybean

Test substance	Germination test % germination (number of seeds germinated / number of seeds tested)		Number of hard seeds	
	Warm germination test	Cold germination test	Warm germination test	Cold germination test
305423 soybean	100% (399/400)	99% (397/400)	0	0
Control (null segregant)	100% (399/400)	100% (398/400)	0	0

VIII-B. Field Trial Evaluations

305423 soybean has been field tested in the United States since 2002 as authorized by USDA permits listed in Appendix 4. The list compiles a number of test sites in diverse regions of the U.S. including the major soybean growing areas of the Midwest and a winter nursery in Hawaii. Agronomic and phenotypic data were collected to assess agronomic comparability as it relates to plant pest potential. Certain agronomic data, for example seed shattering, can also be used for an assessment of enhanced weed potential for 305423 soybean.

Throughout the development process, additional qualitative phenotypic traits such as flower color, pubescence color, pod color, and hila color were also monitored. Flower color in soybean can be purple (dominant) or white (recessive). Pubescence color is based on the short hairs on soybean plant stems and pods at maturity. Pubescence color in soybean can be tawny (dominant) or grey (recessive). Pods on mature soybeans can be brown (dominant) or tan (recessive). The hilum is a scar visible on the soybean seed's coat; colors include yellow, grey, black, imperfect black, buff or brown.

The recipient variety for 305423 soybean, Jack, has white flowers, grey pubescence, brown pods at maturity and yellow hila. Throughout the breeding and development process for 305423 soybean, scientists skilled in the art of plant breeding and agricultural science monitored 305423 soybean for these expected phenotypic traits. Visual observations confirmed these qualitative phenotypic traits for 305423 soybean were unchanged from Jack and were stable through the breeding process over a four-year time period and at least seven generations, when in a Jack genetic background.

Also throughout the development process, 305423 soybean was observed for unexpected differences in responses to abiotic stress (*e.g*, drought, excess moisture, temperature extremes, *etc*.). These monthly observations were qualitative and opportunistic, but 305423 soybean and controls (non-transgenic near isoline Jack, null segregants, and/or conventional soybean lines with similar genetics) were similar with respect to their response to abiotic stress.

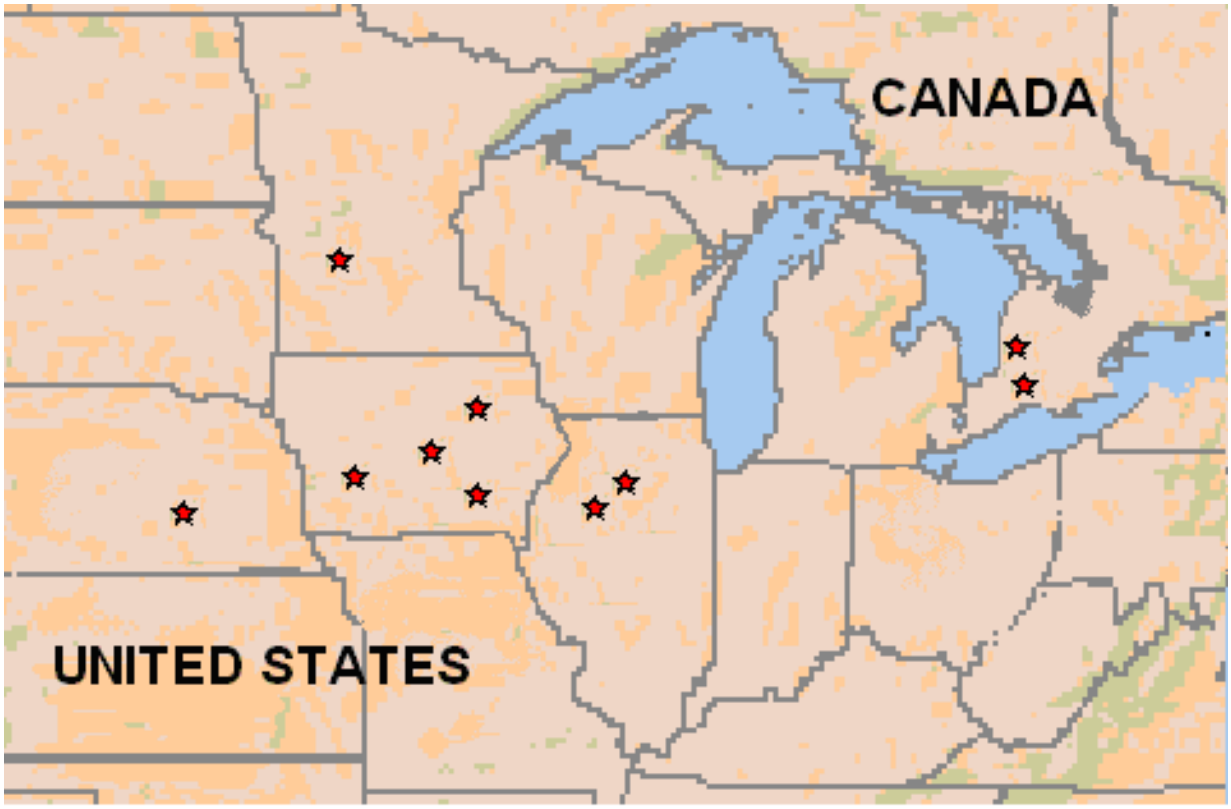
Agronomic data were collected from three experiments (denoted A, B and C) conducted at 13 total field locations over two consecutive years (Figure 23). Categorical and quantitative data were collected from 305423 and control soybeans from nine field locations in 2005 and an additional four field locations in 2006. The trial locations provided a range of environmental and agronomic conditions representative of the major soybean growing regions where commercial production of 305423 soybean is expected. Agronomic practices used to prepare and maintain each field site were characteristic of each respective region.

Table 7 outlines the quantitative phenotypic characteristics that were collected during the 2005 and 2006 growing seasons.

In a separate experiment, four conventional commercial Pioneer brand soybean varieties, 92M10, 92B12, 92B63, and 92M72 (“reference varieties”) were grown in 2005 at six field locations in soybean-growing areas of North America. Data from the reference varieties was used to help determine the normal variation seen in soybean and to develop the statistical tolerance intervals.

Field trial evaluations provide evidence that 305423 soybean is comparable to its nontransgenic near isoline, Jack, and does not exhibit any unexpected qualitative phenotypic traits or unexpected responses to abiotic stress.

Figure 23. Map of 2005 and 2006 Field Locations for Agronomic Data Collection for 305423 Soybean



Experiment A, 2005 Purpose: Agronomic/yield evaluation 1. Stuart, IA 2. Johnston, IA 3. Cedar Falls, IA
Experiment B, 2005 Purpose: Composition, expression and agronomic evaluation 1. Wyoming, IL 2. Richland, IA 3. Paynesville, MN 4. York, NE 5. Thorndale, ON, Canada 6. Branchton, ON, Canada
Experiment C, 2006 Purpose: Agronomic/yield evaluation 1. Stuart, IA 2. Johnston, IA 3. Cedar Falls, IA 4. Princeton, IL

Table 7. Field Agronomic Characteristics Measured

General characteristic	Characteristic measured	Evaluation timing*	Data description	Scale
Germination / emergence	Emergence score	VC-V2	Visual estimate of plant emergence	From 1 to 9, where 1=0-10% plants emerged, and 9= 90-100% plants emerged
	Early population	VC-V2	Number of plants emerged per plot	Actual count per plot
	Seedling vigor	VC-V2	Visual estimate of average vigor of emerged plants per plot	From 1 to 9, where 1=short plants with small leaves, and 9=tall plants with large leaves
Vegetative parameters	Plant height	Approximately R6	Height in cm from the soil surface to the tip of the highest leaf when extended by hand	Height in cm
	Lodging	Approximately R8	Visual estimate of lodging severity	From 1 to 9, where 1=plants laying flat, and 9=plants standing straight
	Final population	Approximately R8	The number of plants remaining per plot	Actual count per plot
Reproductive parameters	Days to maturity	R8	Difference between maturity date and planting date	Number of days
	Shattering score	Approximately R8	Visual estimate of grain shattering at maturity	From 1 to 9, where 1=high shattering and 9=no shattering
	Seed weight	R8	Weight of 100 random seeds	Grams
	Yield	Approximately R8	Harvest weight per area adjusted to 13% moisture content	Bushels per acre
Ecological interactions	Disease incidence	Approximately R6	Visual estimate of foliar disease incidence	From 1 to 9, where 1=poor disease resistance or high infection, and 9=best disease resistance or low infection
	Insect damage	Approximately R6	Visual estimate of insect damage	From 1 to 9, where 1=poor insect resistance or high damage, and 9=best insect resistance or low damage

* Refer to Gaska, 2006 for a description of soybean growth stages.

Experiment A was planted at three locations in the major soybean growing region of the Midwest during the 2005 growing season. The purpose of Experiment A was to evaluate the agronomic characteristics and yield of 305423 soybean (Figure 23). Seed from the T7 generation was used (Figure 2). The control was Jack, the non-transgenic isoline.

The following characteristics were measured: emergence score, shattering score, yield, days to maturity, and seed weight. Descriptions of the characteristics and their measurement are found in Table 7. Seed was planted in rows 12 feet long and 30 inches apart, with 110 seeds per row. Plants were not thinned. Normal agronomic practices were employed throughout the growing season. There were nine replicates of 305423 soybean and two replicates of control Jack soybean at each location.

Results of Experiment A are summarized in Table 8. Categorical data collected during this study (*i.e.*, emergence score and shattering score) were not statistically analyzed. Other agronomic data (yield, days to maturity and seed weight) were analyzed using Statistical Analysis Software (SAS). Refer to Appendix 3 for the statistical model used.

305423 soybean from the T7 generation was similar to control Jack soybean for all characteristics measured except for days to maturity and seed weight. No statistically significant differences were observed for mean yield between the 305423 and control soybean when data were evaluated across locations. A statistically significant difference was observed for days to maturity and seed weight however, these differences are small in magnitude and not biologically meaningful to breeders. Means obtained for both 305423 soybean and control Jack soybean were within the normal variation of seed weight of commercial soybeans (Hartwig, 1973). Results of Experiment A indicate 305423 soybean is agronomically comparable to control Jack soybean that does not contain the *gm-fad2-1* gene fragment and *gm-hra* gene.

Table 8. Summary of Experiment A: 2005 Agronomic Performance of 305423 Soybean across Three Locations

Agronomic characteristic	Control (Jack)	305423 soybean	P-value
Emergence score (1-9 score)	9	9	Not applicable
Shattering score (1-9 score)	9	9	Not applicable
Yield (bushels/acre)	49.4	46.2	0.0714
Days to maturity (days)	126	124	<0.0001[1]
Seed weight (grams per 100 seeds)	15.4	13.7	<0.0001[1]

[1] P-value < 0.05 denotes a statistically significant difference.

Experiment B was planted at six locations in commercial soybean growing regions of North America during the 2005 growing season. The purpose of Experiment B was to evaluate the agronomic characteristics of 305423 soybean and collect tissue samples for determining the transgenic protein levels and nutrient composition (Figure 23). Seed from the BC1F5 generation was used and the control plants were null segregants of the BC1F5 generation (Figure 2).

In a separate experiment, four conventional commercial soybean varieties ("reference varieties") were grown in 2005 at six field locations in soybean-growing areas of North America (Bagley, IA, York, NE, Glen Allen, VA, Germansville, PA, Larned, KS and Branchton, ON, Canada). The purpose of this experiment was to obtain agronomic characteristics that were used to help determine the normal variation seen in soybean. The reference varieties were planted, harvested, and analyzed using the same methods as were used for the 305423 soybean and the null segregant control isoline.

In both experiments, seed was planted in rows 25 feet long and 30 inches apart, with 112 seeds per row. Plants were not thinned. Normal agronomic practices were employed throughout the growing season. There were three replicates at each location, planted in a randomized complete block design of two-row plots. Each two-row plot was bordered on each side by one row of non-transgenic, commercial soybeans of a similar relative maturity. The following characteristics were measured: early population, final population, seedling vigor, lodging, shattering score, disease incidence, insect damage, plant height, days to maturity and yield. Descriptions of the characteristics and their measurement are found in Table 7. The statistical analysis of agronomic data, including categorical data, was conducted to test for differences in the mean values between the 305423 soybean and the null segregant control.

Using the data obtained from the reference varieties, a tolerance interval was calculated to contain, with 95% confidence, 99% of the values contained in the population of commercial soybean varieties. This statistical tolerance interval for each agronomic characteristic was used for interpreting the agronomic results for 305423 soybean. In interpreting the agronomic data, emphasis was placed on the agronomic characteristic means (NRC/IOM, 2004). Means of agronomic characteristic that fell within the tolerance interval for that analyte were considered to be within the normal variability of commercial soybean varieties.

Because a greater number of characteristics were recorded in Experiment B, the statistical analysis of data was refined relative to that described for Experiment A and C in order to adjust for making multiple comparisons. When numerous comparisons are being made, it is important to control the rate of false positive results. Since the introduction of the false discovery rate (FDR) approach in the mid-1990's, it has been widely employed across a number of scientific disciplines, including genomics, ecology, medicine, plant breeding, epidemiology, dairy science and signal/image processing (*e.g.*, Pawitan *et al*., 2005; Spelman and Bovenhuis, 1998). A false positive result occurs when two means are deemed significantly different when, in fact, they are not. If one uses a 5% type I error rate for each agronomic characteristic measured, then the number of false positives increases as the number of characteristics increase. In order to help manage the false positive rate, the FDR method was applied to account for making multiple comparisons (Benjamini and Hochberg, 1995; Westfall *et. al*., 1999). P-values were adjusted accordingly. This resulted in the false positive rate being held to 5%. Both adjusted and unadjusted P-values are provided for Experiment B (Table 9). For a more detailed description of the statistical model used to analyze these data, refer to Appendix 3.

Results of Experiment B are summarized in Table 9. With the exception of early population, all characteristics measured (final population, seedling vigor, lodging, shattering score, disease incidence, insect damage, plant height, days to maturity and yield), showed no statistical differences in mean values between 305423 soybean and the null segregant control across locations (adjusted P-value > 0.05). The early population mean for 305423 soybean was

statistically significantly different from the control, however it was within the tolerance interval. The results from Experiment B indicate 305423 soybean is agronomically comparable to null segregants that do not contain the *gm-fad2-1* gene fragment and *gm-hra* gene.

Table 9. Summary of Experiment B: 2005 Agronomic Performance of 305423 Soybean across Six Locations

Agronomic characteristic		Control (null segregant)	305423 soybean	Tolerance interval[1]
Early population (number of plants)	Mean[2]	132	116	0 – 224
	Range[3]	77 - 192	64 - 169	
	Adjusted P-value[4]		0.0090[6]	
	P-value[5]		0.0009	
Final population (number of plants)	Mean	126	117	0 – 224
	Range	67 - 189	57 - 174	
	Adjusted P-value		0.1635	
	P-value		0.0327	
Seedling vigor (1-9 score)	Mean	8	8	1 – 9
	Range	6 - 9	6 - 9	
	Adjusted P-value		0.5163	
	P-value		0.2065	
Lodging (1-9 score)	Mean	6	6	3 – 9
	Range	1 - 9	1 - 9	
	Adjusted P-value		0.9942	
	P-value		0.5965	
Shattering score (1-9 score)	Mean	9	9	7 – 9
	Range	8 - 9	8 - 9	
	Adjusted P-value		0.7478	
	P-value		0.3739	
Disease incidence (1-9 score)	Mean	7	7	4 – 9
	Range	5 - 8	5 - 9	
	Adjusted P-value		1	
	P-value		0.7316	
Insect damage (1-9 score)	Mean	8	8	5 – 9
	Range	6 - 8	6 - 8	
	Adjusted P-value		1	
	P-value		1	
Plant height (cm)	Mean	74	72	0 – 183
	Range	30 - 104	27 - 94	
	Adjusted P-value		0.5163	
	P-value		0.1827	
Days to maturity (days)	Mean	117	117	58.1 – 167
	Range	102 - 126	99 - 126	
	Adjusted P-value		1	
	P-value		1	
Yield (bushels/acre)	Mean	34.6	34.4	0 – 133
	Range	12.7 – 52.2	10.4 – 50.1	
	Adjusted P-value		1	
	P-value		0.8984	

[1] Tolerance intervals have been adjusted to the minimum and maximum values per observation when necessary.
[2] Least Squares Mean
[3] Range denotes the lowest and highest individual values across locations.
[4] False Discovery Rate (FDR) adjusted P-value
[5] Non-adjusted P-value
[6] Statistically significant difference, FDR adjusted P-value <0.05.

Experiment C was planted at four locations in the major soybean growing region of the Midwest during the 2006 growing season. The purpose of Experiment C was to evaluate the agronomic characteristics and yield of 305423 soybean (Figure 23). Seed from the T8 generation was used (Figure 2). The control was Jack, the non-transgenic isoline.

The following characteristics were measured: emergence score, shattering score, yield, days to maturity, and seed weight. Descriptions of the characteristics and their measurement are found in Table 7. Seed was planted in rows 12 feet long and 30 inches apart, with 110 seeds per row. Plants were not thinned. Normal agronomic practices were employed throughout the growing season. There were nine replicates of 305423 soybean and three replicates of control Jack soybean at each location.

Results of Experiment C are summarized in Table 10. Categorical data collected during this study (*i.e.*, emergence score and shattering score) were not statistically analyzed. Other agronomic data (yield, days to maturity and seed weight) were analyzed using Statistical Analysis Software (SAS). Refer to Appendix 3 for the statistical model used.

305423 soybean from the T8 generation was similar to control Jack soybean for all characteristics measured except for seed weight. No statistically significant differences were observed for mean yield and days to maturity between the 305423 and control soybean when data were evaluated across locations. A statically significant difference was observed for seed weight however, these differences are small in magnitude and not biologically meaningful to breeders. Means obtained for both 305423 soybean and control Jack soybean were within the normal variation of seed weight of commercial soybeans (Hartwig, 1973). Results of Experiment C indicate 305423 soybean is agronomically comparable to control Jack soybean that does not contain the *gm-fad2-1* gene fragment and *gm-hra* gene.

Table 10. Summary of Experiment C: 2006 Agronomic Performance of 305423 Soybean across Four Locations

Agronomic characteristic	Control (Jack)	305423 soybean	P-value
Emergence score (1-9 score)	9	9	Not applicable
Shattering score (1-9 score)	9	9	Not applicable
Yield (bushels/acre)	49.6	47.2	0.1398
Days to maturity (days)	127	126	0.0814
Seed weight (grams per 100 seeds)	14.5	13.5	<0.0001[1]

[1] P-value < 0.05 denotes a statistically significant difference.

VIII-C. Ecological Observations

Ecological observations (plant interactions with insect pests and diseases) were recorded for all USDA-APHIS permitted field trials of 305423 soybean during the 2002, 2003, 2004 and 2005 growing seasons. Plant breeders, plant pathologists and entomologists, and others skilled in the art of agricultural science observed 305423 soybean and control lines at least every four weeks for insect and disease pressure and recorded the severity of any stressor seen. Any unexpected qualitative differences in response between 305423 soybean and various control lines (non-transgenic near isoline Jack, null segregants, and/or conventional soybean lines) were recorded.

A summary of the ecological observations is presented in Table 11 for insect stressors and in Table 12 for disease stressors. This data is also presented at a more detailed level in Appendix 4, Tables 2-4 for experiments A, B and C. In every case, the severity of insect or disease stress on 305423 soybeans was not qualitatively different from various control lines growing at the same location. These results support the conclusion that the ecological interactions for 305423 soybeans were similar to control soybean lines with similar genetics or to conventional soybean lines.

Table 11. Insect Stressor Incidence Comparison of 305423 Soybean and Control Soybean Lines Across All Locations in 2002, 2003, 2004 and 2005[1]

Insect stressor	State (number of counties)	Range of severity in 305423 soybean[2]	Differences with control?
Bean leaf beetle (*Certoma trifurcata*)	IA (5)	mild to moderate	no
	IL (1)	mild	no
	MN (1)	mild	no
	NE (1)	very mild to mild	no
Grasshopper (Orthoptera)	IA (1)	mild	no
	IL (1)	mild	no
	MN (1)	mild	no
	NE (1)	very mild to mild	no
Corn leaf aphid (*Rhopalosiphum maidis*)	HI (1)	mild	no
Rose beetle (*Adoretus sinicus*)	HI (1)	mild to moderate	no
Soybean aphid (*Aphis glycines*)	IA (4)	mild to moderate	no
	IL (1)	very mild	no
	MN (1)	mild	no
Soybean leaf miner (*Odontota horni*)	HI (1)	mild to moderate	no
Stink bug (Pentatomidae)	HI (1)	mild to moderate	no
Whitefly (*Bemisia* sp.)	HI (1)	mild to moderate	no

[1] In USDA field data reports, event DP-3Ø5423-1 is called EAFS 3054.2.3.

[2] Scale: Mild – very little insect injury (<10%) visible;
Moderate –noticeable plant tissue damage (10% to 30%);
Severe – significant plant tissue damage (>30%).

Table 12. Disease Stressor Incidence Comparison of 305423 Soybean and Control Soybean Lines Across All Locations in 2002, 2003, 2004 and 2005[1]

Disease stressor	State (number of counties)	Range of severity in 305423 soybean[2]	Differences with control?
Alternaria (*Alternaia* spp.)	HI (1)	mild	no
Bacterial blight (*Pseudomonas savastanoi* pv. *glycinea*)	IA (3)	mild	no
Bacterial pustule (*Xanthomonas axonopodis* pv. *glycines*)	IA (1)	mild	no
Brown spot (*Septoria glycines*)	IA (4)	mild to moderate	no
	NE (1)	very mild to mild	no
Downey mildew (*Peronospora manshurica*)	HI (1)	mild to moderate	no
	MN (1)	mild	no
Frogeye leaf spot (*Cercospora sojin*a)	IA (1)	mild	no
Powdery mildew (*Microsphaera diffusa*)	IA (2)	mild	no
	MN(1)	mild	no

[1] In USDA field data reports, event DP-3Ø5423-1 is called EAFS 3054.2.3.
[2] Scale: Mild – very little disease injury (<10%) visible;
Moderate – noticeable plant tissue damage (10% to 30%);
Severe – significant plant tissue damage (>30%).

VIII-D. Conclusions

The 305423 soybean was observed in laboratory experiments and at 13 field locations over two years to measure agronomic and phenotypic data. Data generated from these studies represent observations that are typically recorded by plant breeders and agronomists to evaluate the qualities of soybean over a broad range of environmental conditions that 305423 soybean would encounter. The measured characteristics provide crop biology data useful in establishing a basis to assess phenotypic comparability and familiarity of 305423 soybean compared to conventional soybean in the context of ecological risk assessment.

The agronomic and phenotypic data showed no biologically meaningful differences between 305423 soybean and control soybean (the near isoline control Jack, null segregants of 305423 soybean, and conventional soybean lines) with respect to phenotype, germination, vegetative growth, reproductive parameters and response to biotic stressors. These data support the conclusion that 305423 soybean is comparable in agronomic characteristics to conventional soybean. The data also support the conclusion that 304523 soybean is unaltered with respect to weediness and plant pest risk. Likewise, assessment of the ecological data detected no biologically significant differences between 305423 soybean and control soybean lines, indicating no selective advantage that would result in increased weed potential for 305423 soybean.

IX. Compositional Assessment

Compositional analysis of 305423 soybean was used to confirm the high oleic fatty acid profile and evaluate any changes in the levels of key nutrients, isoflavones, or antinutrients compared to the null segregant control. Along with agronomic data, compositional analysis is a general indicator that 305423 soybean will not exhibit unexpected effects with respect to plant pest risk. The U.S. FDA is reviewing the details of the compositional analysis as a component of the safety assessment of 305423 soybean.

Comprehensive compositional analyses were performed on grain and forage tissues collected from the BC1F5 generation of 305423 soybean and the null segregant control grown in 2005 at six field locations in soybean-growing areas of North America (Experiment B, Figure 23).

In a separate experiment, grain and forage tissues were also collected from four conventional commercial soybean varieties ("reference varieties") grown in 2005 at six field locations in soybean-growing areas of North America (refer to Section VIII-B, Experiment B). The reference varieties were planted, harvested, processed, and analyzed using the same methods as were used for the 305423 soybean and the null segregant control. Compositional analysis of the reference varieties was used to help determine the normal variation for the measured analytes.

In both experiments, seed was planted in rows 25 feet long and 30 inches apart, with 112 seeds per row. Normal agronomic practices were employed throughout the growing season. There were three replicates at each location, planted in a randomized complete block design of two-row plots. Each two-row plot was bordered on each side by one row of non-transgenic, commercial soybeans of a similar relative maturity.

The compositional assessment was conducted in accordance with the OECD consensus document on compositional considerations for new varieties of soybean (OECD, 2001). Compositional analysis of forage samples included proximates (protein, fat and ash), acid detergent fiber (ADF), and neutral detergent fiber (NDF). Compositional analysis of grain samples included proximates (protein, fat and ash), ADF, NDF, fatty acids, amino acids, isoflavones, and key antinutrients (stachyose, raffinose, lectins, phytic acid, and trypsin inhibitor).

Statistical analysis of nutrient composition data was conducted to test for differences in the analyte mean values between the 305423 soybean and the null segregant control (for details, refer to Appendix 3). Both adjusted and non-adjusted P-values are provided in this composition assessment section (refer to Section VIII-B, Experiment B and Appendix 3 for more information about the use of the false discovery rate (FDR) adjustment). A significant difference between the mean of 305423 soybean and that of the null segregant control was established with a FDR-adjusted P-value <0.05.

Further context for data interpretation was provided through the use of tolerance intervals and published literature. Using the data obtained from the reference varieties, a tolerance interval was calculated to contain, with 95% confidence, 99% of the values contained in the population of commercial soybean varieties. This statistical tolerance interval and the combined range of values for each analyte available from the published literature (OECD, 2001; ILSI 2004; Taylor *et al*., 1999; Kim *et al*., 2005) were used for interpreting the composition results for 305423 soybean. In interpreting the compositional data, emphasis was placed on the analyte means (NRC/IOM, 2004). Analyte means that fell within the tolerance interval and/or combined literature range for that analyte were considered to be within the normal variability of commercial soybean varieties.

IX-A. Proximates and Fiber in Soybean Forage

Composition of forage was analyzed as soybeans are occasionally used as an alternative forage source when alfalfa or clover are in short supply due to winter-killing or drought conditions. No statistically significant (adjusted P-value >0.05) differences were observed between the 305423 and control soybean line mean values for protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF) and ash (Table 13). For each analyte measured, all means were found to be within the statistical tolerance intervals and/or the combined literature ranges.

In conclusion, proximate and fiber analysis of soybean forage demonstrated that 305423 soybean is comparable to null segregant control and reference soybean lines. No unexpected differences in the nutrient composition of 305423 soybean forage were seen.

Table 13. Proximates and Fiber in Soybean Forage

Analyte (% dry weight)		Control (null segregant)	305423 soybean	Tolerance interval[1]	Combined literature range[2]
Protein	Mean[3]	24.6	24.6	14.2 - 34.1	11.2 - 24.7
	Range[4]	22.2 - 26.3	22.4 - 26.5		
	Adjusted P-value[5]		0.9983		
	P-value[6]		0.9983		
Fat	Mean	4.66	4.27	2.53 - 6.80	1.30 - 5.13
	Range	3.74 - 5.35	3.89 - 4.82		
	Adjusted P-value		0.0807		
	P-value		0.0249		
ADF	Mean	27.9	27.7	14.3 - 40.8	32.0 – 38.0
	Range	25.2 - 33.5	20.7 - 31.3		
	Adjusted P-value		0.8866		
	P-value		0.8428		
NDF	Mean	48.8	48.3	23.8 - 61.5	34.0 – 40.0
	Range	37.6 - 62.5	41.0 - 61.6		
	Adjusted P-value		0.7997		
	P-value		0.7074		
Ash	Mean	8.99	9.07	0 - 19.5	6.72 - 10.8
	Range	7.24 - 10.7	6.95 - 11.2		
	Adjusted P-value		0.7921		
	P-value		0.6845		

[1] Negative tolerance limits were set to zero.
[2] Literature ranges are taken from published literature for soybeans (OECD, 2001; ILSI 2004; Taylor *et al.*, 1999).
[3] Least Squares Mean
[4] Range denotes the lowest and highest individual value across locations.
[5] False Discovery Rate (FDR) adjusted P-value
[6] Non-adjusted P-value

IX-B. Proximates and Fiber in Soybean Grain

Proximates and fiber were analyzed in soybean grain (Table 14). No statistically significant (adjusted P-value >0.05) differences were observed between the 305423 and control soybean line mean values for protein, fat, acid detergent fiber (ADF), and neutral detergent fiber (NDF). The mean value for ash was slightly lower in 305423 soybean as compared to the control line (adjusted P-value <0.05). Mean values of 305423 and control soybean for all analytes were within both the statistical tolerance intervals and literature ranges.

In conclusion, proximate and fiber analysis of soybean grain demonstrate that 305423 soybean is comparable to near isoline and reference soybean lines. No unexpected differences in the proximate and fiber composition of 305423 soybean grain were seen.

Table 14. Proximates and Fiber in Soybean Grain

Analyte (% dry weight)		Control (null segregant)	305423 soybean	Tolerance interval	Combined literature range[1]
Protein	Mean[2]	40.7	41.2	29.9 - 48.7	32.0 - 47.4
	Range[3]	38.3 - 42.6	37.6 - 42.9		
	Adjusted P-value[4]		0.3907		
	P-value[5]		0.1833		
Fat	Mean	15.9	14.9	7.01 - 24.2	8.10 - 24.7
	Range	12.2 - 18.8	12.4 - 17.7		
	Adjusted P-value		0.1091		
	P-value		0.0377		
ADF	Mean	14.3	14.0	8.51 - 22.1	7.81 - 18.6
	Range	10.1 - 17.7	8.49 - 18.8		
	Adjusted P-value		0.7997		
	P-value		0.7108		
NDF	Mean	13.5	13.6	8.07 - 21.9	4.50 - 21.3
	Range	9.91 - 16.9	9.61 - 17.7		
	Adjusted P-value		0.8378		
	P-value		0.7625		
Ash	Mean	5.23	4.91	3.19 - 7.67	3.89 - 6.99
	Range	4.59 - 6.20	4.35 - 5.69		
	Adjusted P-value		0.0007[6]		
	P-value		0.0001		

[1] Literature ranges are taken from published literature for soybeans (ILSI 2004; Taylor *et al*., 1999).
[2] Least Squares Mean
[3] Range denotes the lowest and highest individual value across locations.
[4] False Discovery Rate (FDR) adjusted P-value
[5] Non-adjusted P-value
[6] Statistically significant difference; adjusted P-value < 0.05

IX-C. Fatty Acids in Soybean Grain

Soybean has many uses in the food and industrial sectors and represents one of the major sources of edible vegetable oil. Triglycerides make up 99% of soybean oil. Soybean oil is noted for its high content of linoleic (C18:2) and linolenic (C18:3) polyunsaturated fatty acids. It also contains sizeable amounts of another unsaturated fatty acid, oleic (C18:1) and moderate amounts of the saturated fatty acids palmitic (C16:0) and stearic (C18:0) (OECD, 2001). As described previously, 305423 soybean has a modified fatty acid composition including increased levels of oleic acid and decreased levels of linoleic and linolenic acids, and to a lesser extent, palmitic acid when compared to commodity soybeans. The fatty acid profile of 305423 soybean is presented in this section.

Levels of 25 fatty acids were measured in 305423 and control soybean grain. Levels of eleven fatty acids were near or below the lower limit of quantitation (LLOQ) for the assay: caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristoleic acid (C14:1), pentadecanoic acid (C15:0), pentadecenoic acid (C15:1), γ-linolenic acid (C18:3), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3), arachidonic acid (C20:4), and erucic acid (C22:1). Therefore, no statistical analyses were conducted on these fatty acids and data are not shown. Results of the analysis for the 14 remaining fatty acids are presented in Table 15.

Fatty acid analysis confirmed the expected high oleic phenotype. The mean value for oleic acid (C18:1) was significantly increased, and the mean values for palmitic (C16:0), linoleic acid (C18:2) and linolenic acid (C18:3) were significantly decreased between the 305423 and control soybean (adjusted P-values <0.05). The increase in the oleic acid content and the decrease in linoleic acid content in 305423 soybean are intended effects achieved through introduction of the *gm-fad2-1* gene fragment (refer to Section VI-A for a molecular basis of the high oleic phenotype, Figure 11). Linolenic acid is produced directly from conversion of linoleic acid (see Section VI-A, Figure 11) and therefore the decrease in the linoleic acid content was expected to cause a decrease in the linolenic acid content in 305423 soybean. The mean value for palmitic acid in 305423 soybean was within the statistical tolerance interval and/or the combined literature range

The (9,15) isomer of linoleic acid (cis-9, cis-15-octadecadienoic acid) was measured as this isomer has been previously reported in high oleic soybean oil at less than 1% of the total fatty acid content (Kinney and Knowlton, 1997). The 9,15-linoleic acid isomer was detected in 305423 soybean at the mean concentration of 0.341% of the total fatty acids and in control soybeans at 0.247% (no statistically significant difference (adjusted P-value >0.05)), while the reference varieties did not contain measurable concentrations of this analyte. This isomer is also found, at concentrations ranging from 0.02% to 5.4% of the total fatty acids, in many edible sources of fat including butterfat, cheese, beef and mutton tallow, partially hydrogenated vegetable oils, human milk and mango pulp (Kinney and Knowlton, 1997, and references therein). The 9,15-linoleic acid isomer is likely a result of the activity of the fatty acid desaturase encoded by the *FAD3* gene that normally inserts a δ-15 double bond into 9,12-linoleic acid (also referred to as linoleic acid (C18:2)) to produce 9,12,15-linolenic acid (Section VI-A, Figure 11). In the 305423 soybean, the linoleic acid content is significantly reduced (Table 15) so that the *FAD3*-encoded desaturase likely creates a small amount of the 9,15-linoleic acid isomer by desaturating the abundant 9-oleic acid substrate at the δ-15 position.

The mean values for myristic acid (C14:0), palmitoleic acid (C16:1), stearic acid (C18:0), arachidic acid (C20:0), eicosenoic acid (C20:1) and lignoceric acid (C24:0) were statistically significantly different (adjusted P-values <0.05) from control soybeans. The mean value for behenic acid (C22:0) was not statistically significantly different. These are relatively minor fatty acids, together comprising less than 6% of the total fatty acids in 305423 soybean. These fatty acids are common constituents of vegetable oils and common foodstuffs and are present at levels similar to those observed in 305423 soybean (USDA, 2006). The mean values for all these fatty acids in 305423 soybean were within the statistical tolerance intervals and/or the combined literature ranges.

The mean values of two minor fatty acids, heptadecanoic acid (C17:0) and heptadecenoic acid (C17:1) were statistically significantly different (adjusted P-value <0.05) between the 305423 and control soybean. Mean values for C17:0 and C17:1 in 305423 soybean were above the upper range of the statistical tolerance intervals and literature ranges for conventional soybean varieties. However, levels of heptadecanoic and heptadecenoic acid are in general still very low; together representing less than 2% of the total fatty acid content in 305423 soybean.

The detected increase in heptadecanoic acid (C17:0) and heptadecenoic acid (C17:1) in 305423 soybean is not unexpected, as expression of the GM-HRA protein likely results in a slight shift in availability of the GM-HRA enzyme substrates, pyruvate and 2-ketobutyrate. These two compounds are also substrates for the enzyme complex that initiates oil biosynthesis. Refer to Appendix 7-1 for information regarding biosynthesis of 17-carbon fatty acids in soybean.

Both C17:0 and C17:1 are found in vegetable oils (USDA, 2006 and Pioneer data). C17:0 is found in corn, soybean, sunflower, peanut and olive oils, as well as butter. C17:1 is found in olive oil. Both C17:0 and C17:1 fatty acids are also found in many different kinds of commonly consumed foods. The USDA (2006) nutrition database can be searched to identify the foods highest in various components. C17:0 is commonly found in meat (lamb, beef, pork) and butter at levels comparable to those seen in the 305423 samples. The highest concentrations of C17:0 in foods are found in lamb, tofu, and butter. C17:1 is also found in a wide variety of foods such as tofu, beef, cheese, and various baked products. The highest concentrations of C17:1 in foods are found in tofu and ground beef. Levels of C17:0 and C17:1 in 305423 soybean are comparable to those already found in the diet and there is no evidence to indicate that exposure to either C17:0 or C17:1 from these dietary sources is associated with adverse effects in humans. These fatty acids, like all other fatty acids, are metabolized through the process of β-oxidation (refer to Appendix 7.2. for a description of the 17-carbon fatty acid metabolism in humans and animals). Based on their metabolism, their wide distribution in nature as well as the fact that fatty acids are not toxins nor do they cause allergic reactions, no safety or nutritional issues are expected as a result of this slight increase in exposure. A detailed assessment of the food and feed safety and nutritional value of 305423 soybean has been submitted to FDA.

In conclusion, fatty acid analysis of soybean grain confirmed that 305423 soybean had the intended changes in the fatty acid profile due to introduction of the *gm-fad2-1* gene fragment. An increase in two minor fatty acids, C17:0 and C17:1, was detected in 305423 soybean, but this was not unexpected. This observation is linked to the introduction of the *gm-hra* gene and is likely a result of a shift in substrate availability (Appendix 7.1.).

Table 15. Major Fatty Acids in Soybean Grain

Fatty acid (% total)		Control (null segregant)	305423 soybean	Tolerance interval[1]	Combined literature ranges[2]
Myristic acid (C14:0)	Mean[3]	0.0742	0.0451	0 - 0.174	0.0710 - 0.238
	Range[4]	0.0676 - 0.0807	0.0419 - 0.0522		
	Adjusted P-value[5]		0.0007[7]		
	P-value[6]		0.0001		
Palmitic acid (C16:0)	Mean	10.3	6.28	2.93 - 19.6	7.00 - 15.8
	Range	9.77 - 10.7	5.71 - 7.27		
	Adjusted P-value		0.0007[7]		
	P-value		0.0001		
Palmitoleic acid (C16:1)	Mean	0.0860	0.0946	0.0110 - 0.177	0.0860 - 0.194
	Range	0.0751 - 0.0948	0.0835 - 0.105		
	Adjusted P-value		0.0248[7]		
	P-value		0.0053		
Heptadecanoic acid (C17:0)	Mean	0.113	0.798	0.0722 - 0.131	0.0850 - 0.146
	Range	0.0993 - 0.127	0.703 - 0.890		
	Adjusted P-value		0.0007[7]		
	P-value		0.0001		
Heptadecenoic acid (C17:1)	Mean	0.0614	1.19	0.0351 - 0.0732	0.0730 - 0.0870
	Range	0.0513 - 0.0762	1.01 - 1.51		
	Adjusted P-value		0.0007[7]		
	P-value		0.0001		
Stearic acid (C18:0)	Mean	4.98	4.36	0.852 - 8.34	2.00 - 5.88
	Range	4.36 - 5.89	3.90 - 5.01		
	Adjusted P-value		0.0007[7]		
	P-value		0.0001		
Oleic acid (C18:1)	Mean	21.1	76.5	11.3 - 32.6	14.3 – 34.0
	Range	18.0 - 24.1	68.7 - 79.4		
	Adjusted P-value		0.0007[7]		
	P-value		0.0001		
Linoleic acid (C18:2)	Mean	52.5	3.62	41.7 - 64.3	42.3 - 60.0
	Range	50.2 - 54.3	1.53 - 8.98		
	Adjusted P-value		0.0007[7]		
	P-value		0.0001		
Linoleic acid (C18:2) isomer (9,15)	Mean	0.247	0.341	NA[8]	NR[9]
	Range	0 - 0.532	0.143 - 0.456		
	Adjusted P-value		0.1787		
	P-value		0.0699		
Linolenic acid (C18:3)	Mean	9.35	5.39	1.15 - 14.7	2.00 - 12.5
	Range	7.83 - 11.2	4.03 - 7.32		
	Adjusted P-value		0.0007[7]		
	P-value		0.0001		

Table 15, continued. Major Fatty Acids in Soybean Grain

Fatty acid (% total)		Control (null segregant)	305423 soybean	Tolerance interval[1]	Combined literature ranges[2]
Arachidic acid (C20:0)	Mean	0.396	0.450	0.103 - 0.619	0 - 1.00
	Range	0.348 - 0.479	0.393 - 0.528		
	Adjusted P-value		0.0007[7]		
	P-value		0.0001		
Eicosenoic acid (C20:1)	Mean	0.170	0.347	0.0549 - 0.319	0.140 - 0.350
	Range	0.135 - 0.201	0.290 - 0.394		
	Adjusted P-value		0.0007[7]		
	P-value		0.0001		
Behenic acid (C22:0)	Mean	0.414	0.427	0.188 - 0.458	0.277-0.595
	Range	0.349 - 0.566	0.382 - 0.546		
	Adjusted P-value		0.5468		
	P-value		0.3779		
Lignoceric acid (C24:0)	Mean	0.114	0.143	0 - 0.310	NR[9]
	Range	0.0845 - 0.139	0.115 - 0.173		
	Adjusted P-value		0.0017[7]		
	P-value		0.0003		

[1] Negative tolerance limits were set to zero.
[2] Literature ranges are taken from published literature for soybeans (OECD, 2001; ILSI 2004).
[3] Least Squares Mean
[4] Range denotes the lowest and highest individual value across locations.
[5] False Discovery Rate (FDR) adjusted P-value
[6] Non-adjusted P-value
[7] Statistically significant difference; adjusted P-value < 0.05
[8] Statistical analysis was not available (NA), due to lack of measurable concentrations detected for this analyte.
[9] Analyte ranges were not reported (NR) in the published literature references.

IX-D. Amino Acids in Soybean Grain

Soybeans are a source of complete protein in human diets. A complete protein is one that contains significant amounts of all the essential amino acids that must be provided to the human body because of the body's inability to synthesize them. Soybean meal is also fed to animals primarily as a source of protein. Soybeans contain relatively high levels of certain essential amino acids that are deficient in many other common feedstuffs.

Levels of 18 amino acids were measured in soybean grain (Table 16). No statistically significant (adjusted P-value >0.05) differences were observed between the 305423 and control soybean line for any of the amino acid mean values. The mean values for all amino acids analyzed were within the statistical tolerance intervals and/or the combined literature ranges.

In conclusion, amino acid analysis of soybean grain demonstrates that 305423 soybean is comparable to near isoline and reference soybean lines. No unexpected differences in the amino acid composition of 305423 soybean grain were seen.

Table 16. Amino Acids in Soybean Grain

Amino acid (% dry weight)		Control (null segregant)	305423 soybean	Tolerance interval	Combined literature range[1]
Methionine	Mean[2]	0.714	0.712	0.488 – 0.852	0.431 - 0.681
	Range[3]	0.644 - 0.848	0.641 - 0.766		
	Adjusted P-value[4]		0.9311		
	P-value[5]		0.9196		
Cystine	Mean	0.638	0.614	0.378 – 0.869	0.370 - 0.808
	Range	0.489 - 0.730	0.554 - 0.689		
	Adjusted P-value		0.3013		
	P-value		0.1285		
Lysine	Mean	2.56	2.58	1.98 – 3.10	2.29 - 2.86
	Range	2.34 - 2.76	2.27 - 2.83		
	Adjusted P-value		0.8378		
	P-value		0.7681		
Tryptophan	Mean	0.496	0.507	0.359 – 0.632	0.356 - 0.670
	Range	0.449 - 0.597	0.436 - 0.605		
	Adjusted P-value		0.5468		
	P-value		0.3648		
Threonine	Mean	1.91	1.95	1.57 – 2.21	1.14 - 1.89
	Range	1.78 - 2.02	1.77 - 2.06		
	Adjusted P-value		0.0797		
	P-value		0.0236		
Isoleucine	Mean	1.78	1.79	1.56 – 2.09	1.46 - 2.12
	Range	1.69 - 1.91	1.59 - 1.90		
	Adjusted P-value		0.6926		
	P-value		0.5729		
Histidine	Mean	1.17	1.21	0.897 - 1.41	0.878 - 1.22
	Range	0.982 - 1.36	1.07 - 1.39		
	Adjusted P-value		0.5156		
	P-value		0.2893		
Valine	Mean	1.84	1.87	1.58 - 2.18	1.50 - 2.44
	Range	1.72 - 2.01	1.66 - 2.02		
	Adjusted P-value		0.5468		
	P-value		0.36		
Leucine	Mean	2.97	2.99	2.53 - 3.52	2.20 - 4.00
	Range	2.85 - 3.15	2.73 - 3.16		
	Adjusted P-value		0.6778		
	P-value		0.5439		
Arginine	Mean	2.81	2.99	2.01 - 3.60	2.29 - 3.49
	Range	2.57 - 3.11	2.69 - 3.44		
	Adjusted P-value		0.1787		
	P-value		0.0723		
Phenylalanine	Mean	2.07	2.10	1.74 - 2.43	1.60 - 2.35
	Range	1.92 - 2.28	1.87 - 2.23		
	Adjusted P-value		0.6926		
	P-value		0.5727		

Table 16, continued. Amino Acids in Soybean Grain

Amino acid (% dry weight)		Control (null segregant)	305423 soybean	Tolerance interval	Combined literature range[1]
Glycine	Mean	1.89	1.93	1.54 - 2.18	1.46 - 2.02
	Range	1.75 - 2.05	1.77 - 2.06		
	Adjusted P-value		0.5468		
	P-value		0.3717		
Alanine	Mean	1.66	1.73	1.35 - 2.07	1.49 - 2.10
	Range	1.50 - 1.82	1.47 - 1.98		
	Adjusted P-value		0.4559		
	P-value		0.2195		
Aspartic acid	Mean	5.01	4.91	3.67 - 6.33	3.81 - 5.12
	Range	4.58 - 5.41	4.51 - 5.38		
	Adjusted P-value		0.1763		
	P-value		0.0653		
Glutamic acid	Mean	7.69	7.92	6.04 - 9.54	5.84 - 8.72
	Range	6.87 - 8.48	7.49 - 8.38		
	Adjusted P-value		0.0703		
	P-value		0.0191		
Proline	Mean	2.27	2.32	1.85 - 2.70	1.69 - 2.61
	Range	2.14 - 2.51	2.00 - 2.56		
	Adjusted P-value		0.3907		
	P-value		0.1805		
Serine	Mean	2.26	2.28	1.85 - 2.71	1.11 - 2.48
	Range	2.04 - 2.52	2.10 - 2.48		
	Adjusted P-value		0.5468		
	P-value		0.3492		
Tyrosine	Mean	1.34	1.36	0.908 - 1.69	1.02 - 1.62
	Range	1.13 - 1.59	1.14 - 1.48		
	Adjusted P-value		0.7921		
	P-value		0.6661		

[1] Literature ranges are taken from published literature for soybeans (OECD, 2001; ILSI 2004; Taylor *et al.*, 1999).
[2] Least Squares Mean
[3] Range denotes the lowest and highest individual value across locations.
[4] False Discovery Rate (FDR) adjusted P-value
[5] Non-adjusted P-value

IX-E. Isoflavones in Soybean Grain

Isoflavones were measured in 305423 and control soybean grain. Isoflavones in soybean have three basic types: daidzein, genistein and glycitein. Each of these three isomers, known as aglucones or free forms, can also exist in three conjugate forms: glucoside, acetylglucoside, or malonylglucoside. Therefore, there are 12 isomers of isoflavones in soybeans (OECD, 2001).

Levels of acetylgenistin, acetyldaidzin, and acetylglycitin were below the limit of quantitation for the assay used in this analysis. Therefore, no statistical analyses were conducted on these analytes and data are not shown.

No statistically significant (adjusted P-value >0.05) differences were observed between the 305423 and control soybean line mean values for genistin, genistein, daidzein, glycitin, glycitein and malonylglycitin (Table 17). The mean values for malonylgenistin, daidzin and malonyldaidzin were statistically significant (adjusted P-value <0.05) between the 305423 and control soybean. For each analyte measured, all means were found to be within the statistical tolerance intervals and/or the combined literature ranges.

In conclusion, isoflavone analysis of soybean grain demonstrates that 305423 soybean is comparable to near isoline and reference soybean lines. No unexpected differences in the isoflavone composition of 305423 soybean grain were seen.

Table 17. Isoflavones in Soybean Grain

Analyte (mg/kg dry weight)		Control (null segregant)	305423 soybean	Tolerance interval[1]	Combined literature range[2]
Genistin	Mean[3]	147	176	0 - 402	11.7 - 143
	Range[4]	88.9 - 225	106 - 308		
	Adjusted P-value[5]		0.1086		
	P-value[6]		0.0362		
Genistein	Mean	11.1	12.2	0 - 32.3	0.5 – 22.6
	Range	<4.00[7] - 26.2	<4.00[7] - 37.1		
	Adjusted P-value		0.5531		
	P-value		0.4097		
Malonylgenistin	Mean	987	1100	0 - 2810	136 - 603
	Range	565 - 1540	740 - 1530		
	Adjusted P-value		0.0017[8]		
	P-value		0.0003		
Daidzin	Mean	75.6	90.8	0 - 343	13.1 – 83.6
	Range	52.4 - 106	55.4 - 127		
	Adjusted P-value		0.0248[8]		
	P-value		0.0055		
Daidzein	Mean	13.0	12.6	0 - 47.1	0.1 – 21.2
	Range	<4.00[7] - 33.2	<4.00[7] - 49.7		
	Adjusted P-value		0.9288		
	P-value		0.9059		
Malonyldaidzin	Mean	769	830	0 - 2880	61.9 - 558
	Range	535 - 1090	508 - 1110		
	Adjusted P-value		0.0298[8]		
	P-value		0.007		
Glycitin	Mean	43.7	48.9	0 - 115	1.1 – 33.5
	Range	17.7 - 85.2	18.5 - 104		
	Adjusted P-value		0.5343		
	P-value		0.31		
Glycitein	Mean	5.02	4.35	0 - 12.0	ND[9]
	Range	<4.00[7] - 9.63	<4.00[7] - 5.60		
	Adjusted P-value		0.6269		
	P-value		0.4876		
Malonylglycitin	Mean	114	119	0 - 295	6.6 – 71.2
	Range	57.7 - 206	55.4 - 238		
	Adjusted P-value		0.5468		
	P-value		0.3837		

[1] Negative tolerance limits were set to zero.
[2] Literature ranges are taken from published literature for soybeans (Kim *et al*., 2005).
[3] Least Squares Mean
[4] Range denotes the lowest and highest individual value across locations.
[5] False Discovery Rate (FDR) adjusted P-value
[6] Non-adjusted P-value
[7] Values of the sample or samples were detected below the assay's Lower Limit of Quantitation (LLOQ). Sample results that were below the LLOQ are assigned a value equal to the LLOQ for statistical analysis.
[8] Statistically significant difference; adjusted P-value < 0.05
[9] Analyte ranges were not detected (ND) in the published literature references.

IX-F. Key Antinutrients in Soybean Grain

Soybean grain contains several key antinutrients, such as oligosaccharides, lectins, phytic acid and protease inhibitors (OECD, 2001). Low molecular weight carbohydrates stachyose and raffinose are non-digestible oligosaccharides and are considered antinutrients. Lectins are proteins that bind to carbohydrate-containing molecules. Lectins in raw soybeans can inhibit animal growth and cause death. Phytic acid binds most of the phosphorus in soybeans which results in reduced bioavailability of phosphorus for nonruminant animals. In addition, phytic acid chelates mineral nutrients including calcium, magnesium, potassium, iron and zinc, rendering them unavailable to monogastric animals consuming beans. Protease inhibitors such as trypsin inhibitor interfere with the digestion of proteins, resulting in decreased animal growth. There are two major types of protease inhibitors in soybean: the Kunitz trypsin inhibitor and the Bowman-Birk inhibitor.

Levels of key antinutrients were measured in 305423 soybean grain (Table 18). No statistically significant (adjusted P-value >0.05) differences were observed between the 305423 and control soybean line mean values for stachyose, raffinose, lectins, and phytic acid. Mean values of 305423 and control soybean for all antinutrients measured were within both the statistical tolerance intervals and literature ranges.

The mean value for trypsin inhibitor was statistically significantly different (lower) in 305423 soybean as compared to the control line (adjusted P-value <0.05) although remaining within the statistical tolerance interval and the combined literature range. This was an expected difference as the promoter for the KTi3 gene encoding a Kunitz trypsin inhibitor (one constituent of the trypsin inhibitor family of proteins) was used to drive the transcription of the *gm-fad2-1* gene fragment in 305423 soybean. As has been reported in the literature, the insertion of a promoter fragment can effectively silence expression of the corresponding endogenous gene (Morino *et al*., 2004; Cigan *et al*., 2005; Eike *et al*., 2005; Yang *et al*., 2005). Northern blot analysis was conducted to evaluate expression of the endogenous KTi3 gene in 305423 soybean and results confirmed that the KTi3 gene was silenced by the introduction of the KTi3 promoter in 305423 soybean. Refer to section VI-C for details on the expression analysis of the endogenous KTi3 gene in 305423 soybean.

In conclusion, antinutrient analysis of soybean grain demonstrates that 305423 soybean is comparable to near isoline and reference soybean lines. No unexpected differences in the antinutrient composition of 305423 soybean grain were seen.

Table 18. Antinutrients in Soybean Grain

Analyte		Control (null segregant)	305423 soybean	Tolerance interval[1]	Combined literature range[2]
Stachyose (% dry weight)	Mean[3]	3.05	3.35	2.65 - 4.78	1.21 - 3.50
	Range[4]	2.57 - 3.52	2.46 - 3.81		
	Adjusted P-value[5]		0.0708		
	P-value[6]		0.0201		
Raffinose (% dry weight)	Mean	0.720	0.755	0 - 1.99	0.634 - 1.96
	Range	0.592 - 0.917	0.583 - 1.05		
	Adjusted P-value		0.5468		
	P-value		0.3619		
Lectins (hemagglutinating units/mg)	Mean	3.06	3.65	0 - 11.4	0.105 - 9.04
	Range	0 - 8.53	1.24 - 7.48		
	Adjusted P-value		0.1787		
	P-value		0.0728		
Phytic acid (% dry weight)	Mean	1.23	1.17	0.459 - 1.78	0.634 - 2.74
	Range	0.893 - 1.80	0.948 - 1.61		
	Adjusted P-value		0.5468		
	P-value		0.3637		
Trypsin inhibitor (trypsin inhibitor units/mg)	Mean	50.2	32.9	8.71 - 80.4	19.6 - 119
	Range	43.1 - 59.9	28.2 - 36.9		
	Adjusted P-value		0.0014[7]		
	P-value		0.0002		

[1] Negative tolerance limits were set to zero.
[2] Literature ranges are taken from published literature for soybeans (OECD, 2001; ILSI 2004).
[3] Least Squares Mean
[4] Range denotes the lowest and highest individual value across locations.
[5] False Discovery Rate (FDR) adjusted P-value
[6] Non-adjusted P-value
[7] Statistically significant difference; adjusted P-value < 0.05

IX-G. Conclusions

Extensive compositional analyses of forage and grain were conducted to evaluate the composition of 305423 soybean compared to a null segregant control and four conventional soybean varieties (reference lines). In total, data from 51 different analytical components (51 in grain and five of those in forage) were presented. Compositional analysis of 305423 soybean was used to evaluate any changes in the levels of key nutrients, isoflavones, and antinutrients.

Compositional analyses of forage included protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), and ash. Compositional analyses of grain included protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), ash, fatty acids, amino acids, isoflavones, and key antinutrients.

Based on the compositional evaluation, the grain and forage of 305423 soybean are considered to be comparable to conventional soybean varieties except for the fatty acid composition of the grain. Analysis of soybean grain demonstrated that 305423 soybean has a modified fatty acid profile including an increase in the content of oleic acid (C18:1), and a decrease in the content of the linoleic acid (C18:2), linolenic acid (C18:3), and to a lesser extent, in palmitic acid (C16:0). Also, there is a trace amount of the 9,15-linoleic acid isomer in 305423 soybean grain; this isomer is also found in many commonly consumed foods. An increase in two minor fatty acids, heptadecanoic acid (C17:0) and heptadecenoic acid (C17:1), in 305423 soybean was observed and is likely a result of a slight shift in availability of the GM-HRA enzyme substrates, pyruvate and 2-ketobutyrate (Appendix 7-1). Both C17:0 and C17:1 fatty acids are also found in many different kinds of commonly consumed foods, and there is no evidence to indicate that exposure to either C17:0 or C17:1 from these sources is associated with adverse effects in humans.

In conclusion, 305423 soybean has the high oleic phenotype and is otherwise nutritionally comparable to commercially available soybean. Results of these compositional analyses correspond with the earlier observations that high oleic soybeans generated by introduction of the *FAD2-1* gene (OECD identifier DD-Ø26ØØ5-3, AGBIOS database) were similar to commodity soybeans (Kinney and Knowlton, 1997; USDA-APHIS petition number 97-008-01p nonregulated status granted May 7, 1997).

X. Environmental Assessment and Impact on Agronomic Practices

X-A. Environmental Assessment of the GM-HRA Protein

The *gm-hra* gene which is used solely as a selectable marker in 305423 soybean encodes a modified version of the acetolactate synthase (ALS) enzyme (refer to Section VII for the details on the protein biochemistry and mode of action). ALS proteins are ubiquitously present in nature, as ALS genes have been isolated from bacteria, fungi, algae and plants (Friden *et al.,* 1985; Falco *et al*., 1985; Reith and Munholland, 1995; Mazur *et al*., 1987). Moreover, naturally occurring mutations in plant ALS proteins that confer herbicide tolerance have also been identified (for a review, see Duggleby and Pang, 2000; Tan *et al*., 2006). Several crops where herbicide tolerant *als* genes are utilized to confer commercial level of herbicide tolerance have been commercialized and do not display any unexpected environmental consequences (for example, Clearfield®[1] wheat, Clearfield® sunflower, Clearfield® lentils, STS®[2] soybean). None of these ALS enzymes or herbicide tolerant crops is known to pose an environmental safety concern. This indicates a previous history of exposure to and safe use of proteins similar to GM-HRA.

The GM-HRA protein introduced into 305423 soybean has minimal modifications compared to the endogenous soybean ALS enzyme from which it was derived. It has only two amino acid differences from the corresponding endogenous soybean ALS protein, plus an additional five amino acids at the N-terminus derived from translation of 15 nucleotides from the *als* 5' UTR (refer to Section VII). In addition to being derived from a soybean protein and having a high degree of similarity to other ALS enzymes, the GM-HRA amino acid sequence does not have any homology to proteins that are toxic to humans or animals, as described in Section VII-D. Therefore, the GM-HRA protein is highly unlikely to pose a safety risk to beneficial organisms or the environment.

In conclusion, there are not likely to be any environmental effects due to the presence of the GM-HRA protein introduced into 305423 soybean.

X-B. Fate of Transgenic DNA in Humans and Animals

Transgenic DNA is no different from other DNA consumed as part of the normal diet. Genetically engineered organisms have been used in drug production (insulin, http://pubs.acs.org/cen/coverstory/83/8325/8325insulin.html) and microbial fermentation (cheese) since the late 1970's (http://www.ncbe.reading.ac.uk/NCBE/GMFOOD/chymosin.html, and http://vm.cfsan.fda.gov/~lrd/biopolcy.html#summary). More than 1.4 billion cumulative acres of engineered food and feed crops have been grown and consumed worldwide over the past seven years (ISAAA, 2006). The FDA has not reported any significant concerns with bioengineered food and feed currently on the market. The EPA has exempted from a tolerance DNA that encodes currently registered plant incorporated protectants because of a lack of toxicity (Federal Register, 2001).

Studies in humans and animals following the fate of DNA once consumed have shown that the majority of DNA is degraded in the gastrointestinal tract. There is evidence that DNA can move from the gastrointestinal tract lumen to other areas of the body, but this is considered to be a normal occurrence and no risks have been identified as a result of absorption (Einspanier *et al*., 2001; Duggan *et al*., 2003).

[1] Clearfield® is a registered trademark of BASF.
[2] STS® is a registered trademark of DuPont or its affiliates.

X-C. Weediness Potential of 305423 Soybean

Commercial soybean varieties in the United States are not considered weeds and are not effective in invading established ecosystems. Soybean has been grown throughout the world without any report that it is a serious weed. Cultivated soybean is unlikely to become a weed. Soybean seed rarely displays any dormancy characteristics and only under certain environmental conditions grows as a volunteer in the year following cultivation. If this should occur, volunteers do not compete well with the succeeding crop and can easily be controlled mechanically or chemically. The soybean plant has no weedy tendencies and is non-invasive in natural habitats in the United States. It does not grow in unmanaged habitats.

Soybeans are not an overwintering crop: they are not frost tolerant and do not survive freezing winter conditions (OECD, 2000). Studies by Kodama and co-authors (1994, 1995) indicate that increases in levels of trienoic fatty acids such as hexadecatrienoic acid (C16:3) and linolenic acid (C18:3) can enhance cold tolerance in model plants such as *Arabidopsis thaliana* and tobacco. In the case of 305423 soybean, levels of linolenic acid are significantly decreased. Therefore, we would not expect 305423 soybean plants to exhibit any enhanced cold tolerance. Moreover, in-season and post-trial monitoring of 305423 soybean experiments confirmed there were no unexpected changes relative to weediness and seed ability to survive over winter.

There is little probability that 305423 soybean could become a problem weed. Although 305423 soybean contains the *gm-hra* gene, it is only used as a selectable marker gene in 305423 soybean and does not confer commercial levels of herbicide tolerance in this transgenic event. Normal agronomic practices and weed control measures can therefore be used. Various characteristics that might impart weediness potential were evaluated for 305423 and control soybean in comparative studies (Section VIII). No differences were seen in characteristics such as seed germination, emergence, seedling vigor, seed shattering, yield, and disease/insect susceptibility. Assessment of these data detected no biologically significant differences between 305423 and control soybean indicative of a selective advantage that would result in increased weediness potential. Furthermore, post-harvest monitoring of field trial plots containing 305423 soybean has shown no differences in survivability or persistence of 305423 soybean as compared to control or conventional soybeans. The ecological fitness of 305423 soybean is therefore similar to that of conventional soybeans.

X-D. Gene Flow Assessment

Vertical Transfer of the Introduced Genetic Material

Due to the reproductive morphology (papilionaceous flower) of soybeans, this crop exhibits a high percentage of self-fertilization. Natural outcrossing levels in soybean range from less than 0.5% to about 1% (Carlson and Lersten, 1987). As a reflection of this low potential for cross-pollination, Certified Seed Regulations allow foundation seed to be grown adjacent to other soybean varieties as long as the distance is adequate to prevent mechanical mixing of the harvested seeds (see 7 CFR §201.76, http://www.access.gpo.gov/nara/cfr/waisidx_01/7cfr201_01.html). Hence, the probability of gene transfer from 305423 soybean to other commercial soybean varieties is very low.

Cultivated soybean can cross only with other members of its subgenus, *Soja* (reviewed in OECD, 2000). However, the potential for such gene flow to wild soybean relatives is limited by geographic isolation. Wild soybean species are native to China, Korea, Japan, Taiwan and the former USSR, and do not exist naturally in the United States. These species are not naturalized in North America, and although they could occasionally be grown in research plots, there are no reports of their escape from such plots to unmanaged habitats. There is therefore no potential for gene flow from cultivated 305423 soybean plants to wild soybean relatives in the United States.

Horizontal Transfer of the Introduced Genetic Material

There is no known mechanism for, or definitive demonstration of, DNA transfer from plants to microbes (Nap *et al.*, 1992; Redenbaugh *et al.*, 1994). Many genomes have been sequenced from bacteria that are closely associated with plants (*e.g*. *Agrobacterium* and *Rhizobium*), and there is no evidence that these organisms contain genes derived from plants (Kaneko *et al.*, 2002, Wood *et al*., 2001). The occurrence of potential horizontal gene transfer in the environment (bacteria, pathogens, *etc*.) has been studied using soil, water, and mammalian digestive tract systems. These studies conclude that the risk of a possible transfer is irrelevant to an environmental risk assessment of transgenic soybean (Bogosian and Kane, 1991; Prins and Zadoks, 1994; Schluter *et al*., 1995; Jonas *et al.,* 2001). Where sequence data indicate that horizontal gene transfer may have occurred, these events are estimated to occur on an evolutionary time scale on the order of millions of years (Koonin, 2001; Brown, 2003). In addition, transgene DNA promoters and coding sequences are optimized for plant expression and not bacterial expression, and it is therefore very unlikely that a protein corresponding to the transgene would be produced. Even if such a transfer were to take place and protein produced, the DNA and protein would not present a human health or plant pest risk. The *gm-fad2-1* gene fragment and the *gm-hra* gene are endogenous to soybean.

X-E. Potential Impact of the Introduction of 305423 Soybean on Agronomic Practices

Soybeans are typically grown in the United States as row crops. Planting usually begins in late April or early-to-mid May, and harvesting generally occurs in late October to early November. Clean tillage has been the traditional method of field preparation, but no tillage and reduced tillage systems have become increasingly common. More than 60% of soybean acres are now grown under some form of conservation tillage (no-till, ridge-till or mulch-till). Conservation tillage practices provide the advantages of decreased soil compaction and fuel costs through reduction in use of heavy machinery, reduced soil erosion and better soil moisture conservation. Irrigation is not usually practiced (Van Doren and Reicosky, 1987); in 2002 only 7.5% of planted soybean acres were irrigated (USDA/ERS, http://www.ers.usda.gov/).

Most soybeans (67% in 2002, USDA/ERS (2002)) are grown in rotation following corn, with another 18% in rotation with other row crops and small grains; 14% are grown continuously. In areas with a longer growing season such as the southern United States, soybeans are also double cropped, after winter wheat is harvested (6% of planted acres in 2004, CTIC (2006)). Crop rotation aids in the management of diseases, insects and weeds and increases organic matter and soil fertility. In addition, crop rotation allows growers to diversify farm production to minimize market risks.

Soybeans are quite intensively managed, as evidenced by the chemical usage data from the 2004 USDA-NASS Agricultural Chemical Usage Report (http://www.nass.usda.gov/). Eleven states (81% of the total US soybean acreage) were included in this report: Arkansas, Illinois, Indiana, Iowa, Kansas, Minnesota, Missouri, Nebraska, North Dakota, Ohio and South Dakota. Phosphate and potash were the most commonly applied fertilizers in soybean; they were used on average in 26% and 23% of the acreage, respectively, of the acreage in the states listed in the report. However, the acreage treated with fertilizer varies greatly by state, with some treating up to 63% of acres, and others only 5%. Because phosphate and potash are stable in soils, many growers don't apply them every year. Phosphate and potash are often applied on the corn crop in a corn-soybean rotation, using the same application for both crops. Insecticides and fungicides were not widely used, with 4% and 1% of the acres treated, respectively. Herbicides were applied on 97% of the soybean acres. Glyphosate dominated herbicide usage, with application on 87% of planted acres. The next most used herbicides were chlorimuron-ethyl (7% of acres treated), sulfentrazone (6%), trifluralin (5%), and pendimethalin (4%).

Similar to other value-added soybeans (low linolenic, clear-hilum, non-GMO), 305423 soybean is intended to be marketed under an identity preservation program. Identity preservation is a stringent process by which a crop is grown, handled, delivered, and processed under controlled conditions that insure the purity and maintenance of a unique, value-added trait from the farm-gate to end use. Specially bred high oleic soybeans will be offered under a premium to growers who contract their crop with local participating elevators under specified production, delivery, and sampling standards. Other than this, management and production practices for growing high oleic 305423 soybeans are much the same as growing any variety of conventional soybeans. No impact on current soybean cultivation practices is expected from introduction of the high oleic 305423 soybean. With exception of the intended changes in fatty acid composition of the seed, 305423 soybean were shown to be substantially equivalent to conventional soybean varieties in agronomic, ecological and compositional characteristics and have the same levels of resistance to insects and diseases.

X-F. Potential Impact on Organic or Conventional Farming

Growers choose to grow organic, conventional or biotechnology-derived soybean primarily based on economic and market factors. Growers of organic soybean and conventional soybean for non-biotechnology markets are generally paid a premium for their products, justifying the additional production costs. Conventional and organic soybean seeds are readily available to growers who decide to plant them. In addition to the market segments that produce organic or conventional soybean, distinct value-added specialty soybeans (low linolenic, clear hilum or high protein) have also been grown and successfully marketed for specific food uses in domestic and export markets for many years (Cui *et al.*, 2004). The introduction of 305423 soybean offers growers another value-added specialty soybean option. Value-added specialty soybean products are grown under identity preservation programs with grower premiums similar to conventional and organic products (see Section X-E for details). Growers will continue to have the option of growing conventional, organic or biotechnology-derived soybeans based on market factors.

X-G. Potential Impacts on Raw or Processed Agricultural Commodities

Data submitted on agronomic performance, disease and insect susceptibility, and compositional analyses of 305423 soybean show no significant differences between 305423 soybean and non-transgenic control soybean that would be expected to cause either a direct or indirect plant pest effect on any raw or processed plant commodity. The 305423 soybean will also be reviewed by the FDA for use in food and feed. It is not anticipated that 305423 soybeans would be processed any differently from the way conventional soybeans are processed. Meal produced from 305423 soybean will be used in the same manner as conventional soybean meal. Oil derived from 305423 soybean will be a value-added oil intended as a replacement for hydrogenated fats containing trans fatty acids (Kinney and Knowlton, 1997). Based on the analyses above, we expect no significant impact on raw or processed agricultural commodities other than the intended fatty acid changes in identity-preserved oil products based on the introduction of 305423 soybean.

X-H. Potential Impact on Non-target Organisms, Including Beneficial Organisms and Threatened or Endangered Species

Based on the safety of the GM-HRA protein expressed in 305423 soybean described in Section X-A and the compositional analysis described in Section IX, we would expect no effect on non-target organisms, including beneficial organisms and threatened or endangered species. A wide variety of ALS proteins are already present in the environment. The GM-HRA protein is not a potential food allergen. Based on tests conducted in rodents (mouse acute study), the GM-HRA

protein is not toxic to mammals. Observations made during field testing have revealed no effects on invertebrate populations.

We would expect that 305423 soybean would replace some of the soybean acres currently planted, but do not expect that 305423 soybean will cause new soybean acres to be planted in areas that are not already in agricultural use. Threatened or endangered species are generally found outside of agricultural fields. Any habitat disruption within fields will be comparable to any other cropping systems. Based on this information, we would not expect cultivation of 305423 soybean to have an effect on threatened or endangered species, or expect it to adversely change designated critical habitats compared to current agricultural practices.

X-I. Potential Impact on Biodiversity

The 305423 soybean does not have an increased weediness potential, and unconfined cultivation of 305423 soybean should not lead to increased weediness of other sexually compatible relatives, as non-cultivated *Glycine* species are not found in the United States (Section X-C). Therefore, it is unlikely to have effects on non-target organisms common to the agricultural ecosystem or threatened or endangered species recognized by the U.S. Fish and Wildlife Service and the National Marine Fishery Services, and there is no apparent potential for significant impact to biodiversity.

X-J. Overall Environmental and Agronomic Practices Conclusions

A thorough characterization of 305423 soybean was performed, including molecular analysis, GM-HRA protein level analysis, phenotypic and ecological evaluation, and nutrient composition evaluation. Assessment of the data generated supports the conclusion of no increased plant pest potential, phenotypic comparability, and familiarity as they relate to ecological risk assessment.

Due to the previous history of exposure to and safe use of organisms containing proteins similar to GM-HRA, as well as the safety assessment on the GM-HRA protein, no environmental effects due to the presence of the GM-HRA protein introduced in 305423 soybean are expected. Likewise, there is no impact on public health or safety expected due to the DNA introduced in 305423 soybean.

The 305423 soybean has been shown to be agronomically and ecologically similar to conventional soybeans, which have no weedy tendencies and are non-invasive in natural habitats. No differences were seen in characteristics such as seed germination, dormancy, emergency, seedling vigor, seed shattering, yield, and disease/insect susceptibility. Assessment of these data detected no biologically significant differences between 305423 and control soybean indicative of a selective advantage that would result in increased weediness or outcrossing potential. On the basis of these data, it is concluded that there is no increased plant pest potential of 305423 soybeans.

Because of the agronomic similarity of 305423 soybean to conventional soybean, there is no significant impact expected on raw or processed agricultural commodities (other than the intended fatty acid changes in identity-preserved oil products), on non-target, beneficial organisms (including threatened and endangered species), or on biodiversity. Impacts on organic or conventional farming are also expected to be minimal, as growers' decisions to plant biotechnology-derived, organic or conventional soybean are driven largely by market dynamics. Market dynamics, grower choice, and existing soybean production practices will not change due to the availability of 305423 soybean other than offering growers another value-added specialty soybean option.

With the introduction of high oleic 305423 soybean, we do not expect any change in agronomic practices.

XI. Adverse Consequences of Introduction

Pioneer Hi-Bred International, Inc. is unaware of any information indicating that 305423 soybean may pose a greater plant pest risk than conventional soybean. There are no adverse environmental consequences anticipated with its introduction. Thus we make the statement "unfavorable information: NONE", and on the basis of the substantial benefits that this product offers for the food and industrial oil sectors, Pioneer requests that 305423 soybean be granted nonregulated status under 7 CFR Part 340.

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XIII. Appendices 1-7

Appendix 1. Materials and Methods for Molecular Characterization

1-1. Materials and Methods for Southern Blot Analysis

To characterize the DNA insertion in 305423 soybean, Southern blot analysis was conducted. Individual plants of the T4 generation were analyzed to determine the number of each of the genetic elements of the cassettes on fragments PHP19340A and PHP17752A inserted and to verify the integrity of the fragments upon integration. The integration pattern of the insertion in 305423 soybean was investigated with several restriction enzymes and various combinations. Southern blot analysis was conducted on individual plants of two generations, T4 and T5, to confirm insert stability across generations and to examine for plasmid backbone sequences from PHP19340 and PHP17752. The F2 generation was analyzed to verify insertion stability after crossing to an elite line and to confirm Mendelian segregation of the insertion. All probes used for the analysis are indicated in Section V on the maps of PHP19340A, PHP17752A, PHP19340, and PHP17752 (Figures 3 through 6) and outlined in Appendix 2, Table 1.

1.a. 305423 Soybean Material

Seeds from the T4, T5, and F2 generations of 305423 soybean were planted (Figure 2) and leaf tissue harvested from individual plants was used for genomic DNA extraction.

1.b. Control Soybean Material

Seeds from the unmodified Jack soybean variety and the Elite line were planted and leaf tissue harvested from individual plants was used for genomic DNA extraction. Jack control DNA was used as a negative control to confirm hybridization due to soybean endogenous sequences from all probes of the *gm-fad2-1* and *gm-hra* cassettes. The Jack and elite line control DNA were used to confirm hybridization due to endogenous sequences for analysis of the F2 generation.

1.c. Reference Material

PHP19340 and PHP17752 plasmid DNA

PHP19340 and PHP17752 plasmid DNA was used as a positive control for Southern analysis to verify probe hybridization and to verify sizes of internal fragments. The plasmid stock was a copy of the plasmid used for microprojectile bombardment experiments to produce 305423 soybean and was digested with restriction enzymes to confirm the plasmid map. The probes used in this study (Appendix 2, Table 1) were derived from plasmid PHP19340, PHP17752, or from a plasmid containing equivalent genetic elements.

Molecular Weight Markers

DNA molecular weight markers for gel electrophoresis and Southern blot analysis were used to determine approximate molecular weights. For Southern analysis, DNA Molecular Weight Marker VII, digoxigenin (DIG) labeled (Roche, Indianapolis, IN), was used as a size standard for hybridizing fragments. ΦX174 RF DNA/*Hae* III Fragments (Invitrogen, Carlsbad, CA) was used as a molecular weight standard to determine sufficient migration and separation of the fragments on the gel.

1.d. Confirmation of 305423 Soybean Phenotype

Prior to planting as described below, small seed chips (~2 mg.) were removed from the seed cotyledons using a razor blade. Fatty acid methyl esters (FAMES) were prepared from single, matured, soybean seed chips by transesterification using trimethylsulfonium hydroxide (TMSH) (Butte, 1983). Seed chips were placed in a 1.5 ml glass gas chromatography vial containing 50 µl of TMSH and 0.5 ml of heptane and were incubated for 10 minutes at room temperature while shaking. Vials were then transferred to the vial racks on the Gas Chromatograph. Fatty acid methyl esters (3 µL injected from heptane layer) were separated and quantified using a Hewlett-Packard 6890-2 Gas Chromatograph fitted with an Omegawax 320 fused silica capillary column (Supelco Inc., Bellfonte, PA) and a Flame Ionization Detector (FID). The oven temperature was programmed to hold at 220°C for 5 min, increase to 240°C at 20 C /min and hold for an additional minute. A Whatman hydrogen generator supplied carrier gas and supplied hydrogen for the FID. Retention times were compared to those for methyl esters of standards commercially available (Nu-Chek Prep, Inc., Elysian, MN).

Oil profiles for all seeds were reviewed for elevated oleic acid (18:1) levels as confirmation of the phenotype.

A preliminary Southern blot analysis of DNA isolated from all 305423 soybean plants was used to verify the presence of both the *gm-fad2-1* gene fragment and the *gm-hra* gene. Methods for this preliminary characterization are described below. Final Southern blot analysis was carried out on a subset of 305423 soybean plants.

1.e. Genomic DNA Extraction and Quantitation

Genomic DNA was extracted from leaf tissue harvested from individual plants as described above. The tissue was pulverized in tubes containing grinding beads using a Geno/Grinder™ (SPEX CertiPrep, Inc., Metuchen, NJ) instrument and the genomic DNA isolated using a urea-based procedure (modification from Chen and Delalporta, 1994). Approximately 1 gram ground tissue was extracted with 5 ml Urea Extraction Buffer (7 M Urea, 0.34 M NaCl, 0.05 M Tris-HCl, pH 8.0, 0.02 M EDTA, 1% N-Lauroylsarcosine) for 12-30 minutes at 37°C, followed by two extractions with phenol/chloroform/isoamyl alcohol (25:24:1) and one extraction with water saturated chloroform. The DNA was precipitated from the aqueous phase by the addition of 1/10 volume of 3 M NaOAc (pH 5.2) and 1 volume of isopropyl alcohol, followed by centrifugation to pellet the DNA. After washing the pellet twice with 70% ethanol, the DNA was dissolved in 0.5 ml TE buffer (10mM Tris, 1 mM EDTA, pH 7.5) and treated with 10 µg Ribonuclease A for 15 minutes at 37°C. The sample was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with water saturated chloroform, followed by precipitation with isopropyl alcohol and washing with 70% ethanol. After drying, the DNA was re-dissolved with 0.5 ml TE buffer and stored at 4°C.

Following extraction, the DNA was quantified on a spectrofluorometer using PicoGreen® reagent (Molecular Probes, Inc., Eugene, OR) following a standard procedure. The DNA was also visualized on an agarose gel to confirm quantitation values from the PicoGreen® analysis and to determine DNA quality.

1.f. Digestion of DNA for Southern Blot Analyses

Genomic DNA samples extracted from 305423 soybean and control soybean plants were digested with restriction enzymes following a standard procedure. Approximately 2 µg of genomic DNA was digested in a volume of 100 µl using 50 units of enzyme according to manufacturer's recommendations. The digestions were carried out at 37°C for three hours,

followed by ethanol precipitation with 1/10 volume of 3 M NaOAc (pH 5.2) and 2 volumes of 100% ethanol. After incubation at 4°C and centrifugation, the DNA was allowed to dry and re-dissolved in TE buffer. The reference plasmids, PHP19340 or PHP17752, were spiked into a control plant DNA sample in an amount equivalent to approximately one or two gene copies per soybean genome and digested with the same enzyme to serve as a positive control for probe hybridization and to verify sizes of internal fragments on the Southern blot.

1.g. Electrophoretic Separation of DNA and Southern Blot Transfer

Following restriction enzyme digestion, the DNA fragments produced were electrophoretically separated by size through an agarose gel and a molecular weight standard [ΦX174 RF DNA/*Hae* III Fragments (Invitrogen)] was used to determine sufficient migration and separation of the fragments on the gel. DIG labeled DNA Molecular Weight Marker VII (Roche), visible after DIG detection as described below, was used to determine hybridizing fragment size on the Southern blots.

Agarose gels containing the separated DNA fragments were depurinated, denatured, and neutralized *in situ*, and transferred to a nylon membrane in 20x SSC buffer (3M NaCl, 0.3 M Sodium Citrate) using the method as described for the TURBOBLOTTER™ Rapid Downward Transfer System (Schleicher & Schuell, Keene, NH). Following transfer to the membrane, the DNA was bound to the membrane by UV crosslinking (Stratalinker, Stratagene, La Jolla, CA).

1.h. DNA Probe Labeling for Southern Blot Hybridization

Probes for the KTi3 promoter, *gm-fad2-1* gene fragment, KTi3 terminator, SAMS regulatory region, *gm-hra* gene, and *als* terminator were used to detect genes and elements within the insertion (Appendix 2, Table 1). Backbone, hygromycin resistance gene cassette, and plasmid origin of replication regions (*backbone, hyg,* and *plasmid ori* probes, respectively) of the PHP19340 and PHP17752 plasmids were used to examine plasmid backbone DNA in 305423 soybean (Appendix 2, Table 1). DNA fragments of the probe elements were generated by PCR from plasmids PHP19340 or PHP17752 or a plasmid with equivalent elements using specific primers. PCR fragments were electrophoretically separated on an agarose gel, excised and purified using a gel purification kit (Qiagen, Valencia, CA). DNA probes were generated from these fragments by PCR that incorporated a DIG labeled nucleotide, [DIG-11]-dUTP, into the fragment. PCR labeling of isolated fragments was carried out according to the procedures supplied in the PCR DIG Probe Synthesis Kit (Roche).

1.i. Probe Hybridization and Visualization

The DNA fragments bound to the nylon membrane were detected as discrete bands when hybridized to a labeled probe. Labeled probes were hybridized to the target DNA on the nylon membranes for detection of the specific fragments using the procedures essentially as described for DIG Easy Hyb solution (Roche). After stringent washes, the hybridized DIG-labeled probes and DIG-labeled DNA standards were visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System with DIG Wash and Block Buffer Set (Roche). Blots were exposed to X-ray film for one or more time points to detect hybridizing fragments and to visualize molecular weight standards. Images were then digitally captured by detection with the Luminescent Image Analyzer LAS-3000 (Fujifilm Medical Systems, Stamford, CT). Digital images were compared to original X-ray film exposures as verification for use in this report. The sizes of detected bands were documented for each digest and each probe.

1.j. Stripping of Probes and Subsequent Hybridizations

Following hybridization and detection, membranes were stripped of DIG-labeled probe to prepare the blot for subsequent re-hybridization to additional probes. Membranes were rinsed briefly in distilled, de-ionized water and then stripped in a solution of 0.2 M NaOH and 1.0% SDS at 37-40°C with constant shaking. The membranes were then rinsed in 2x SSC and either used directly for subsequent hybridizations or stored at 4°C or -20°C for later use. The alkali-based stripping procedure effectively removes probes labeled with the alkali-labile DIG.

1-2. Materials and Methods for Northern Blot Analysis

Northern blot analysis was conducted to evaluate expression levels of *FAD2-1, FAD2-2, FAD3, dapA*, and KTi3 genes in leaf and developing seed of 305423 and control soybean.

1.a. 305423 Soybean Material

Seeds from the T4 generation of 305423 soybean were planted (Figure 2) for harvesting leaf tissue, developing seed at 20 daf (days after flowering), and developing seed at 30 daf, from individual plants. These samples were used for RNA extraction and subsequent analysis.

1.b. Control Soybean Material

Seeds from the unmodified Jack soybean variety were used as a control in these experiments. Seeds were planted for harvesting leaf tissue, developing seed at 20 daf (days after flowering), and developing seed at 30 daf, from individual plants. These samples were used for RNA extraction and subsequent analysis.

1.c. Reference Material

Molecular Weight Markers

RNA molecular weight markers for gel electrophoresis and Northern blot analysis were used to determine approximate molecular weights of detected transcripts. For Northern analysis, RNA Molecular Weight Marker I, digoxigenin (DIG) labeled (Roche, Indiana-polis, IN), was used as a size standard for hybridized transcripts. Unlabeled RNA markers (Sigma, St. Louis, MO), visible with ethidium bromide over UV light, were used as additional molecular weight standards.

In Vitro Sense Transcripts as Hybridization Positive Controls

Sense RNA transcripts generated *in vitro* from *fad2-1*, *fad2-2*, and *dapA* clones were used as positive controls for probe hybridization. The *fad2-1*, *fad2-2* and *dapA* sense transcripts were transcribed from full length expressed sequence tags (ESTs, including 5'and 3' untranslated regions) from a proprietary DuPont soybean library. The *dapA* transcript spans only the 5' half of its EST, whereas the *fad2-1* and *fad2-2* transcripts span their entire ESTs.

1.d. Confirmation of High Oleic Phenotype by Gas Chromatography (GC)

Please refer to Appendix 1-1, section 1.d for the description of the method. The plants used for Northern blot analysis were a subset of the plants from Southern blot characterization studies.

Southern analysis confirmed the presence of the introduced *gm-fad2-1* gene fragment and *gm-hra* gene in all the 305423 soybean plants, their absence in all the control soybean plants, and correlated with the oleic acid results.

1.e. Sample Collection

A total of twenty 305423 soybean seeds and fifteen Jack control seeds were planted in the growth chambers to produce plant tissues for RNA extraction and analysis. One seed was planted per pot, and the pot was uniquely identified. All plants were grown with light, temperature, and water regulated for healthy plant growth.

Harvesting of young leaves from 305423 and control soybean plants occurred three times: first at the V2-V4 growth stage, and then two more times after the plants had re-grown sufficiently and prior to the R1 stage. Sufficient young leaf material was collected and immediately placed on dry ice. The samples were returned to the laboratory and maintained frozen (<-50°C) until processing.

Developing seeds were harvested at 20 daf (20± 2 days after flowering) and again at 30 daf (30± 2 days after flowering). Pods removed from the plants were placed immediately on wet ice and transported to a laboratory or processing area for seed removal. Seeds, with pods removed, were placed immediately in the liquid nitrogen and thereafter maintained frozen (<-50°C) until processing.

1.f. RNA Extraction and Quantitation

The leaf and seed samples were ground to a fine powder in the presence of liquid nitrogen. Total RNA was isolated using standard detergent based extraction processes. The extraction buffer for leaf tissue was 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA,1% SDS, and the extraction buffer for seed tissue was 1 M Tris-HCl, pH 9.0, 1%SDS. Following extraction, the total RNA was visualized on an agarose gel to determine the quality and were quantified on a spectrofluorometer using RiboGreen® reagent (Molecular Probes, Inc., Eugene, OR). Messenger RNA (mRNA) was isolated from known amounts of total RNA using a NucleoTrap® kit (Macherey-Nagel Inc., Easton, PA) and quantified by the same means as for total RNA.

1.g. Electrophoretic Separation of mRNA and Northern Blot Transfer

Denatured mRNA samples were subjected to standard agarose-formaldehyde gel electrophoresis to separate the mRNA transcripts by size. Also loaded on the gel were DIG-labeled (Roche) and unlabeled (Sigma) RNA molecular weight standards, and *in vitro* sense transcripts specific to the probe used for subsequent hybridization. The unlabeled standards were visualized and documented by photographing the gel under UV illumination. Color dyes bromphenol blue and xylene cyanol in the RNA loading buffer were used to determine sufficient migration and separation of the transcripts on the gel.

The separated mRNA transcripts on the agarose-formaldehyde gel were transferred to a nylon membrane in 20x SSC buffer using the method as described for the TURBO-BLOTTER™ Rapid Downward Transfer System (Schleicher & Schuell, Keene, NH). Following transfer, the mRNA was bound to the membrane by UV crosslinking (Stratalinker, Stratagene, La Jolla, CA).

1.h. Probe Labeling and Northern Blot Hybridization

The mRNA transcripts bound to the nylon membrane were detected as discrete bands when hybridized to a labeled probes: *fad2-1* 3'UTR, *fad2-2* 3'UTR*, fad3, dapA,* and *kti3* coding.

RNA probes (riboprobes) *fad2-1* 3'UTR, *fad2-2* 3'UTR*, fad3,* and *dapA* (Section VI-B, Table 4) in the antisense orientation, were prepared to detect the soybean endogenous gene transcripts. All riboprobes were transcribed from T7/Sp6 plasmids containing these gene fragments: 284 bp of *fad2-1* 3' UTR, 171 bp of *fad2-2* 3' UTR, 547 bp of *fad3* (416 bp of protein coding sequence plus 131 bp of 3'UTR), and 538 bp of *dapA* coding sequence. The process of labeling the riboprobes started with linearization, by restriction enzyme digestion, of the corresponding plasmids containing the gene region to be labeled and Sp6/T7 promoters for *in vitro* transcription. Using a DIG RNA Labeling Kit (Roche), antisense probes were transcribed with incorporation of a digoxigenin (DIG) labeled nucleotide (*e.g.* [DIG]-11-UTP) according to the procedures supplied in the DIG RNA Labeling Kit.

To generate the *kti3* coding DNA probe (Section VI-C), detecting the soybean endogenous KTi3 gene, a template of partial KTi3 coding region was PCR amplified from control soybean genomic DNA. Primers used were based on the sequence information on the KTi3 gene (GenBank accession number S45092). The initial *kti3* PCR fragment (470 bp) was electrophoretically separated on an agarose gel, excised and purified using a Gel Extraction Kit (Qiagen, Valencia, CA). The *kti3* coding DNA probe was in turn generated from this purified fragment using the same primer pair in a PCR that incorporated a digoxigenin (DIG)-labeled nucleotide, [DIG-11]-dUTP, into the new product. PCR labeling was carried out according to the procedures supplied in the PCR DIG Probe Synthesis Kit (Roche).

Labeled probes were hybridized to the mRNA on the nylon membranes for detection of the absence or presence of specific transcripts using the procedures essentially as described for DIG Easy Hyb solution (Roche). DIG-labeled RNA Molecular Weight Marker I (Roche), visible after DIG detection as described below, was used to determine the sizes of the hybridized transcripts on the Northern blots.

An individual blot was prepared for each probe hybridization performed in this study. Blots hybridized with *fad2-1* 3'UTR, *fad2-2* 3'UTR and *dapA* probes contained two different quantities of the positive control sense transcript corresponding to the probe used for that blot. For this purpose sense transcripts were generated *in vitro* for *fad2-1*, *fad2-2* and *dapA* probes. With the *fad3* and *kti3* coding probes, the detection of transcripts of expected sizes was indicative of appropriate hybridization.

1.i. Detection of Hybridized Probes

DIG-labeled probes hybridized to mRNA bound to the nylon membrane after stringent washes, and DIG-labeled RNA standards were visualized using the CDP-Star Chemiluminescent Nucleic Acid Detection System with DIG Wash and Block Buffer Set (Roche). Blots were exposed to X-ray film for one or more time points to detect hybridized fragments and to visualize DIG labeled molecular weight standards. Images were then captured with a Luminescent Image Analyzer LAS-3000 (Fujifilm Medical Systems, Stamford, CT). These digital images were compared to original X-ray film exposures as verification. The numbers and sizes of detected bands for each probe were recorded using the DIG RNA I molecular weight marker (Roche) or unlabeled RNA markers (Sigma) as size references.

Appendix 2. Genetic Characterization of 305423 Soybean: Southern Blot Analysis

Southern blot analysis was conducted to characterize the DNA insertion in 305423 soybean.. The 305423 soybean was generated via microprojectile co-bombardment with fragments PHP19340A (Appendix 2, Figure 1) and PHP17752A (Appendix 2, Figure 2) that were isolated from plasmids PHP19340 and PHP17752, respectively (Appendix 2, Figures 3 and 4). Fragment PHP19340A contains the *gm-fad2-1* cassette that is comprised of the KTi3 promoter, the *gm-fad2-1* fragment, and the KTi3 terminator. Fragment PHP17752A contains the *gm-hra* cassette that is comprised of the SAMS regulatory region, *gm-hra* gene, and *als* terminator.

Individual plants of the T4 generation were analyzed by Southern blot to determine the copy number of each of the genetic elements and to examine the integrity of the PHP19340A and PHP17752A fragments inserted into the 305423 soybean genome (Appendix 2-1). Individual plants of the T4 and T5 generations of 305423 soybean were analyzed for plasmid backbone sequence from PHP19340 or PHP17752 outside of the transformation fragments (Appendix 2-2).

An approximate physical map of the insertion region in 305423 soybean was determined based on sequence data and additional Southern blot analysis confirmed the insertions (Appendix 2-3). In addition, Southern blot analysis on three generations of 305423 soybean (T4, T5, and F2) was conducted to confirm the stability of the inserted DNA during soybean breeding (Appendix 2-4).

Genomic DNA from leaf material of the Jack soybean variety was used as a negative control for the Southern blot analysis. Genomic DNA from the Elite soybean line was included as an additional negative control for analysis of the F2 generation. Plasmids PHP19340 and PHP17752 were used as a positive control for probe hybridization and to verify fragment sizes internal to the transformation fragments. Cassette probes used for the analysis are indicated on the maps of PHP19340A and PHP17752A (Appendix 2, Figures 1 and 2, respectively) and outlined in Appendix 2, Table 1. Backbone probes are indicated on the maps of PHP19340 and PHP17752 (Appendix 2, Figures 3 and 4, respectively) and outlined in Appendix 2, Table 1. A breeding diagram of the 305423 soybean generations analyzed is provided in Figure 2 of the petition. The methods used for Southern blot analysis are further described in Appendix 1-1.

2-1. Transgene Copy Number and Insertion Integrity

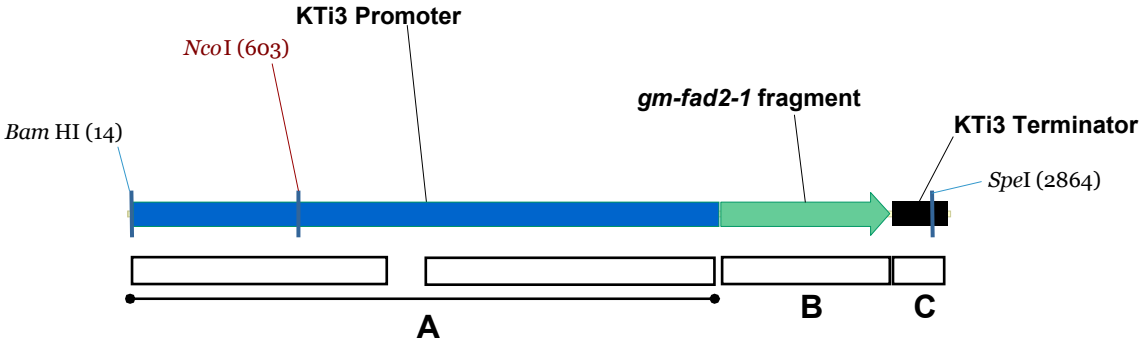
The integration pattern of the inserted DNA in 305423 soybean was investigated with selected restriction enzyme digestions to determine copy number and integrity of the inserted PHP19340A and PHP17752A fragments. To characterize the inserted elements from PHP19340A fragment, a combined *Eco*R V and *Spe* I restriction enzyme digestion (*Eco*R V/*Spe* I analysis) was used to examine copy number of the genetic elements, and a combined *Bam*H I and *Spe* I digestion (*Bam*H I/*Spe* I analysis) was used to examine integrity of the inserted fragment. Location of the restriction enzyme sites on the PHP19340A fragment is shown in Appendix 2, Figure 1. KTi3 promoter, *gm-fad2-1*, and KTi3 terminator probes were used to characterize the *gm-fad2-1* cassette on PHP19340A (Appendix 2, Table 1). Predicted hybridizing band sizes are presented in Appendix 2, Table 2. To characterize the inserted PHP17752A fragment, *Nco* I restriction enzyme digestion was used to examine the copy number of the genetic elements and *Hin*d III was used to examine the integrity of the inserted fragment. The location of the restriction enzyme sites on the PHP17752A fragment is shown in Appendix 2, Figure 2. SAMS, *gm-hra* gene, and *als* terminator probes were used to characterize the *gm-hra* cassette on PHP17752A (Appendix 2, Table 1). Predicted hybridizing band sizes are presented in Appendix 2, Table 3.

The *gm-fad2-1* and *gm-hra* cassette probes used for Southern analysis were either identical to or highly homologous to sequences in the endogenous soybean genome and thus additional hybridizing fragments were expected. The hybridizing bands in 305423 soybean originating from the endogenous soybean genome are indicated by asterisks (*) in the shaded boxes of Appendix

2, Tables 4 and 5. These bands were determined by their presence in the control soybean sample and are thus not associated with the inserted DNA of 305423 soybean.

Based on the Southern blot analysis discussed below and as confirmed by sequence data, it was determined that multiple copies, intact and truncated, of PHP19340A have been inserted into the genome of 305423 soybean comprising eight copies of the KTi3 promoter, seven copies of the *gm-fad2-1* fragment, and five copies of the KTi3 terminator. Two copies of PHP19340A are complete from the *Bam*H I site to the *Spe* I site and hybridize to all three cassette probes (sequencing showed that one of these copies is truncated at the end of the KTi3 terminator). Three copies of PHP19340A have some truncation, removing the *Bam*H I site, but contain portions of all three cassette elements. In addition, two copies of PHP19340A retaining only the KTi3 promoter with the *gm-fad2-1* fragment are present as well as one copy of the KTi3 promoter associated with a small non-functional fragment of backbone (discussed further in Appendix 2-2). For the PHP17752A fragment, a single, intact *gm-hra* cassette has been inserted into the genome of 305423 soybean. An approximate physical map of the inserted elements has been generated based on sequence data and supported by the Southern blot analysis (discussed in Appendix 2-3).

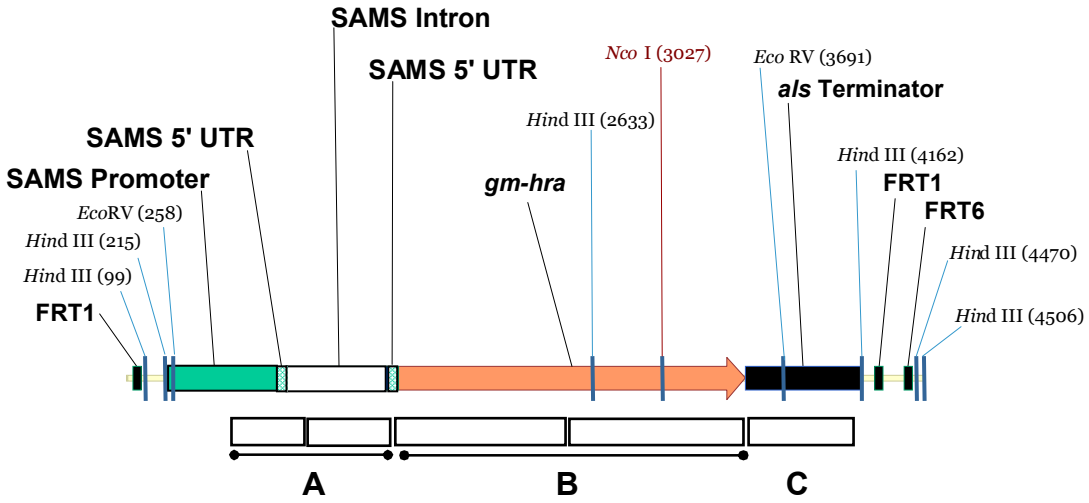
Appendix 2, Figure 1. Map of Fragment PHP19340A with Genetic Element Probes Indicated



Schematic map of the *Asc* I fragment of PHP19340 indicating location of genetic elements and restriction enzyme sites for *Bam*H I, *Spe* I, and *Nco* I. *Eco*R V does not cut the fragment. The size of the fragment is 2924 bp. Probes are indicated as lettered boxes below the fragment map and are identified in the table below. Additional details about these probes are provided in Appendix 2, Table 1.

Letter	Probe name
A	KTi3 promoter
B	*gm-fad2-1*
C	KTi3 terminator

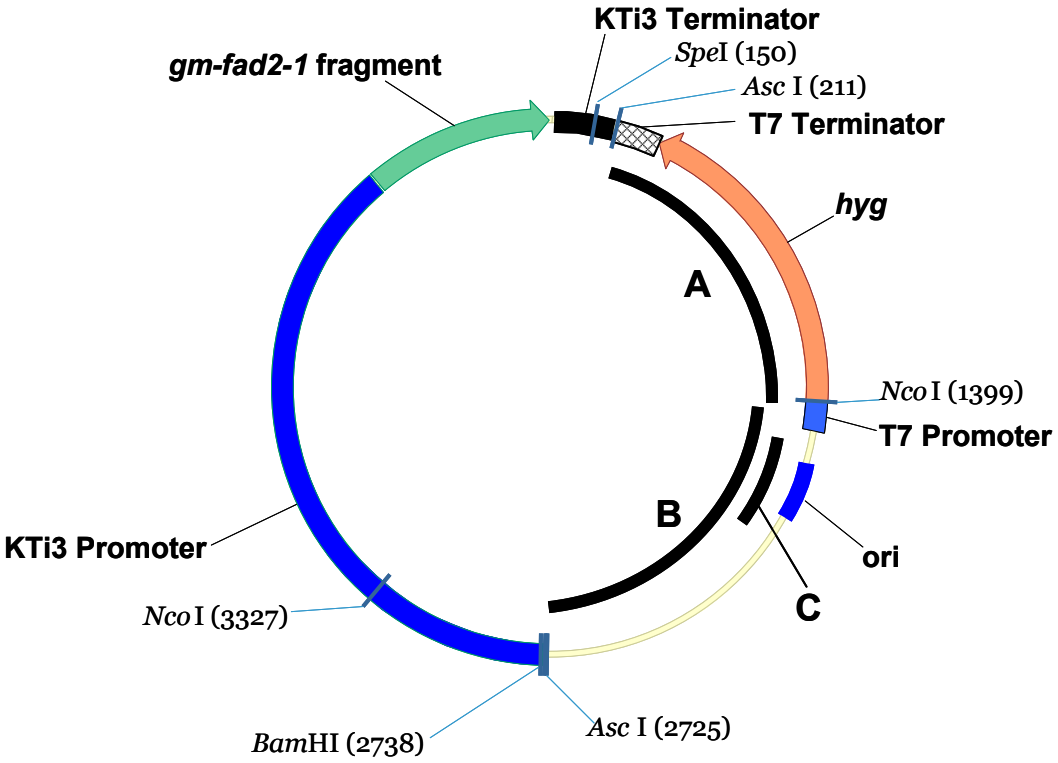
Appendix 2, Figure 2. Map of Fragment PHP17752A with Genetic Element Probes Indicated



Schematic map of the *Asc* I fragment of PHP17752 indicating location of genetic elements and restriction enzyme sites for *Eco*R V, *Nco* I, and *Hin*d III. *Spe* I does not cut the fragment. The size of the fragment is 4512 bp. Probes are indicated as lettered boxes below the fragment map and are identified in the table below. Additional details about these probes are provided in Appendix 2, Table 1.

Letter	Probe name
A	SAMS
B	*gm-hra*
C	*als* terminator

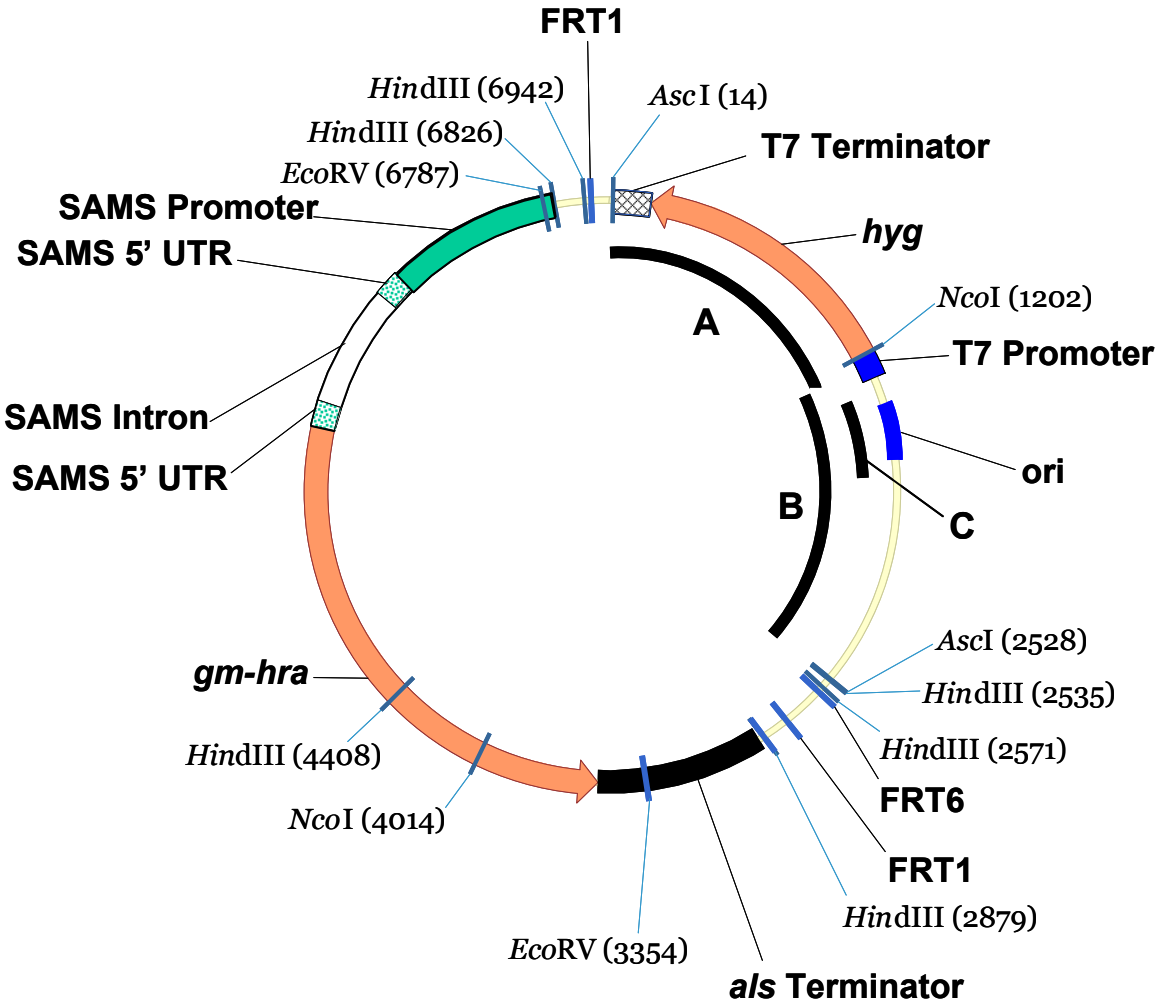
Appendix 2, Figure 3. Map of Plasmid PHP19340 with Backbone DNA Probes Indicated



Schematic map of plasmid PHP19340 indicating location of genetic elements and restriction enzyme sites for *Bam*H I, *Spe* I, *Nco* I, and *Asc* I. *Eco*R V does not cut the plasmid. *Asc* I digestion of PHP19340 was used to isolate PHP19340A. Plasmid size is 5438 bp. Backbone probes are indicated schematically as lines within the plasmid and identified in the table below. Additional details about these probes are provided in Appendix 2, Table 1.

Letter	Probe name
A	*hyg*
B	*backbone*
C	*plasmid ori*

Appendix 2, Figure 4. Map of Plasmid PHP17752 with Backbone DNA Probes Indicated



Schematic map of plasmid PHP17752 indicating location of genetic elements and restriction enzyme sites for *Eco*R V, *Nco* I, *Hin*d III, and *Asc* I. *Spe* I does not cut the plasmid. *Asc* I digestion of PHP17752 was used to isolate PHP17752A. Plasmid size is 7026 bp. Backbone probes are indicated schematically as lines within the plasmid and identified in the table below. Additional details about these probes are provided in Appendix 2, Table 1.

Letter	Probe name
A	*hyg*
B	*backbone*
C	*plasmid ori*

Appendix 2, Table 1. Description of DNA Probes Used for Southern Blot Hybridization

A. Probes for *gm-fad2-1* and *gm-hra* Cassette Analysis

Probe name	Genetic element	Figure	Position on PHP19340A (bp to bp)	Position on PHP17752A (bp to bp)	Length (bp)
KTi3 pro[1]	KTi3 promoter	1	14 to 1065 1158 to 2104	N/A	1052 947
gm-fad2-1	*gm-fad2-1* gene fragment	1	2111 to 2710	N/A	600
KTi3 term	KTi3 terminator	1	2721 to 2916	N/A	196
SAMS[1]	SAMS regulatory region	2	N/A	539 to 983 984 to 1475	445 492
gm-hra[1]	*gm-hra* gene	2	N/A	1537 to 2466 2472 to 3501	930 1030
als term	*als* terminator	2	N/A	3507 to 4156	650

B. Probes for Backbone DNA Analysis

Probe name	Genetic element	Figure	Position on PHP19340 (bp to bp)	Position on PHP17752 (bp to bp)	Length (bp)
backbone[1,2]	plasmid backbone	3, 4	1439 to 2190 2201 to 2724	1242 to 1993 2004 to 2527	752 524
hyg[1]	hygromycin resistance gene	3, 4	211 to 865 871 to 1393	14 to 668 674 to 1196	655 523
plasmid ori	bacterial origin of replication (*Hae* II fragment)	3, 4	1541 to 1943	1344 to 1746	403

N/A - Not Applicable, these are not present in the fragment
[1] Two non-overlapping segments were generated for this probe and were combined for hybridization.
[2] Plasmid backbone probe used for detailed physical mapping of 305423 soybean.

Appendix 2, Table 2. Predicted Hybridizing Band Sizes Based on the Map of Fragment PHP19340A and Plasmid PHP19340

A. Predicted hybridizing band sizes (in base pairs) per single copy of PHP19340A fragment inserted into genome[1]

	Probe		
Enzyme digestion	**KTi3 promoter**	***gm-fad2-1***	**KTi3 terminator**
*Eco*R V/*Spe* I **OR** *Spe* I	>2900[2]	>2900[2]	>2900[2] >60[2]
*Bam*H I/*Spe* I	2850	2850	2850 >60[2]
Nco I	>600[2] >2300[2]	>2300[2]	>2300[2]
*Eco*R V	>2900[2]	>2900[2]	>2900[2]
*Bam*H I	>2900[2]	>2900[2]	>2900[2]

Note: size prediction for *backbone* probe not able to be determined because the element is not present on PHP19340A.

B. Predicted hybridizing band sizes (in base pairs) for Plasmid PHP19340

	Probe				
Enzyme digestion	**KTi3 promoter**	***gm-fad2-1***	**KTi3 terminator**	***backbone, plasmid ori***	***hyg***
*Eco*R V/*Spe* I **OR** *Spe* I	5438	5438	5438	5438	
*Bam*H I/*Spe* I	2850	2850	2850 2588	2588	
Nco I	3510 1928	3510	3510	1928	3510
*Eco*R V	Does not cut	Does not cut	Does not cut	Does not cut	
*Bam*H I	5438	5438	5438	5438	

[1] Predicted fragment sizes for 305423 soybean are based on the map of PHP19340A as shown in Appendix 2, Figure 1.
[2] Minimum fragment size predicted based on an intact insertion of PHP19340A (Appendix 2, Figure 1).

Appendix 2, Table 3. Predicted Hybridizing Band Sizes Based on the Map of Fragment PHP17752A and Plasmid PHP17752

A. Predicted hybridizing band sizes (in base pairs) per single copy of PHP17752A fragment inserted into genome[1]

	Probe		
Enzyme digestion	**SAMS**	***gm-hra***	***als* terminator**
Nco I	>3000[2]	>3000[2] >1500[2]	>1500[2]
*Hin*d III	2418	2418 1529	1529
*Eco*R V **OR** *Eco*R V/*Spe* I	3433	3433	3433 >800[2]
Spe I	>4500[2]	>4500[2]	>4500[2]

Note: size prediction for *backbone* probe not able to be determined because the element is not present on PHP17752A.

B. Predicted hybridizing band sizes (in base pairs) for Plasmid PHP17752

	Probe				
Enzyme digestion	**SAMS**	***gm-hra***	***als* terminator**	***backbone, plasmid ori***	***hyg***
Nco I	4214	4214 2812	2812	2812	4214
*Hin*d III	2418	2418 1529	1529		
*Eco*R V **OR** *Eco*R V/*Spe* I	3433	3433	3593 3433	3593	
Spe I	Does not cut	Does not cut	Does not cut	Does not cut	

[1] Predicted fragment sizes for 305423 soybean are based on the map of PHP17752A as shown in Appendix 2, Figure 2.
[2] Minimum fragment size predicted based on an intact insertion of PHP17752A (Appendix 2, Figure 2).

2-1a. Copy Number of Elements of the *gm-fad2-1* Cassette on PHP19340A

The number of copies of PHP19340A fragment integrated into the genome of 305423 soybean was determined by using a combined *Eco*R V and *Spe* I restriction enzyme digestion (*Eco*R V/*Spe* I) as *Spe* I cuts once at base pair position 2864 in the fragment and *Eco*R V does not cut (Appendix 2, Figure 1) providing a good assessment of the copy number based on the number of hybridizing fragments. For the PHP19340A fragment, hybridization with the KTi3 promoter and *gm-fad2-1* probes would indicate the number of copies of each element found in 305423 soybean based on the number of hybridizing bands (*e.g.* one hybridizing band indicates one copy of the element). For the KTi3 terminator probe, *Spe* I cuts within the element and a second hybridizing band from a 53 bp overlap with the probe may be expected for each copy of the element. With a small region of probe overlap, hybridizations from this region may only be detectable in small fragments on the Southern blot and may only be variably detectable in larger fragments. Predicted fragment sizes for 305423 soybean with *Eco*R V/*Spe* I are given in Appendix 2, Table 2 for the *gm-fad2-1* cassette and observed band sizes are provided in Appendix 2, Table 4. The Southern results were consistent with sequence data obtained for 305423 soybean; predicted hybridizing fragments based on sequence are provided in Appendix 2, Table 4. Each band was identified to be from a particular insertion as labeled in Appendix 2, Table 4. Four insertions were identified (referred to as Insertion 1, 2, 3, and 4) and are discussed further in Appendix 2-3.

Appendix 2, Table 4. Observed Hybridizing Band Sizes in 305423 Soybean: *gm-fad2-1* Cassette Probes and *backbone* Probe

Sizes in base pairs (bp)

Enzyme Digestion	Predicted Sizes from 305423 Sequence	Insertion Number[1]	Observed Hybridizing Band Sizes in Southern Blot Analysis[2]			
			KTi3 promoter	*gm-fad2-1*	KTi3 terminator	*backbone*
*Eco*R V/*Spe* I (Copy number)	5906[3]	1	~5500[3]	~5500[3]		
	5101	4	~4900	~4900	~4900	
		(4)[4]	~4600[4]	~4600[4]		
	4211	3	~4300			~4300
	3120	1	~3200	~3200	~3200	
	2924	1	~2900	~2900	~2900	
	2787	4	~2600	~2600	~2600	
	2333	2	~2400	~2400	~2400	
	984	2			~990	
			~3000*	~7400* ~6200*	~3000*	
*Bam*H I/*Spe* I (Insertion integrity)	5101	4	~4900[5]	~4900	~4900	
	5006	3	~4900[5]			~4900
	>2974[6]	1	~4600	~4600		
	2850	1	2850[7]	2850[7]	2850[7]	
	2850	1	2850[7]	2850[7]	2850[7]	
	2787	4	2787[7]	2787[7]	2787[7]	
	2333	2	~2400	~2400	~2400	
	2169	1	~2200	~2200		
	984	2			~990	
			~3000*	2 bands ~3200*	~3000* ~1800*	

***** Hybridizing bands that were also present in the control sample. These bands are determined to be from sequences endogenous to the Jack variety background and are not related to the inserted DNA in 305423 soybean.

[1]Insertions are discussed in further detail in Appendix 2-3.

[2]Observed fragment sizes are approximated in the analysis from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, an approximate error of +/- 10% may be observed from sequence prediction.

[3]This hybridizing band contains the two copies of the KTi3 promoter and the *gm-fad2-1* fragment at the 3' end of Insertion 1

[4]Band observed determined to be an artifact of digestion from secondary structure and incomplete digestion of Insertion 4. KTi3 terminator does not hybridize well due to inverted repeat structure.

[5]Two bands co-migrate as one on the blot due to similar size (5006 bp from Insertion 3 and 5101 bp from Insertion 4).

[6]Sequence data does not extend far enough to determine exact size of this fragment.

[7]Three bands co-migrate on the blot due to similar size (two 2850 bp bands from Insertion 1 and 2787 bp band from Insertion 4).

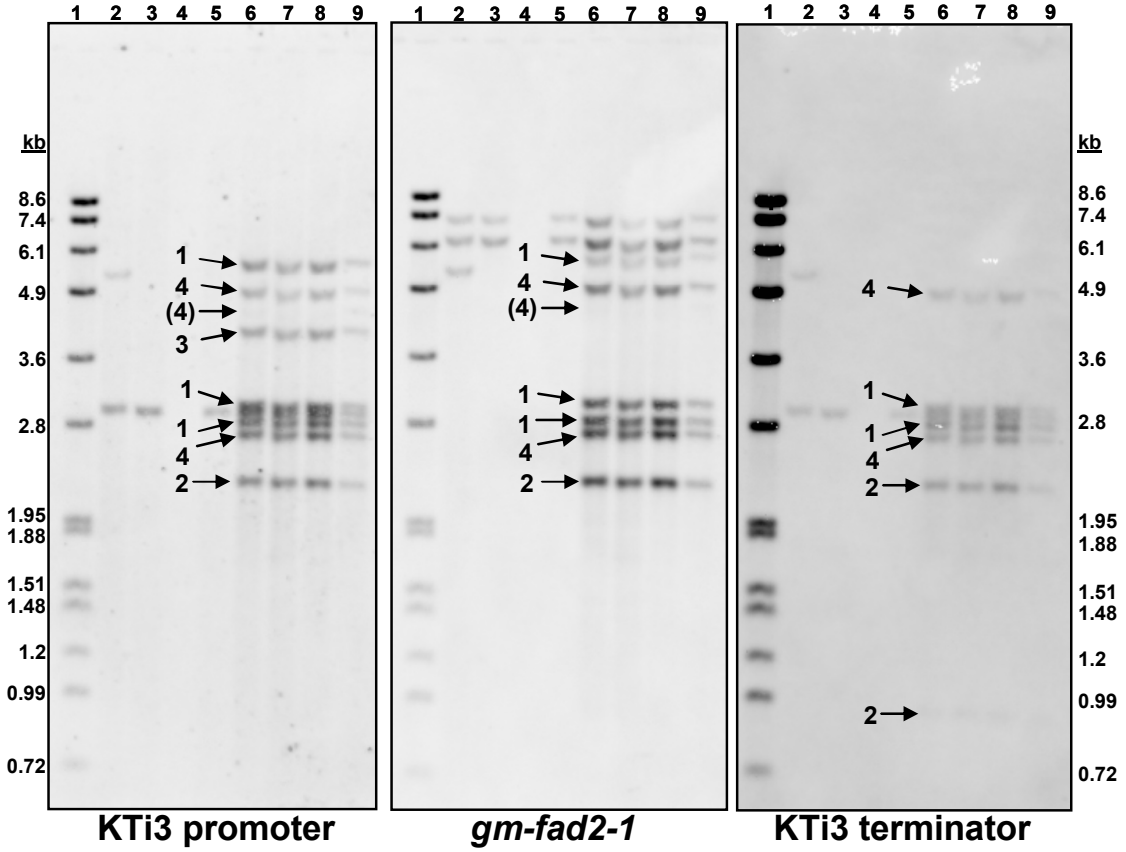
Eight bands hybridized to the KTi3 promoter probe, indicating multiple copies of the element inserted in 305423 soybean (Appendix 2, Table 4 and Appendix 2, Figure 5, KTi3 promoter panel). Bands of approximately 5500 bp, 4900 bp, 4600 bp (faint), 4300 bp, 3200 bp, 2900 bp, 2600 bp, and 2400 bp were observed (Appendix 2, Table 4). Based on the sequence data, seven bands would be expected from the four insertions and would represent the eight inserted copies of the KTi3 promoter (Appendix 2, Table 4). Two copies of the KTi3 promoter are found on the approximately 5500 bp band based on sequence data of 305423 soybean (Appendix 2, Table 4). The Southern data confirms these insertions (Appendix 2, Figure 5). The extra band of approximately 4600 bp is determined to be an artifact of digestion from Insertion 4 and not due to an additional insertion of the element; the inverted repeat structure of Insertion 4 (discussed in Appendix 2-3) likely inhibits reliable restriction enzyme digestion and thus creates an additional hybridizing DNA fragment with secondary structure that migrates abnormally on the gel. An additional band of approximately 3000 bp was observed that was determined to be due to hybridization to endogenous soybean sequences based on its presence in the control sample and was not related to the inserted DNA in 305423 soybean (Appendix 2, Table 4).

Seven bands hybridized to the *gm-fad2-1* probe, indicating multiple copies of this element inserted in 305423 soybean (Appendix 2, Table 4 and Appendix 2, Figure 5, *gm-fad2-1* panel). Bands were common with seven of those described for the KTi3 promoter probe and were approximately 5500 bp, 4900 bp, 4600 bp (faint), 3200 bp, 2900 bp, 2600 bp, and 2400 bp (Appendix 2, Table 4). Based on the sequence data, six bands would be expected from the four insertions and would represent the seven inserted copies of the *gm-fad2-1* fragment (Appendix 2, Table 4). Two copies of the *gm-fad2-1* fragment are found on the approximately 5500 bp band based on sequence data of 305423 soybean (Appendix 2, Table 4). The Southern data confirms these insertions (Appendix 2, Figure 5). As with the KTi3 promoter probe, the extra 4600 bp band was likely an artifact from incomplete restriction enzyme digestion and secondary structure of Insertion 4. Additional bands of approximately 7400 bp and 6200 bp were determined to be due to hybridization to endogenous soybean sequences based on their presence in the control sample and were not related to the inserted DNA in 305423 soybean (Appendix 2, Table 4).

Five hybridizing bands were observed indicating multiple copies of the KTi3 terminator element from PHP19340A are present in 305423 soybean (Appendix 2, Table 4 and Appendix 2, Figure 5, KTi3 terminator panel). Five bands common to those described for the KTi3 promoter and *gm-fad2-1* probes were observed: 4900 bp, 3200 bp, 2900 bp, 2600 bp, and 2400 bp (Appendix 2, Table 4) and are consistent with expected sequence data. A sixth band of approximately 990 bp was observed that was an additional hybridizing band due to the *Spe* I site in this element and not from an additional copy of the element (Appendix 2, Table 4 and Appendix 2, Figure 5) and is also consistent with the sequence of 305423 soybean. In this case, the 4600 bp band was not observed from Insertion 4, as the secondary structure of this band would not allow efficient hybridization of the KTi3 terminator probe. An additional band of approximately 3000 bp was observed that was determined to be due to hybridization to endogenous soybean sequences based on its presence in the control sample and was not related to the inserted DNA in 305423 soybean (Appendix 2, Table 4).

The results of the Southern blot analysis indicate that multiple copies (intact and truncated) of PHP19340A have been inserted into the genome of 305423 soybean and were in agreement with the sequence data. The inserted DNA in 305423 soybean contains eight copies of the KTi3 promoter, seven copies of the *gm-fad2-1* fragment, and five copies of the KTi3 terminator.

Appendix 2, Figure 5. Southern Blot Analysis of 305423 Soybean: *gm-fad2-1* Cassette Probes and *Eco*R V/*Spe* I Digest



Genomic DNA isolated from leaf tissue from individual plants of 305423 soybean (T4 generation) and of control soybean (Jack) was digested with a combination of *Eco*R V and *Spe* I and hybridized to the probes from the *gm-fad2-1* cassette. Probes used are indicated below each panel. Approximately 3 µg of genomic DNA was digested and loaded per lane. Approximately one gene copy of plasmid PHP19340 (Lane 2) or plasmid PHP17752 (Lane 3) was spiked into 3 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). **Note:** A band at approximately 990 bp was observed on original film data with KTi3 terminator hybridization, but is only faintly visible in the panel above.

Numbers and arrows indicate the Insertion number in 305423 soybean for each hybridizing fragment. This information is also noted in Appendix 2, Table 6. Number 4 in parentheses indicates the faint band that is an artifact of digestion from Insertion 4.

Lane	Sample
1	DNA molecular weight markers DIG VII
2	PHP19340 + control (Jack)
3	PHP17752 + control (Jack)
4	blank
5	control (Jack)
6	305423 soybean/ plant 7
7	305423 soybean/ plant 8
8	305423 soybean/ plant 9
9	305423 soybean/ plant 19

2-1b. Copy Number of Elements of the *gm-hra* Cassette of PHP17752A

Nco I restriction enzyme digestion was used to examine copy number of the genetic elements as it cuts the PHP17752A fragment once at bp position 3027, within the *gm-hra* gene (Appendix 2, Figure 2). The elements comprising this cassette - the SAMS regulatory region, *gm-hra* gene, and *als* terminator - were used as probes to determine number of copies inserted. Predicted fragment sizes for PHP17752A insertion with *Nco* I are given in Appendix 2, Table 3. The number of hybridizing bands with the SAMS and the *als* terminator probes would indicate the number of copies of each element in 305423 soybean. One hybridizing band would be expected in hybridizations with the SAMS and *als* terminator probes for one copy of the genetic element inserted (Appendix 2, Table 3). Since the *gm-hra* probe region spans the *Nco* I site in PHP17752A (Appendix 2, Figure 2), two bands would be expected for one copy of the *gm-hra* gene (Appendix 2, Table 3). Observed hybridizing bands with the *gm-hra* cassette probes are provided in Appendix 2, Table 5. Hybridization to bands endogenous to the soybean genome are indicated by asterisks (*) in the shaded boxes of Appendix 2, Table 5. Based on the Southern blot analysis, a single copy of the PHP17752A was determined to be present in 305423 soybean and this was consistent with the sequence data.

Appendix 2, Table 5. Observed Hybridizing Band Sizes in 305423 Soybean: *gm-hra* Cassette Probes

Sizes in base pairs (bp)

Enzyme Digestion	Predicted Sizes from 305423 Sequence	Insertion Number[1]	Observed Hybridizing Band Sizes in Southern Blot Analysis[2]		
			SAMS	*gm-hra*	*als* terminator
Nco I (Copy number)	3628 3053	1 1	~3600	~3600 ~3200	~3200
			~1800*	>8600* ~8000* ~6900* ~6100* ~5200* ~4900* ~4500* ~1600*	~4900*
*Hin*d III (Insertion integrity)	2418 1529	1 1	2418	2418 1529	1529
			~8600*	>8600 (faint)* ~8000 (faint)* ~7000* ~5000* ~4300* ~2300* ~2100* ~900*	~6800* ~5500 (faint)*

***** Hybridizing bands that were also present in the control sample. These bands are determined to be from sequences endogenous to the Jack variety background and are not related to the inserted DNA in 305423 soybean.

[1]Insertions are discussed in further detail in Appendix 2-3.

[2]Observed fragment sizes are approximated in the analysis from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, an approximate error of +/- 10% may be observed from sequence prediction.

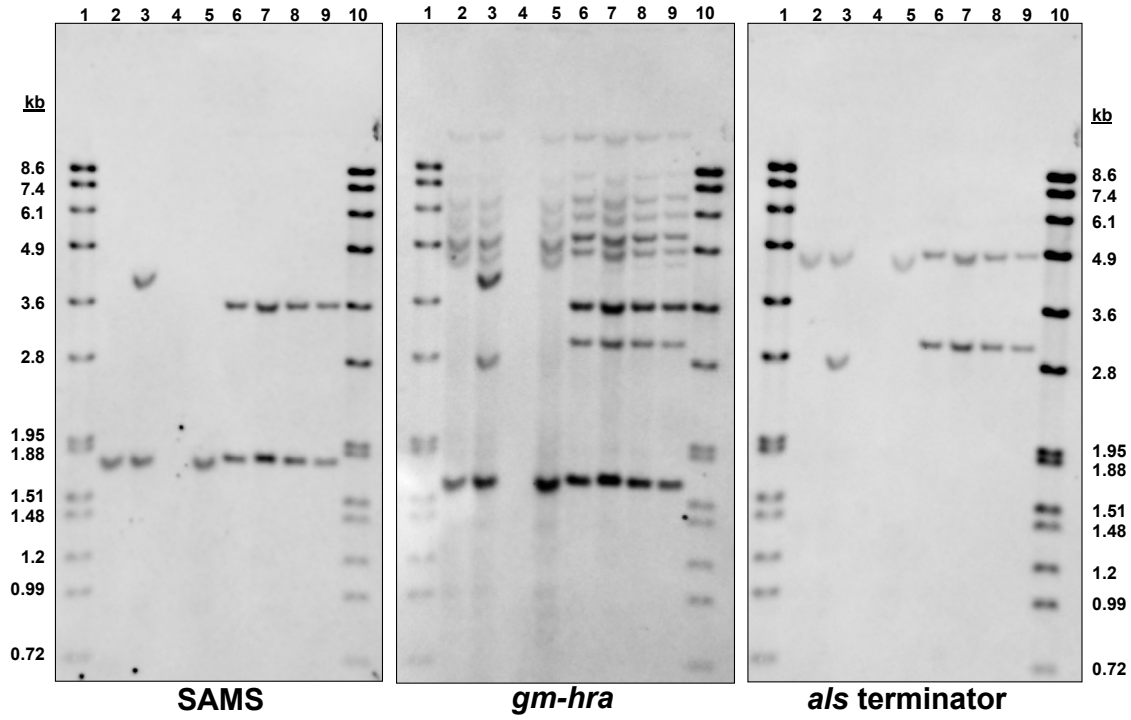
The SAMS probe hybridized to one band of approximately 3600 bp in 305423 soybean (Appendix 2, Table 5 and Appendix 2, Figure 6, SAMS panel), indicating one copy of the element. An additional band was observed at approximately 1800 bp that was observed in all 305423 soybean and in the control sample, indicating that this band was not related to the inserted DNA in 305423 soybean (Appendix 2, Table 5).

The *gm-hra* gene probe hybridized to the 3600 bp band described above and also to a band of approximately 3200 bp (Appendix 2, Table 5 and Appendix 2, Figure 6, *gm-hra* panel). Because the *Nco* I site is located in the probe region (Appendix 2, Figure 2), two bands were expected for a single element and the presence of two bands confirms one copy of the gene in 305423 soybean. The 3600 bp band is the same that hybridized to the SAMS probe (Appendix 2, Table 5), indicating the expected arrangement of elements on the inserted fragment (Appendix 2, Figure 2). Additional bands were observed at approximately 1600 bp and between 4500 bp and greater than 8600 bp that are not related to the inserted DNA and are present in the endogenous soybean background (Appendix 2, Table 5).

The *als* terminator probe hybridized to an approximately 3200 bp fragment (Appendix 2, Table 5 and Appendix 2, Figure 6, *als* terminator panel). This fragment is the same one that hybridized to the *gm-hra* probe, indicating the expected order of elements on the inserted fragment (Appendix 2, Figure 2). An additional band of approximately 4900 bp was observed in all 305423 soybean and in the control sample indicating that the hybridizing fragment was in the endogenous soybean genome and not related to the inserted DNA in 305423 soybean (Appendix 2, Table 5).

All hybridizations confirmed a single copy of the elements of the *gm-hra* cassette of PHP17752A and the expected arrangement of these elements in the 305423 soybean insertion.

Appendix 2, Figure 6. Southern Blot Analysis of 305423 Soybean: *gm-hra* Cassette Probes and *Nco* I Digest



Genomic DNA isolated from leaf tissue from individual plants of 305423 soybean (T4 generation) and of control soybean (Jack) was digested with *Nco* I and hybridized to the probes from the *gm-hra* cassette. Probes used are indicated below each panel. Approximately 4 µg of genomic DNA was digested and loaded per lane. Approximately one gene copy of plasmid PHP19340 (Lane 2) or plasmid PHP17752 (Lane 3) was spiked into 4 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	DNA molecular weight markers DIG VII
2	PHP19340 + control (Jack)
3	PHP17752 + control (Jack)
4	blank
5	control (Jack)
6	305423 soybean/ plant 16
7	305423 soybean/ plant 17
8	305423 soybean/ plant 18
9	305423 soybean/ plant 19
10	DNA molecular weight markers DIG VII

2-1c. Insertion Integrity of PHP19340A

A combined *Bam*H I and *Spe* I digestion (*Bam*H I/*Spe* I) was used to examine the integrity of the inserted DNA deriving from fragment PHP19340A. The *Bam*H I site is located at the beginning of fragment PHP19340A at bp position 14 and the *Spe* I site is located at the end of the fragment at bp position 2864 (Appendix 2, Figure 1). Predicted hybridizing fragment sizes are given in Appendix 2, Table 2. As discussed earlier, the *Spe* I site is located within the KTi3 terminator and, in addition to the internal 2850 bp band expected, a second faint band may be expected for each copy of the element (Appendix 2, Table 2).

The three probes of the *gm-fad2-1* cassette, KTi3 promoter, *gm-fad2-1*, and KTi3 terminator, hybridized to several common bands in the *Bam*H I/*Spe* I digested 305423 soybean DNA as can be seen in Appendix 2, Figure 7. Observed bands from these hybridizations are provided in Appendix 2, Table 4. Each probe hybridized to bands endogenous to the soybean genome (Appendix 2, Figure 7) and these bands are also provided in Appendix 2, Table 4. The three probes hybridized strongly to a band migrating equivalently to the plasmid band at 2850 bp indicating the presence of PHP19340A copies inserted into the genome of 305423 soybean that likely retain the *Bam*H I and *Spe* I sites (Appendix 2, Figure 7 and Appendix 2, Table 4). Based on these observations and as clarified by sequence data (discussed in Appendix 2-3), two 2850 bp bands, retaining the *Bam*H I and *Spe* I sites, and a closely co-migrating 2787 bp band, with a truncation that removes the *Bam*H I site, were present in 305423 soybean (Appendix 2, Figure 7 and Appendix 2, Table 4). Two fragments, approximately 4900 bp and 2400 bp, hybridized to all three elements (Appendix 2, Table 4 and Appendix 2, Figure 7) confirming the presence of truncated copies that had lost the *Bam*H I site on the PHP19340A fragment; the presence of these fragments was also confirmed by 305423 soybean sequence data. The KTi3 promoter and the *gm-fad2-1* probe hybridized to a 2200 bp and a 4600 bp fragment that did not hybridize with the KTi3 terminator (Appendix 2, Table 4 and Appendix 2, Figure 7), indicating two inserted regions in 305423 soybean contained only the KTi3 promoter and the *gm-fad2-1* fragment; these observations were consistent with sequence data. In addition to the hybridizing bands discussed above, the KTi3 terminator probe hybridized to another band of approximately 990 bp (Appendix 2, Table 4 and Appendix 2, Figure 7) that was due to the *Spe* I site within this region; this band was also observed in the *Eco*R V/*Spe* I analysis (Appendix 2, Table 4 and Appendix 2, Figure 5).

As expected based on copy number observations from *Eco*R V/*Spe* I analysis, two bands of approximately 4900 bp, one containing the KTi3 promoter and a non-functional backbone fragment and another containing the *gm-fad2-1* cassette, discussed above for *Bam*H I/*Spe* I analysis, were determined to be co-migrating. These two bands were determined to be fragments of 5006 bp and of 5101 bp from sequence data of 305423 soybean (Appendix 2, Table 4). The physical map of the inserted DNA in 305423 soybean is further discussed in Appendix 2-3.

Based on the Southern blot analysis which supports the sequence data, the inserted DNA in 305423 soybean consists of two copies of PHP19340A retaining *Bam*H I and *Spe* I sites (sequencing showed that one of these copies is truncated at the end of the KTi3 terminator), three PHP19340A fragments with a truncation of the *Bam*H I site, two truncated PHP19340A fragments containing only KTi3 promoter and *gm-fad2-1*, and one KTi3 promoter associated with a non-functional backbone DNA fragment (discussed in Appendices 2-2 and 2-3).

2-1d. Insertion Integrity of PHP17752A

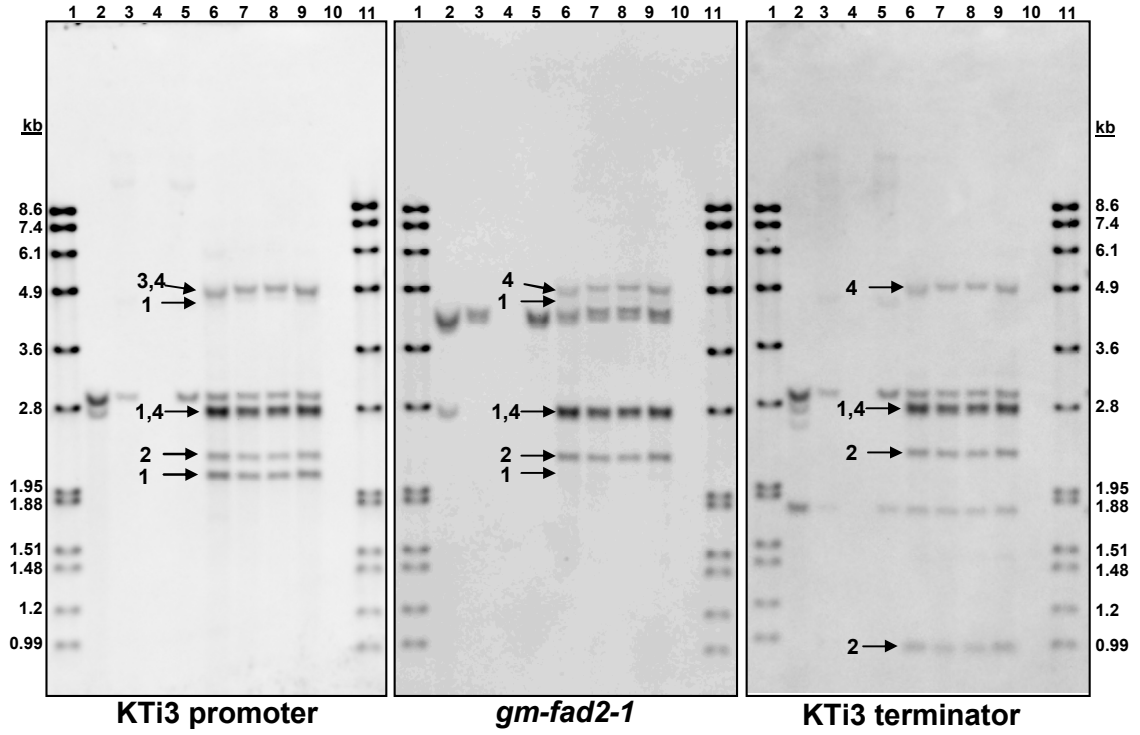
*Hin*d III was used to examine the integrity of the PHP17752A fragment inserted in 305423 soybean. *Hin*d III cuts at three critical sites on the PHP17752A fragment: bp positions 215, 2633, and 4162 (Appendix 2, Figure 2). This digestion excises the intact *gm-hra* expression cassette in two fragments thus verifying its integrity (Appendix 2, Figure 2). SAMS, *gm-hra* gene, and *als* terminator probes were used to characterize the *gm-hra* cassette of PHP17752A (Appendix 2,

Table 1 and Appendix 2, Figure 2). Predicted hybridizing band sizes are given in Appendix 2, Table 3.

The SAMS probe hybridized to the expected 2418 bp band (Appendix 2, Table 5 and Appendix 2, Figure 8). The *gm-hra* probe hybridized to the expected 2418 bp and 1529 bp bands (Appendix 2, Table 5 and Appendix 2, Figure 8). The *als* terminator hybridized to the expected 1529 bp band (Appendix 2, Table 5 and Appendix 2, Figure 8). All probes hybridized to bands endogenous to the soybean genome (Appendix 2, Figure 8) and these bands are further clarified in Appendix 2, Table 5. All three probes hybridized as expected confirming the integrity of the inserted PHP17752A fragment and the presence of the elements of the *gm-hra* cassette.

Based on the Southern blot analysis, a single, intact *gm-hra* cassette from fragment PHP17752A has been inserted into the genome of 305423 soybean.

Appendix 2, Figure 7. Southern Blot Analysis of 305423 Soybean: *gm-fad2-1* Cassette Probes and *Bam*H I/*Spe* I Digest

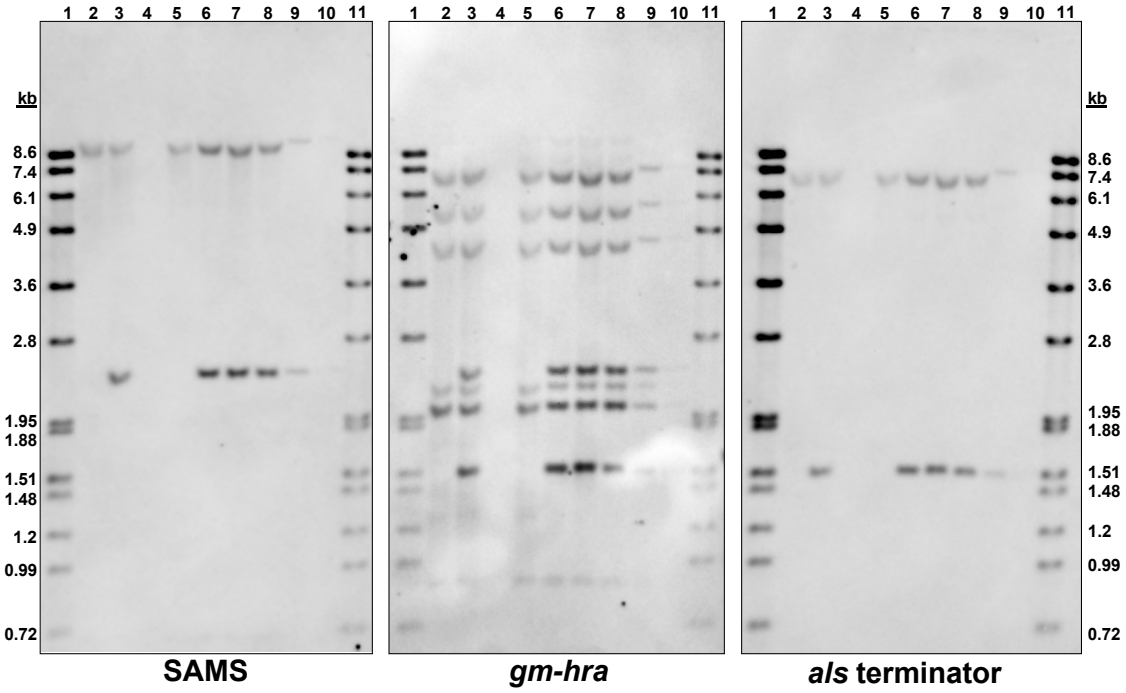


Genomic DNA isolated from leaf tissue from individual plants of 305423 soybean (T4 generation) and of control soybean (Jack) was digested with a combination of *Bam*H I and *Spe* I and hybridized to the probes from the *gm-fad2-1* cassette. Probes used are indicated below each panel. Approximately 3 µg of genomic DNA was digested and loaded per lane. Approximately one gene copy of plasmid PHP19340 (Lane 2) or plasmid PHP17752 (Lane 3) was spiked into 3 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). **Note:** Samples in Lanes 3, 5 and 6 did not digest completely and additional hybridizing bands are visible.

Numbers and arrows indicate the Insertion number in 305423 soybean for each hybridizing fragment. This information is also noted in Appendix 2, Table 6.

Lane	Sample
1	DNA molecular weight markers DIG VII
2	PHP19340 + control (Jack)
3	PHP17752 + control (Jack)
4	blank
5	control (Jack)
6	305423 soybean/ plant 1
7	305423 soybean/ plant 2
8	305423 soybean/ plant 16
9	305423 soybean/ plant 17
10	blank
11	DNA molecular weight markers DIG VII

Appendix 2, Figure 8. Southern Blot Analysis of 305423 Soybean: *gm-hra* Cassette Probes and *Hind* III Digest



Genomic DNA isolated from leaf tissue from individual plants of 305423 soybean (T4 generation) and of control soybean (Jack) was digested with *Hin*d III and hybridized to the probes from the *gm-hra* cassette. Probes used are indicated below each panel. Approximately 4 µg of genomic DNA was digested and loaded per lane. Approximately one gene copy of plasmid PHP19340 (Lane 2) or plasmid PHP17752 (Lane 3) was spiked into 4 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	DNA molecular weight markers DIG VII
2	PHP19340 + control (Jack)
3	PHP17752 + control (Jack)
4	blank
5	control (Jack)
6	305423 soybean/ plant 16
7	305423 soybean/ plant 17
8	305423 soybean/ plant 18
9	305423 soybean/ plant 19
10	blank
11	DNA molecular weight markers DIG VII

2-2. Plasmid Backbone DNA Analysis

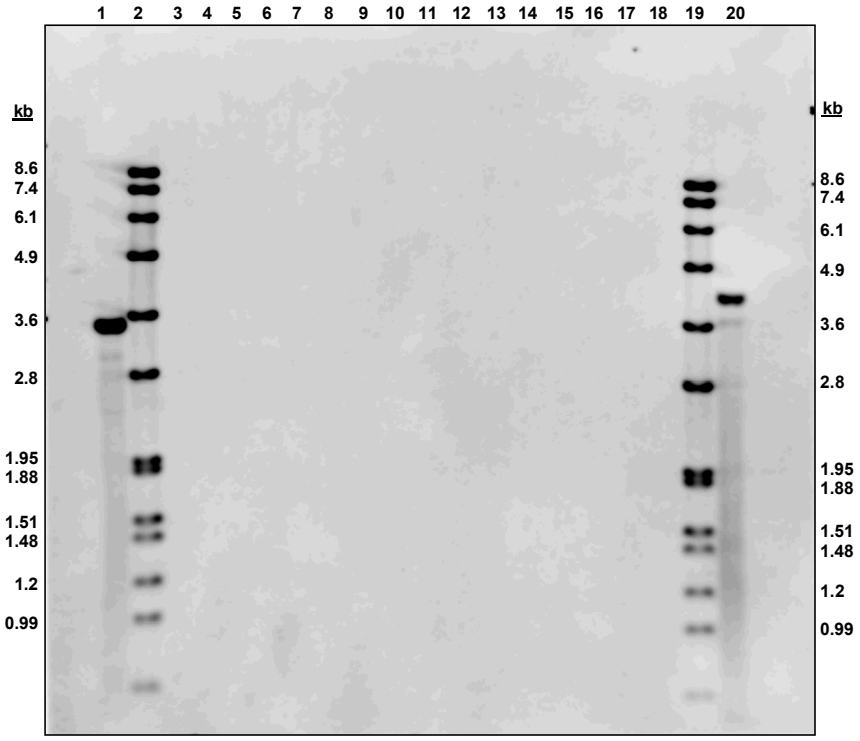
305423 soybean genomic DNA digested with *Nco* I was hybridized to the *hyg* and *backbone* probes representing the complete backbone DNA of plasmids PHP19340 and PHP17752 (Appendix 2, Figures 3 and 4). As the backbones of PHP19340 and PHP17752 are identical, the same set of probes were used in this analysis to examine the presence of regions outside the two transformation fragments, PHP19340A and PHP17752A, used to produce 305423 soybean. Southern blot analysis with the *hyg* probe confirms no hybridization with 305423 soybean DNA (Appendix 2, Figure 9), confirming that the hygromycin resistance gene was not present.

The results with the *backbone* probe are shown in Appendix 2, Figure 10. A hybridizing band of greater than 8600 bp was observed on the *Nco* I blot. This indicates the presence of a small fragment of backbone DNA in 305423 soybean from one of the two plasmids PHP19340 or PHP17752. As will be discussed further in Appendix 2-3, this fragment is associated with a truncated copy of the KTi3 promoter and is part of Insertion 3 in 305423 soybean.

Encompassed within the *backbone* probe region of both PHP19340 and PHP17752 is the bacterial origin of replication. Additional Southern blot analysis was conducted to confirm its absence from 305423 soybean. For each of these plasmids, the origin of replication was identified as a 370 base pair *Hae* II fragment on the backbone based on previously published work by Tomizawa *et al.* (1977). A probe was generated to encompass this *Hae* II region of the plasmid backbone (*plasmid ori*) and is marked on plasmids PHP19340 and PHP17752 (Appendix 2, Figures 3 and 4, respectively). Appendix 2, Figure 11 shows the results of the *plasmid ori* probe hybridization. This blot shows no hybridization of the probe to 305423 soybean DNA, confirming that the plasmid origin of replication was not present.

The Southern blot analysis confirmed the absence of all functional elements from the plasmid backbone in 305423 soybean (*i.e.* the absence of the hygromycin resistance gene and the plasmid origin of replication) and the presence of a small non-functional fragment of the plasmid backbone DNA.

Appendix 2, Figure 9. Southern Blot Analysis of 305423 Soybean: *hyg* Probe and *Nco* I Digest

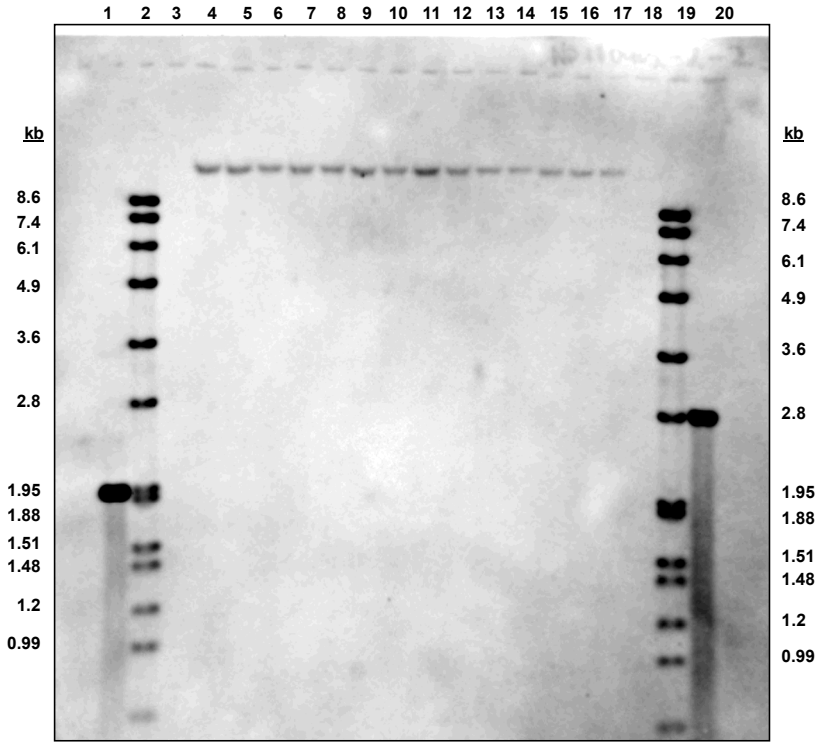


Genomic DNA isolated from leaf tissue of individual plants of 305423 soybean (T5 and T4 generation) and of unmodified control soybean (Jack) was digested with *Nco* I and probed with the *hyg* probe. Approximately 2 µg of genomic DNA was digested and loaded per lane. Approximately two gene copies of plasmid PHP19340 (Lane 1) or plasmid PHP17752 (Lane 20) was spiked into 2 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	2 copies PHP19340 + control (Jack)
2	DNA molecular weight markers DIG VII
3	control (Jack)
4	305423 soybean/ plant 8 (T5)
5	305423 soybean/ plant 9 (T5)
6	305423 soybean/ plant 10 (T5)
7	305423 soybean/ plant 11 (T5)
8	305423 soybean/ plant 12 (T5)
9	305423 soybean/ plant 13 (T5)
10	305423 soybean/ plant 14 (T5)

Lane	Sample
11	305423 soybean/ plant 38 (T4)
12	305423 soybean/ plant 39 (T4)
13	305423 soybean/ plant 40 (T4)
14	305423 soybean/ plant 41 (T4)
15	305423 soybean/ plant 42 (T4)
16	305423 soybean/ plant 43 (T4)
17	305423 soybean/ plant 44 (T4)
18	control (Jack)
19	DNA molecular weight markers DIG VII
20	2 copies PHP17752 + control (Jack)

Appendix 2, Figure 10. Southern Blot Analysis of 305423 Soybean: *backbone* Probe and *Nco* I Digest

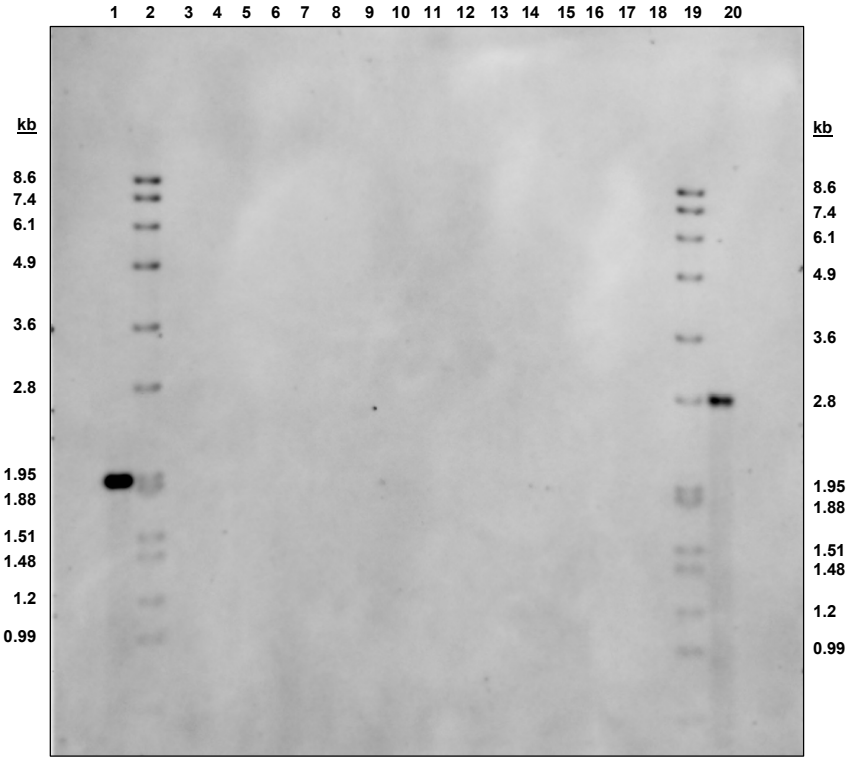


Genomic DNA isolated from leaf tissue of individual plants of 305423 soybean (T5 and T4 generation) and of unmodified control soybean (Jack) was digested with *Nco* I and probed with the *backbone* probe. Approximately 2 µg of genomic DNA was digested and loaded per lane. Approximately two gene copies of plasmid PHP19340 (Lane 1) or plasmid PHP17752 (Lane 20) was spiked into 2 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	2 copies PHP19340 + control (Jack)
2	DNA molecular weight markers DIG VII
3	control (Jack)
4	305423 soybean/ plant 8 (T5)
5	305423 soybean/ plant 9 (T5)
6	305423 soybean/ plant 10 (T5)
7	305423 soybean/ plant 11 (T5)
8	305423 soybean/ plant 12 (T5)
9	305423 soybean/ plant 13 (T5)
10	305423 soybean/ plant 14 (T5)

Lane	Sample
11	305423 soybean/ plant 38 (T4)
12	305423 soybean/ plant 39 (T4)
13	305423 soybean/ plant 40 (T4)
14	305423 soybean/ plant 41 (T4)
15	305423 soybean/ plant 42 (T4)
16	305423 soybean/ plant 43 (T4)
17	305423 soybean/ plant 44 (T4)
18	control (Jack)
19	DNA molecular weight markers DIG VII
20	2 copies PHP17752 + control (Jack)

Appendix 2, Figure 11. Southern Blot Analysis of 305423 Soybean: plasmid *ori* Probe and *Nco* I Digest



Genomic DNA isolated from leaf tissue of individual plants of 305423 soybean (T5 and T4 generation) and of unmodified control soybean (Jack) was digested with *Nco* I and probed with the *plasmid ori* probe. Approximately 2 µg of genomic DNA was digested and loaded per lane. Approximately two gene copies of plasmid PHP19340 (Lane 1) or plasmid PHP17752 (Lane 20) was spiked into 2 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	2 copies PHP19340 + control (Jack)
2	DNA molecular weight markers DIG VII
3	control (Jack)
4	305423 soybean/ plant 8 (T5)
5	305423 soybean/ plant 9 (T5)
6	305423 soybean/ plant 10 (T5)
7	305423 soybean/ plant 11 (T5)
8	305423 soybean/ plant 12 (T5)
9	305423 soybean/ plant 13 (T5)
10	305423 soybean/ plant 14 (T5)

Lane	Sample
11	305423 soybean/ plant 38 (T4)
12	305423 soybean/ plant 39 (T4)
13	305423 soybean/ plant 40 (T4)
14	305423 soybean/ plant 41 (T4)
15	305423 soybean/ plant 42 (T4)
16	305423 soybean/ plant 43 (T4)
17	305423 soybean/ plant 44 (T4)
18	control (Jack)
19	DNA molecular weight markers DIG VII
20	2 copies PHP17752 + control (Jack)

2-3. Physical Map of the Inserted DNA

As discussed in Appendix 2-1, intact and truncated copies of PHP19340A have been inserted into 305423 soybean comprising eight copies of the KTi3 promoter, seven copies of the *gm-fad2-1* fragment, and five copies of the KTi3 terminator. As determined in Appendix 2-2, a non-functional fragment of the plasmid backbone DNA was inserted in 305423 soybean. In addition, a single copy of the *gm-hra* cassette of PHP17752A has also been integrated.

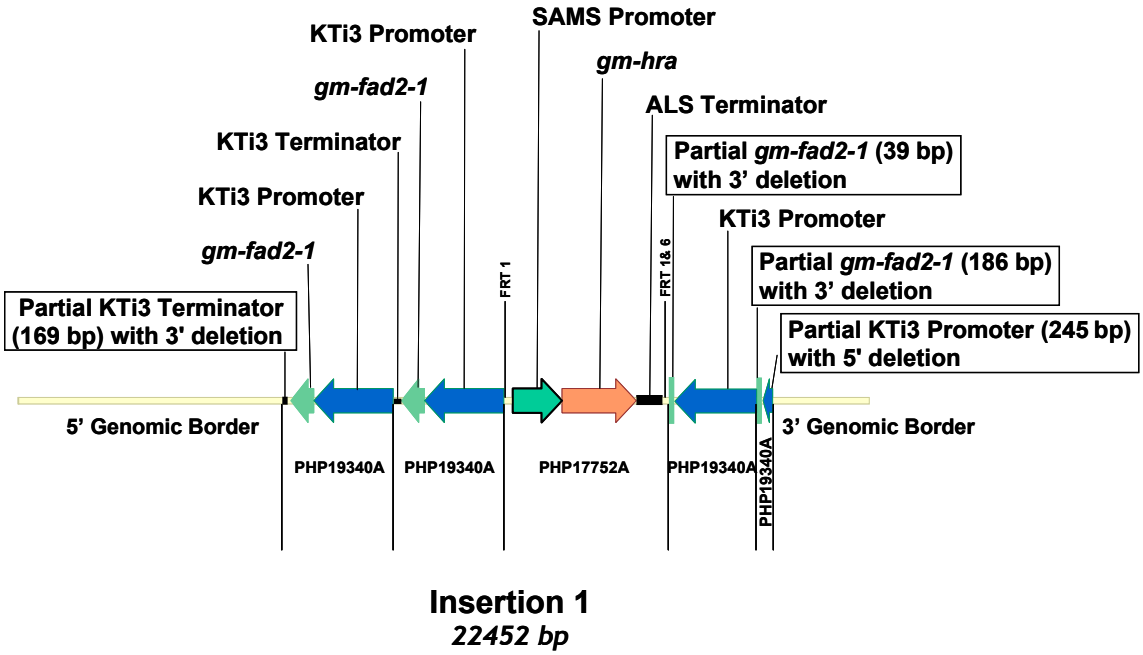
As determined by sequence data and as supported by the Southern blot analysis discussed in Appendices 2-1, 2-2 and below, the inserted DNA of 305423 soybean contains the following four insertions:

- Insertion 1: one intact PHP19340A, one intact PHP17752A, and three truncated PHP19340A fragments (one with all three elements and two with only the KTi3 promoter and the *gm-fad2-1* fragment)
- Insertion 2: one truncated PHP19340A fragment (with all three elements)
- Insertion 3: one truncated copy of the KTi3 promoter with a non-functional 495 bp fragment of the plasmid backbone
- Insertion 4: two inverted truncated copies of the PHP19340A fragment (with all three elements) connected by the KTi3 terminators.

A schematic overview map of these insertions in 305423 soybean is presented in Appendix 2, Figures 12 through 15. Orientation of the inserted PHP19340A fragments, the PHP17752A fragment, and elements is clarified by arrows in these figures.

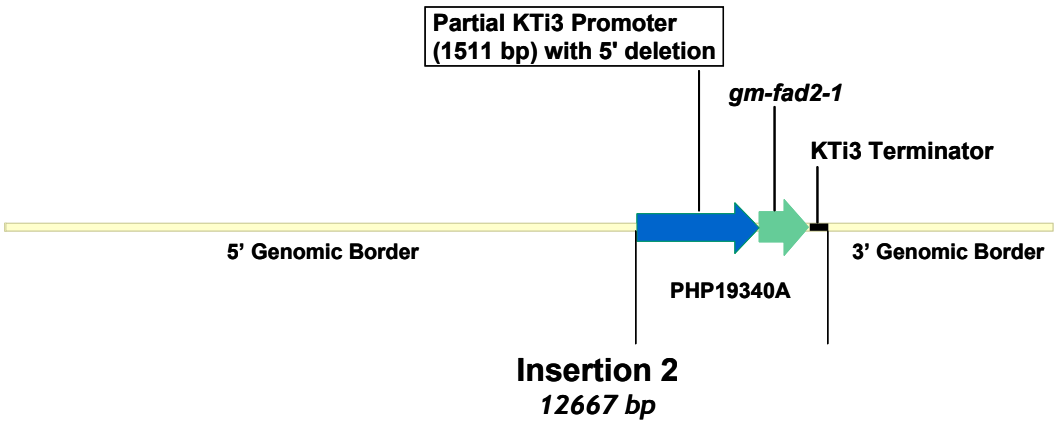
As diagrammed in Appendix 2, Figure 12 from 5' end to 3' end (left to right), Insertion 1 contains one PHP19340A fragment truncated at the KTi3 terminator, one intact copy of PHP19340A, one intact copy of PHP17752A, one truncated PHP19340A fragment with an intact KTi3 promoter and a truncated *gm-fad2-1* fragment, and one truncated PHP19340A fragment with a truncated KTi3 promoter and truncated *gm-fad2-1* fragment. As diagrammed in Appendix 2, Figure 13, Insertion 2 contains one PHP19340A fragment truncated at the KTi3 promoter. Insertion 3, shown in Appendix 2, Figure 14, contains a truncated copy of the KTi3 promoter with a non-functional 495 bp fragment of the plasmid backbone. Insertion 4 diagrammed in Appendix 2, Figure 15 contains, from 5' end to 3' end (left to right): one PHP19340A fragment truncated at the KTi3 promoter followed by an inverted copy of the PHP19340A fragment, also truncated at the KTi3 promoter. All insertions in 305423 soybean are present in a segregating out-crossed population based on Southern blot analysis (Appendix 2-4), indicating that the insertions are genetically linked.

Appendix 2, Figure 12. Schematic Map of Insertion 1 in 305423 Soybean



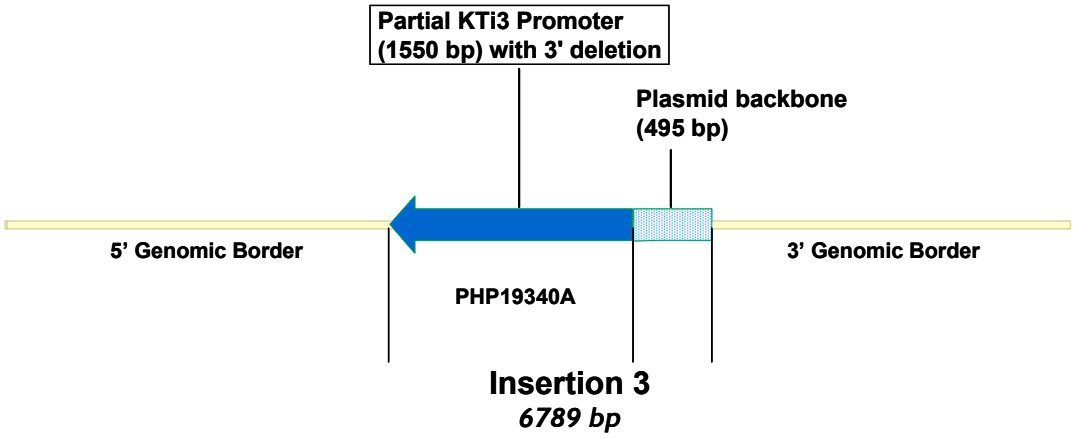
An overview of Insertion 1 in 305423 soybean. From the 5' end to 3' end (left to right), this insertion contains one PHP19340A fragment truncated at the KTi3 terminator, one intact copy of PHP19340A, one intact copy of PHP17752A, and two truncated PHP19340A fragments with only the KTi3 promoter and the *gm-fad2-1* fragment. Arrows indicate the orientation of each inserted element (arrows pointing to the right match orientation provided in the fragment maps in Appendix 2, Figures 1 and 2).

Appendix 2, Figure 13. Schematic Map of Insertion 2 in 305423 Soybean



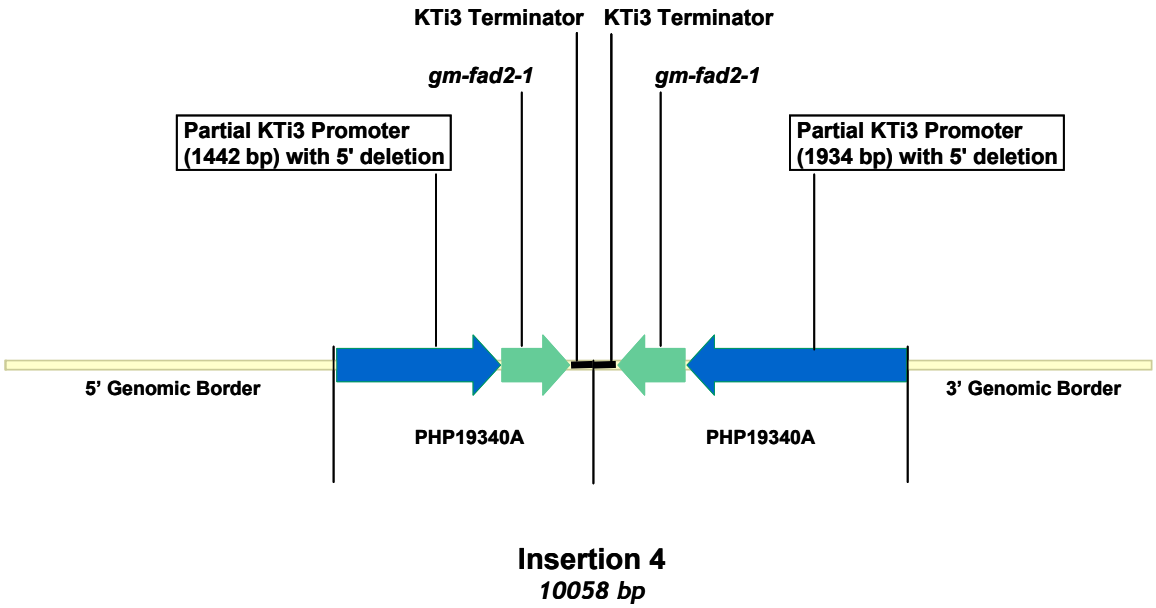
Overview of Insertion 2 in 305423 soybean. Insertion 2 contains one PHP19340A fragment truncated at the KTi3 promoter. Arrows indicate the orientation of each inserted element (arrows pointing to the right match orientation provided in the fragment maps in Appendix 2, Figures 1 and 2).

Appendix 2, Figure 14. Schematic Map of Insertion 3 in 305423 Soybean



Overview of Insertion 3 in 305423 soybean. Insertion 3 contains a truncated copy of the KTi3 promoter with a non-functional 495 bp fragment of the plasmid backbone. Arrows indicate the orientation of each inserted element (arrows pointing to the right match orientation provided in the fragment maps in Appendix 2, Figures 1 and 2).

Appendix 2, Figure 15. Schematic Map of Insertion 4 in 305423 Soybean



Overview of Insertion 4 in 305423 soybean. Insertion 4 contains, from 5' to 3' end (left to right), one PHP19340A fragment truncated at the KTi3 promoter and an inverted copy of the PHP19340A fragment also truncated at the KTi3 promoter. Arrows indicate the orientation of each inserted element (arrows pointing to the right match orientation provided in the fragment maps in Appendix 2, Figures 1 and 2).

Southern blot analysis of 305423 soybean using *Nco* I, *Eco*R V, *Bam*H I, *Spe* I, and *Eco*R V/*Spe* I restriction enzyme digestion, as described below, provided additional information to support the physical maps of the insertions.

Appendix 2, Figure 16 shows the *Nco* I analysis with the *gm-fad2-1* cassette probes and the *gm-hra* and *backbone* elements (Appendix 2, Table 6) and is described in detail below. The *gm-hra* and the *backbone* probes, because they are single copy elements in 305423 soybean, provided information on linking the insertions or elements together. Many of the bands observed in the *Nco* I Southern blots can be assigned to one of the insertions described based on the combination of probes that hybridized to that fragment (Appendix 2, Table 6 and Appendix 2, Figure 16). Some bands were not able to be assigned specifically to one insertion, as *Nco* I sites are located outside of the sequenced region and an exact size of the band could not be determined. However, the number of bands observed are fewer than would be expected, indicating an association of two or more of the insertions in 305423 soybean (*i.e.* two associated insertions resulting in only one hybridizing band). The association of Insertion 1 and Insertion 3 is most likely confirmed with this data as described below, however there are other associations of the insertions that are not confirmed but are indicated by the detection of fewer hybridizing bands than expected (Appendix 2, Table 6). These results indicate that the Southern data is in agreement with the sequence data obtained from 305423 soybean. Further description of the *Nco* I analysis is described in Appendix 2-4, where *Nco* I was used to determine inserted DNA stability.

Nine bands were observed in the T4 generation of the 305423 plants with the KTi3 promoter probe (Appendix 2, Figure 16); the sizes of these bands are indicated in Appendix 2, Table 6. Two of these bands, one of approximately 3600 bp and one of approximately 3200 bp also hybridized to the *gm-hra* probe (Appendix 2, Table 6 and Appendix 2, Figure 16). These common bands indicated an association of the KTi3 promoter elements with the *gm-hra* cassette. This association is supported by sequence data of 305423 soybean as diagrammed for Insertion 1 (Appendix 2, Figure 12).

Six bands were observed with the *gm-fad2-1* probe and their sizes are indicated in Appendix 2, Table 6 (Appendix 2, Figure 16). These six bands are also observed in hybridizations with the KTi3 promoter (Appendix 2, Table 6). The band that is greater than 8600 bp is common also to the *backbone* probe hybridization indicating that Insertion 1 and Insertion 3 are most likely linked to one another (Appendix 2, Table 6 and Appendix 2, Figure 16).

With the KTi3 terminator probe, five bands were observed (Appendix 2, Table 6 and Appendix 2, Figure 16). All five are common to both KTi3 promoter and *gm-fad2-1* hybridizations (Appendix 2, Table 6 and Appendix 2, Figure 16). For all the hybridizations, a very faint band of approximately 6100 bp was observed that was determined to be an artifact of digestion from Insertion 4 (Appendix 2, Table 6 and Appendix 2, Figure 16). It is likely that the inverted repeat structure of Insertion 4 may prevent complete restriction enzyme digestion and secondary structure created by the inverted repeat may cause anomalous migration on the blot.

Appendix 2, Table 6. Predicted and Observed Hybridizing Band Sizes from 305423 Soybean: *Nco* I Analysis

A. Predicted hybridization results based on 305423 Sequence

Predicted Size from 305423 Sequence (bp)	Insertion Number	Predicted Southern Hybridization Results[1]				
		KTi3 promoter	*gm-fad2-1*	KTi3 terminator	*gm-hra*	*backbone*
>9284[2]	1	+	+	+		
2924		+	+	+		
3628		+			+	
3053		+	+[3]		+	
>3563[2]		+	+			
>5058[2]	2	+	+	+		
>3400[2]	3	+				
>3389[2]		+				+
>7472[2]	4	+	+	+		
>2586[2]		+				

B. Observed hybridizing band sizes[4] (in base pairs) for *Nco* I Analysis (Appendix 2, Figure 16)

Insertion	KTi3 promoter	*gm-fad2-1*	KTi3 terminator	*gm-hra*	*backbone*
2	>8600	>8600	>8600		
3 and 1[5]	>8600	>8600			>8600
1	~8600	~8600[6]	~8600		
4	~7400	~7400	~7400		
3 or 4[7]	~6900[7]				
(4)[8]	~6100[8]	~6100[8]	~6100[8]		
1	~3600			~3600	
1	~3200			~3200	
1	~2900	~2900	~2900		
	~4000*	>8600*	~4000*	>8600*	
		~900*		~8000*	
				~6900*	
				~6100*	
				~5200*	
				~4900*	
				~4500*	
				~1600*	

*****Hybridizing bands that were also present in the control sample. These bands are determined to be from sequences endogenous to the Jack variety background and are not related to the inserted DNA in 305423 soybean.

[1]Plus sign (+) indicates probe expected to hybridize to this fragment.

[2]Sequence data does not extend far enough to determine exact size of this fragment.

[3]39 bp portion of *gm-fad2-1* in this fragment. No detection or weak detection is expected.

[4]Observed fragment sizes are approximated in the analysis from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, an approximate error of +/- 10% may be observed from sequence prediction.

[5]These two Insertions are most likely associated in a single hybridizing fragment.

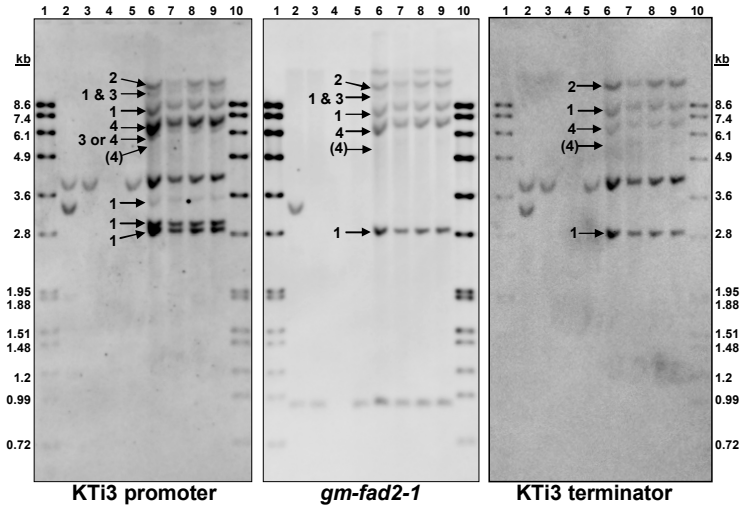
[6]This hybridizing band appears greater than 8600 bp in Appendix 2, Figures 21 and 23, due to the differences in amount of genomic DNA loaded per lane as greater amounts of DNA can decrease observed size on an agarose gel. Per lane, 4 µg of genomic DNA were loaded in Appendix 2, Figure 16 and 2 µg were loaded per lane in Appendix 2, Figures 21 and 23. (Discussed in Appendix 2-4)

[7]Insertion 3 or 4 could be assigned to this fragment. Either insertion could also be associated with other insertions on hybridizing bands ~8600 bp or greater.

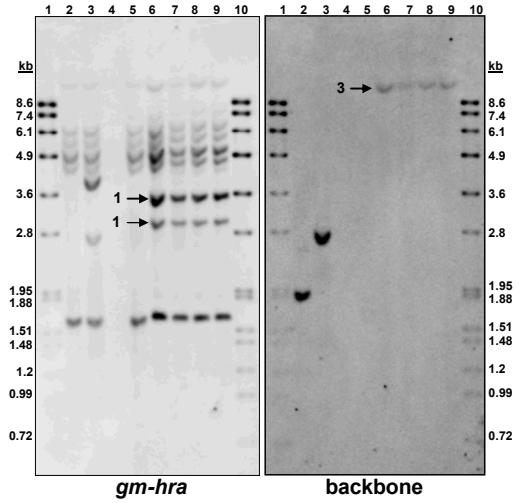
[8]Faintly hybridizing fragment that is an artifact of digestion from Insertion 4.

Appendix 2, Figure 16. Southern Blot Analysis of 305423 Soybean: *Nco* I Analysis

A.



B.



Genomic DNA isolated from leaf tissue from individual plants of 305423 soybean (T4 generation) and of control soybean (Jack) was digested with *Nco* I. Probes used are indicated below each panel. *Panel A:* *gm-fad2-1* cassette; *Panel B:* single copy elements. Approximately 4 µg of genomic DNA was digested and loaded per lane. Approximately one gene copy of plasmid PHP19340 (Lane 2) or plasmid PHP17752 (Lane 3) was spiked into 4 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Numbers and arrows indicate the Insertion number in 305423 soybean for each hybridizing fragment. This information is also noted in Appendix 2, Table 6. Number 4 in parentheses is the faint band that is an artifact of digestion from Insertion 4. As indicated above, Insertion 1 and 3 are likely associated together in the same greater than 8600 bp band.

Lane	Sample	Lane	Sample
1	DNA molecular weight markers DIG VII	6	305423 soybean/ plant 16
2	PHP19340 + control (Jack)	7	305423 soybean/ plant 17
3	PHP17752 + control (Jack)	8	305423 soybean/ plant 18
4	blank	9	305423 soybean/ plant 19
5	control (Jack)	10	DNA molecular weight markers DIG VII

Southern blot results for the *Eco*R V analysis are presented in Appendix 2, Figure 17 and summarized in Appendix 2, Table 7. The probes used for this analysis include those from the *gm-fad2-1* cassette and the *als* terminator and *backbone* probes that are single copy elements in 305423 soybean. Each of the bands observed in the *Eco*R V analysis were able to be assigned to a particular insertion and the number of hybridizing bands observed is consistent with what was expected based on 305423 sequence (Appendix 2, Table 7 and Appendix 2, Figure 17). These bands were determined to be from a particular insertion based on the combination of probes that hybridized to it.

Appendix 2, Table 7. Predicted and Observed Hybridizing Band Sizes from 305423 Soybean: *Eco*R V Analysis

Predicted Size (*in base pairs*) from 305423 Sequence	**Insertion Number**	**Observed Hybridizing Bands in *Eco*R V Analysis (Appendix 2, Figure 17) *sizes in base pairs*[1]**				
		KTi3 promoter	***gm-fad2-1***	**KTi3 terminator**	***als* terminator**	***backbone***
>9332[2]	4	>8600	>8600	>8600		
>13067[2]	1	>8600	>8600	>8600		
>5546[2]	2	~8600	~8600	~8600		
5906	1	~5500	~5500		~5500	
4211	3	~4300				~4300
3433[3]	1				3433[3]	
		~8600*	>8600*	~8600*	~4500* ~2200*	

*****Hybridizing bands that were also present in the control sample. These bands are determined to be from sequences endogenous to the Jack variety background and are not related to the inserted DNA in 305423 soybean.

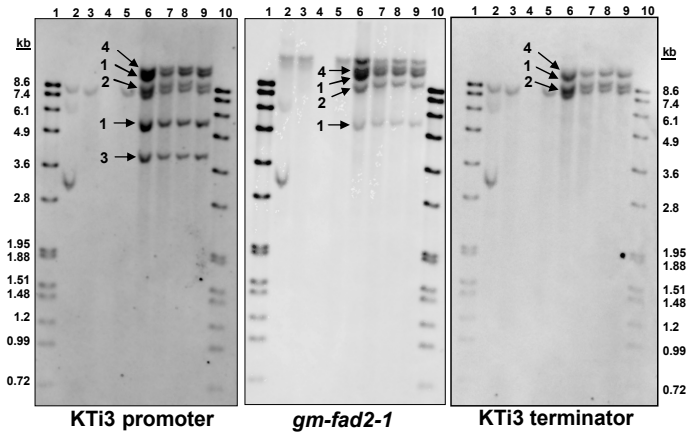
[1]Observed fragment sizes are approximated in the analysis from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, an approximate error of +/- 10% may be observed from sequence prediction.

[2]Sequence data does not extend far enough to determine exact size of this fragment.

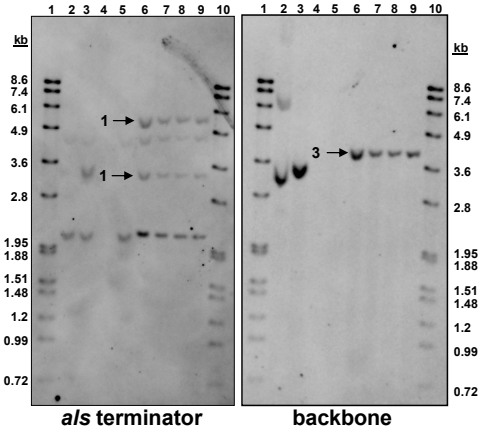
[3]This band is the same size as predicted from fragment PHP17752A (Appendix 2, Figure 2). This band migrated equivalently with the plasmid positive control.

Appendix 2, Figure 17. Southern Blot Analysis of 305423 Soybean: *Eco*R V Analysis

A.



B.



Genomic DNA isolated from leaf tissue from individual plants of 305423 soybean (T4 generation) and of control soybean (Jack) was digested with *Eco*R V. Probes used are indicated below each panel. *Panel A:* *gm-fad2-1* cassette; *Panel B:* single copy elements. Approximately 4 µg of genomic DNA was digested and loaded per lane. Approximately one gene copy of plasmid PHP19340 (Lane 2) or plasmid PHP17752 (Lane 3) was spiked into 4 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Numbers and arrows indicate the Insertion number in 305423 soybean for each hybridizing fragment. This information is also noted in Appendix 2, Table 7.

Lane	Sample	Lane	Sample
1	DNA molecular weight markers DIG VII	6	305423 soybean/ plant 16
2	PHP19340 + control (Jack)	7	305423 soybean/ plant 17
3	PHP17752 + control (Jack)	8	305423 soybean/ plant 18
4	blank	9	305423 soybean/ plant 19
5	control (Jack)	10	DNA molecular weight markers DIG VII

Similarly, each of the bands observed in *Bam*H I analysis were able to be assigned to a particular insertion and were consistent with the number expected (Appendix 2, Table 8 and Appendix 2, Figure 18). The *Bam*H I analysis presented in Appendix 2, Figure 24 and summarized in Appendix 2, Table 8 show that the bands predicted from the 305423 soybean sequence were consistent with observations on the Southern blot. As with previous analysis, the combination of probes that hybridized to each fragment helped to identify the associated insertion number.

Appendix 2, Table 8. Predicted and Observed Hybridizing Band Sizes from 305423 Soybean: *Bam*H I Analysis

Predicted Size (*in base pairs*) from 305423 Sequence	**Insertion Number**	**Observed Hybridizing Bands in *Bam*H I Analysis (Appendix 2, Figure 18) *sizes in base pairs*[1]**			
		KTi3 promoter	***gm-fad2-1***	**KTi3 terminator**	***backbone***
>9958[2]	2	>8600[3]	>8600[3]	>8600[3]	
>10058[2]	4	>8600[4]	>8600[4]	>8600[4]	
>6607[2]	3	~8000			~8000
>2974[2]	1	~6100	~6100		
3943	1	~3900	~3900	~3900	
2924	1	~2800	~2800	~2800	
2169	1	~2200	~2200 (faint)		
			~7400*		
			~6800*		

*Hybridizing bands that were also present in the control sample. These bands are determined to be from sequences endogenous to the Jack variety background and are not related to the inserted DNA in 305423 soybean.

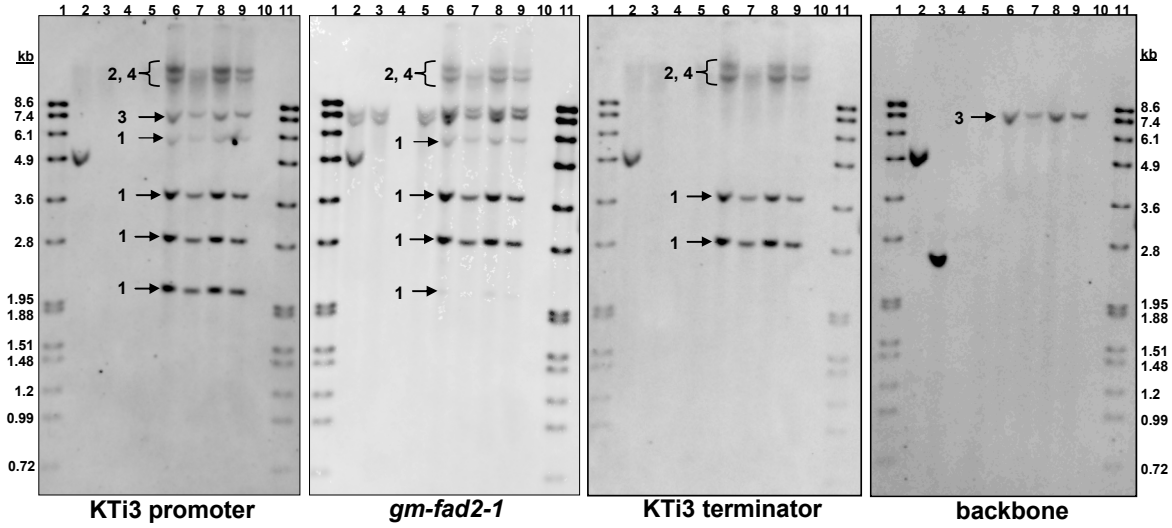
[1]Observed fragment sizes are approximated in the analysis from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, an approximate error of +/- 10% may be observed from sequence prediction.

[2]Sequence data does not extend far enough to determine exact size of this fragment.

[3]This hybridizing band cannot be assigned to one insertion. Insertion 4 (>10058 bp) is also a possibility.

[4]This hybridizing band cannot be assigned to one insertion. Insertion 2 (>9958 bp) is also a possibility.

Appendix 2, Figure 18. Southern Blot Analysis of 305423 Soybean: *Bam*H I Analysis



Genomic DNA isolated from leaf tissue from individual plants of 305423 soybean (T4 generation) and of control soybean (Jack) was digested with *Bam*H I. Probes used are indicated below each panel. Approximately 4 µg of genomic DNA was digested and loaded per lane. Approximately one gene copy of plasmid PHP19340 (Lane 2) or plasmid PHP17752 (Lane 3) was spiked into 4 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Numbers and arrows indicate the Insertion number in 305423 soybean for each hybridizing fragment. This information is also noted in Appendix 2, Table 10. Hybridizing fragments due to Insertion 2 and Insertion 4 were not able to be individually identified and are shown bracketed in the panel above.

Lane	Sample
1	DNA molecular weight markers DIG VII
2	PHP19340 + control (Jack)
3	PHP17752 + control (Jack)
4	blank
5	control (Jack)
6	305423 soybean/ plant 16
7	305423 soybean/ plant 17
8	305423 soybean/ plant 18
9	305423 soybean/ plant 19
10	blank
11	DNA molecular weight markers DIG VII

The bands observed in the *Spe* I Southern blots were similarly able to be assigned to particular insertions based on the sequence data (Appendix 2, Table 9 and Appendix 2, Figure 19). As with the *Bam*H I/*Spe* I analysis discussed in Appendix 2-1, two bands, 5006 bp from Insertion 3 and 5101 bp from Insertion 4, were also observed as a single migrating band of approximately 4900 bp in the KTi3 promoter hybridization (Appendix 2, Table 9 and Appendix 2, Figure 19). As summarized in Appendix 2, Table 9, the predicted hybridizing bands from each of the four insertions in 305423 soybean were able to be directly correlated to the observed hybridizing bands in the *Spe* I analysis and confirm the sequence from 305423 soybean.

Appendix 2, Table 9. Predicted and Observed Hybridizing Band Sizes from 305423 Soybean: *Spe* I Analysis

Predicted Size (*in base pairs*) from 305423 Sequence	**Insertion Number**	**Observed Hybridizing Bands in *Spe* I Analysis (Appendix 2, Figure 19) *sizes in base pairs*[1]**				
		KTi3 promoter	***gm-fad2-1***	**KTi3 terminator**	***gm-hra***	***backbone***
>12505[2]	1	>8600	>8600	>8600	>8600	
5101	4	~4900[3]	~4900	~4900		
5006	3	~4900[3]				~4900
2924	1	~2900	~2900	~2900		
2787	4	~2600	~2600	~2600		
2333	2	~2400	~2400	~2400		
984	2			~990		
		~3000*	~7100* ~6100*	~3000* ~1800* ~1500 (faint)*	>8600* ~8000*	

*Hybridizing bands that were also present in the control sample. These bands are determined to be from sequences endogenous to the Jack variety background and are not related to the inserted DNA in 305423 soybean.

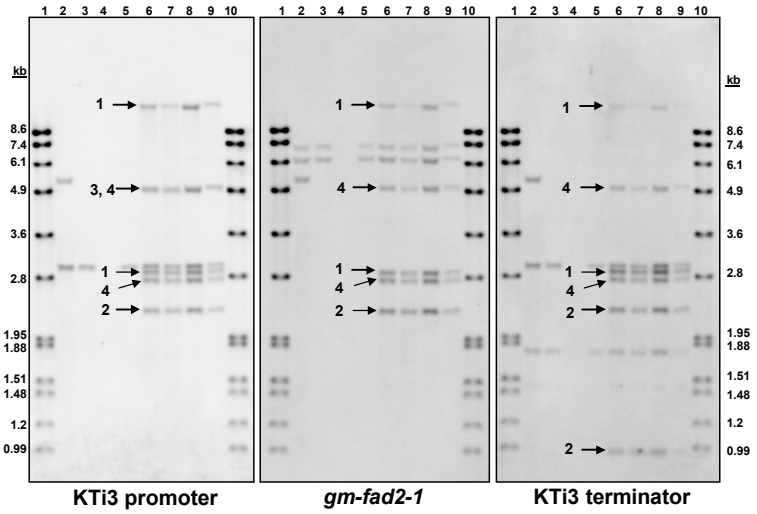
[1]Observed fragment sizes are approximated in the analysis from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, an approximate error of +/- 10% may be observed from sequence prediction.

[2]Sequence data does not extend far enough to determine exact size of this fragment.

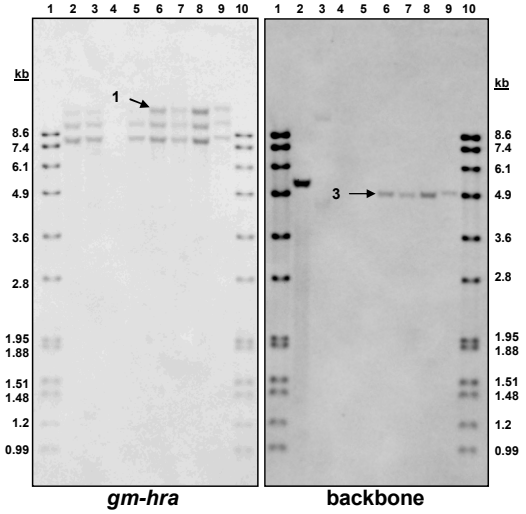
[3]Two bands co-migrate as one on the blot due to similar size (5006 bp from Insertion 3 and 5101 bp from Insertion 4).

Appendix 2, Figure 19. Southern Blot Analysis of 305423 Soybean: *Spe* I Analysis

A.



B.



Genomic DNA isolated from leaf tissue from individual plants of 305423 soybean (T4 generation) and of control soybean (Jack) was digested with *Spe* I. Probes used are indicated below each panel. *Panel A:* *gm-fad2-1* cassette; *Panel B:* single copy elements. Approximately 3 µg of genomic DNA was digested and loaded per lane. Approximately one gene copy of plasmid PHP19340 (Lane 2) or plasmid PHP17752 (Lane 3) was spiked into 3 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Numbers and arrows indicate the Insertion number in 305423 soybean for each hybridizing fragment. This information is also noted in Appendix 2, Table 9.

Lane	Sample	Lane	Sample
1	DNA molecular weight markers DIG VII	6	305423 soybean/ plant 7
2	PHP19340 + control (Jack)	7	305423 soybean/ plant 8
3	PHP17752 + control (Jack)	8	305423 soybean/ plant 9
4	blank	9	305423 soybean/ plant 19
5	control (Jack)	10	DNA molecular weight markers DIG VII

The *Eco*R V/*Spe* I analysis is presented in Appendix 2, Figure 20 and provides additional hybridization data with the *als* terminator and *backbone* probes that are single copy elements in 305423 soybean. As described in Appendix 2-1 and also summarized in Appendix 2, Table 10 and Appendix 2, Figure 20 below, the bands observed in the *Eco*R V/*Spe* I Southern blots were able to be assigned to particular insertions based on 305423 soybean sequence. Additional hybridization data with the *als* terminator and *backbone* probes (Appendix 2, Figure 20) clarify the insertion assignments discussed in Appendix 2-1. As summarized in Appendix 2, Table 10, the predicted hybridizing bands from each of the four insertions in 305423 soybean were able to be directly correlated to the observed hybridizing bands in the *Eco*R V/*Spe* I analysis and confirm the sequence from 305423 soybean.

Appendix 2, Table 10. Predicted and Observed Hybridizing Band Sizes from 305423 Soybean: *Eco*R V/*Spe* I Analysis

Predicted Sizes from 305423 Sequence	Insertion Number	Observed Hybridizing Bands in *Eco*R V/*Spe* I Analysis (Appendix 2, Figure 20) *sizes in base pairs*[1]				
		KTi3 promoter	*gm-fad2-1*	KTi3 terminator	*backbone*	*als* terminator
5906	1	~5500	~5500			~5500
5101	4	~4900	~4900	~4900		
	(4)[2]	~4600[2]	~4600[2]			
4211	3	~4300			~4300	
3433	1					3433[3]
3120	1	~3200	~3200	~3200		
2924	1	~2900	~2900	~2900		
2787	4	~2600	~2600	~2600		
2333	2	~2400	~2400	~2400		
984	2			~990		
		~3000*	~7400* ~6200*	~3000*		~2500* ~2200*

*Hybridizing bands that were also present in the control sample. These bands are determined to be from sequences endogenous to the Jack variety background and are not related to the inserted DNA in 305423 soybean.

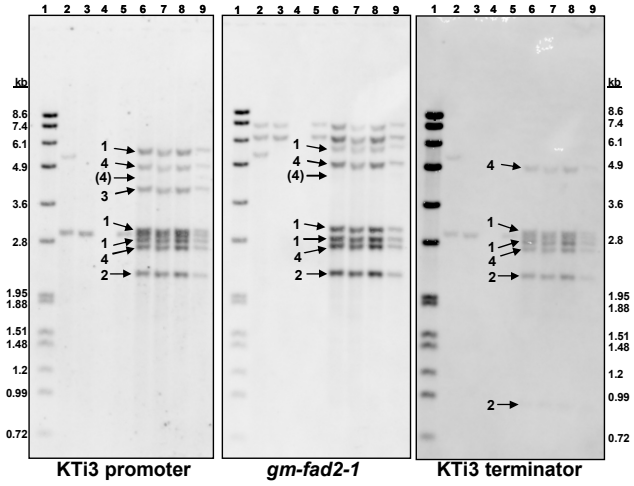
[1]Observed fragment sizes are approximated in the analysis from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, an approximate error of +/- 10% may be observed from sequence prediction.

[2]Band observed determined to be an artifact of digestion from Insertion 4. KTi3 terminator does not hybridize well due to inverted repeat structure.

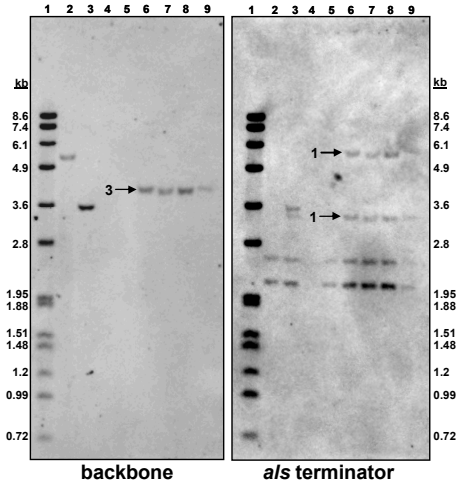
[3]This band is the same size as predicted from fragment PHP17752A (Appendix 2, Figure 2). This band migrated equivalently with the plasmid positive control.

Appendix 2, Figure 20. Southern Blot Analysis of 305423 Soybean: *Eco*R V/*Spe* I Analysis

A.



B.



Genomic DNA isolated from leaf tissue from individual plants of 305423 soybean (T4 generation) and of control soybean (Jack) was digested with a combination of *Eco*R V and *Spe* I. Probes used are indicated below each panel. *Panel A:* *gm-fad2-1* cassette; *Panel B:* single copy elements. Approximately 3 µg of genomic DNA was digested and loaded per lane. Approximately one gene copy of plasmid PHP19340 (Lane 2) or plasmid PHP17752 (Lane 3) was spiked into 3 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Numbers and arrows indicate the Insertion number in 305423 soybean for each hybridizing fragment. This information is also noted in Appendix 2, Table 10. Number 4 in parentheses is the faint band that is an artifact of digestion from Insertion 4.

Lane	Sample	Lane	Sample
1	DNA molecular weight markers DIG VII	6	305423 soybean/ plant 7
2	PHP19340 + control (Jack)	7	305423 soybean/ plant 8
3	PHP17752 + control (Jack)	8	305423 soybean/ plant 9
4	blank	9	305423 soybean/ plant 19
5	control (Jack)		

As discussed above, Southern blot analysis using several restriction enzyme digestions correlated with the sequence data and confirmed the presence of the four insertions in 305423 soybean as listed below:

- Insertion 1: one intact PHP19340A, one intact PHP17752A, and three truncated PHP19340A fragments (one with all three elements and two with only the KTi3 promoter and the *gm-fad2-1* fragment)
- Insertion 2: one truncated PHP19340A fragment (with all three elements)
- Insertion 3: one truncated copy of the KTi3 promoter with a non-functional 495 bp fragment of the plasmid backbone
- Insertion 4: two inverted truncated copies of the PHP19340A fragment (with all three elements) connected by the KTi3 terminators.

Using the sequence data encompassing the 5' and 3' genomic border sequences, each of the four insertions in 305423 soybean was screened for the presence of open reading frames (ORFs) containing both start and stop codons that spanned any novel junctions and that were greater than or equal to 300 bp (100 amino acids) in length. When identified, these ORFs were screened against the FARRP7 allergen dataset (Food Allergy Research and Resource Program – University of Nebraska – Lincoln) using current criteria (FAO/WHO, 2001; Codex, 2003) to identify any potential for cross reactivity with known or putative allergens, and subjected to BLASTP searches against the NCBI Protein dataset (version 158, 2/15/07) in order to ascertain any identities to potentially toxic proteins.

Two junction spanning ORFs greater than 300 bp in length were identified from the search. The first ORF occurs at the 5' insert/border junction of Insertion 2, and is 106 amino acids long, with only 9 amino acids contributed by the 305423 insertion. An *in silico* analysis showed the lack of biologically significant identities to known protein toxins or allergens. Given the low degree of novelty (only 9 amino acids are contributed by the insertion) and the low likelihood of transcription due to the absence of transcriptional elements upstream and adjacent to the ORF, these results indicate that there are no safety concerns with this ORF.

A second ORF comprising 235 amino acids, with 54 residues contributed by the 305423 insertion, extends out from the truncated KTi3 promoter sequence into the 5' genomic border of Insertion 3. The vast majority of alignments produced by this ORF with protein sequences from NCBI Protein database occur predominantly in the genomic portion of the ORF (amino acids 55 – 235), and show a low level of identity to retrotransposon *int* sequences, that likely reflect the presence of an inactive, ancestral *copia*-type transposable element in the genomic border region (Kumar and Bennetzen, 1999). No identities to known allergens as well as biologically significant alignments to toxin proteins were returned. Additionally, the truncated KTi3 promoter upstream of this ORF is missing all the known elements necessary for transcription (Jofuku and Goldberg, 1989). Taken together, these observations indicate that there are no safety concerns with this ORF.

2-4. Stability of the Insertion Across Generations

DNA samples from individual plants of 305423 soybean of the T4, T5, and F2 generations (27, 30, and 100 plants, respectively) were digested with *Nco* I to verify that the hybridization pattern remained consistent across the three generations. A single *Nco* I site is found in PHP19340A at base pair position 603 (Appendix 2, Figure 1) and, using the *gm-fad2-1* probe, the event-specific hybridization pattern could be examined. Likewise for PHP17752A, a single site lies within the *gm-hra* gene at base pair position 3027 (Appendix 2, Figure 2) and the *gm-hra* probe was used to examine the event-specific pattern. As discussed in Appendix 2-3, the *Nco* I digest with the *gm-fad2-1* and *gm-hra* probes provided a means to examine the four insertions in 305423 soybean and to determine if the inserted DNA is stable across these generations. Representative blots of the analysis conducted on the T4, T5, and F2 generations are presented in the discussion below. The Southern blot analysis, as discussed below, determined that the inserted DNA remained stable across these three breeding generations.

As discussed in Appendix 2-3, an event-specific hybridization pattern was determined through Southern blot analysis with *Nco* I digestion and select probes (Appendix 2, Table 6 and Appendix 2, Figure 16). This determined pattern was the basis for further analysis of the generations used to determine the stability of the inserted DNA in 305423 soybean. With the *gm-fad2-1* probe, six bands were observed in the T4 generation of the 305423 soybean plants (discussed in Appendix 2-3, Appendix 2, Table 6 and Appendix 2, Figure 16). There were two bands greater than 8600 bp, one of approximately 8600 bp, one of approximately 7400 bp, one very faintly hybridizing of approximately 6100 bp, and one of approximately 2900 bp (Appendix 2, Table 6 and Appendix 2, Figure 16). Two bands, one at greater than 8600 bp and one at approximately 900 bp were also observed in all 305423 soybean and Jack control samples, indicating the bands were endogenous to the Jack variety background and not related to the insertion (Appendix 2, Table 6 and Appendix 2, Figure 16). When the T4 and T5 generations of 305423 soybean were compared to each other, the same hybridization pattern of the two generations were observed across all the T4 and T5 plants analyzed (representative blot shown in Appendix 2, Figure 21). The approximately 8600 bp band observed in Appendix 2, Figure 16 shifted slightly in size to an estimated size greater than 8600 bp (Appendix 2, Table 6 and Appendix 2, Figure 21), as 2 µg of genomic DNA were loaded per lane for this blot while 4 µg of genomic DNA were loaded per lane in Appendix 2, Figure 16. The extra band of 6100 bp was very faint and variable between the blots covering all T4 and T5 plants tested (data not shown) and was determined to be an artifact of restriction enzyme digestion from Insertion 4 (Appendix 2-3). In addition, the same two hybridizing bands endogenous to the soybean genome, described above, were observed in both 305423 soybean and in the control (Appendix 2, Table 6).

As discussed earlier for the T4 generation, two bands were observed in 305423 soybean plants digested with *Nco* I and probed with *gm-hra*, one of approximately 3600 bp and the other approximately 3200 bp (Appendix 2, Table 5 and Appendix 2, Figure 6). Eight hybridizing bands from the Jack variety background not related to the insertion were also observed in all 305423 and control soybean plants (Appendix 2, Table 5 and Appendix 2, Figure 6). The same hybridization pattern in the T4 and T5 generations of 305423 soybean was observed (Appendix 2, Figure 22).

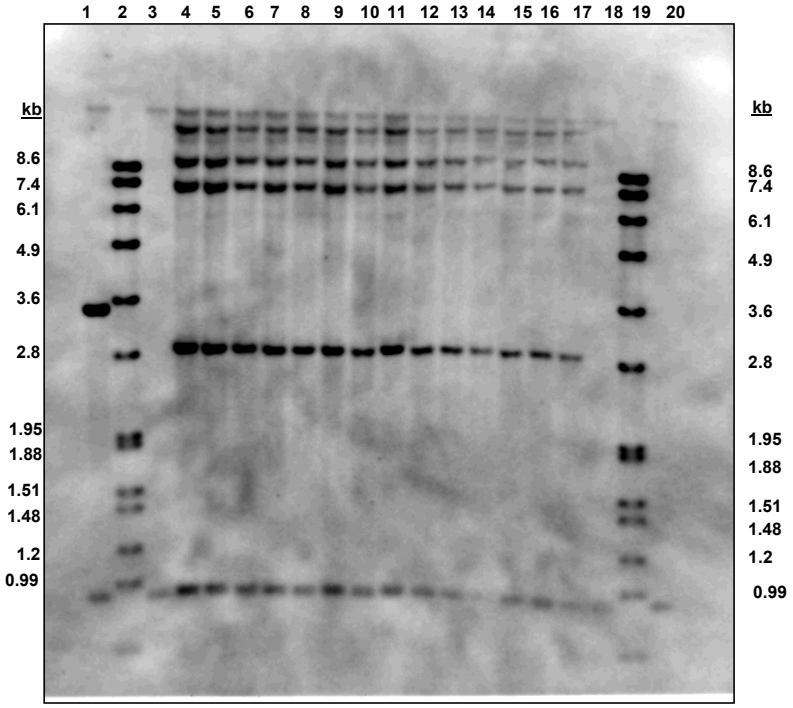
The hybridization pattern discussed above for the T4 and T5 generations was also consistent across the segregating F2 individuals of 305423 soybean. One-hundred individuals of the F2 population of 305423 soybean were examined and exhibited the same hybridization pattern as described for the T4 and T5 generations, except for a single plant with an altered hybridization pattern for *gm-hra*. Representative blots from this analysis are shown in Appendix 2, Figure 23, for hybridization with the *gm-fad2-1* probe and in Appendix 2, Figure 24, for hybridization with the *gm-hra* probe. The single plant with an altered hybridization pattern for *gm-hra* is shown in Lane 15 (Appendix 2, Figures 23 and 24). The same bands that hybridized with the *gm-fad2-1* probe observed in the T4 and T5 plants were also observed for all F2 individuals (Appendix 2, Figure 23). The *gm-hra* probe hybridized to the same bands in the F2 individuals, except for the single

plant shown in Lane 15 (Appendix 2, Figure 24), as were observed in the T4 and T5 plants. Based on further analysis with the SAMS and *als* terminator probes of the *gm-hra* cassette and with the KTi3 promoter probe (data not shown), it was determined that this plant had putatively undergone recombination between two repeated KTi3 promoter elements flanking the *gm-hra* cassette (see Insertion 1, Appendix 2, Figure 12) removing the *gm-hra* cassette and portions of the KTi3 promoter elements.

The frequency of this recombination was examined in other F2 segregating populations representing over 1000 individuals (see Section V-F of the petition for representative F2 sub-population) by examining 305423 soybean event-specific PCR data and PCR data specific to the *gm-hra* gene of these individuals. No other individual plant showing the loss of the *gm-hra* gene was identified, indicating that the frequency of this genetic rearrangement occurs at an extremely low level and that the inserted DNA in 305423 soybean is stable.

Overall, the Southern blot analysis of the T4, T5, and F2 generations shows consistent hybridization results with the *gm-fad2-1* and *gm-hra* probes and confirms the stability of inheritance of the inserted DNA during soybean breeding. In addition, the occurrence of a single recombination event in the F2 population removing the *gm-hra* cassette was determined to be at a very low frequency based on examination of 1000 additional segregating 305423 soybean F2 individuals by PCR-based assays specific for the event and for the *gm-hra* gene. No other recombinant individuals were identified, indicating the 305423 soybean insertion is stable. These analyses show that the inserted DNA in 305423 soybean is stable across multiple breeding generations.

Appendix 2, Figure 21. Southern Blot Analysis of 305423 Soybean: *gm-fad2-1* Probe and *Nco* I Digest

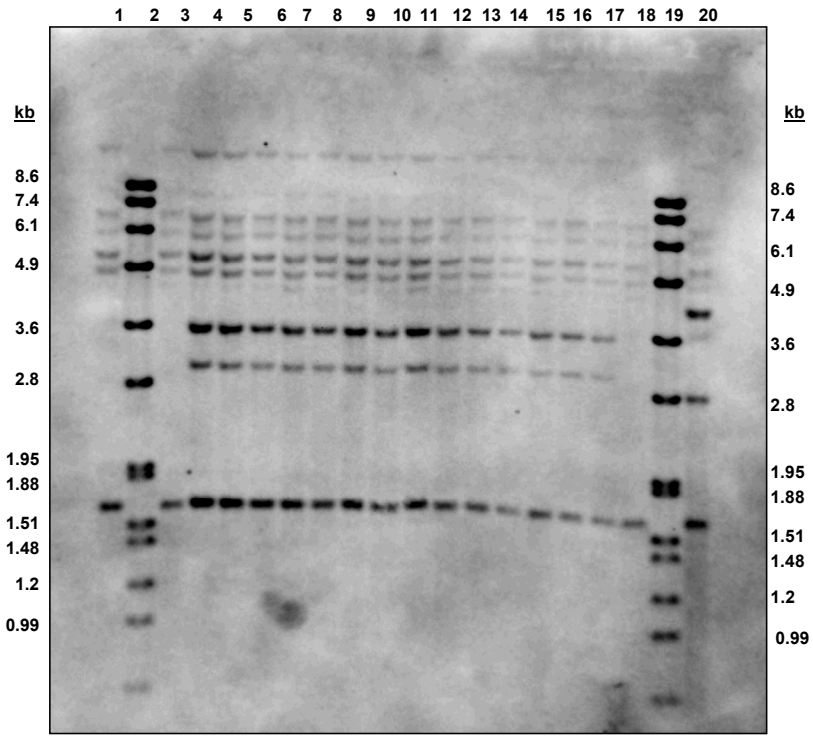


Genomic DNA isolated from leaf tissue of individual plants of 305423 soybean (T5 and T4 generation) and of unmodified control soybean (Jack) was digested with *Nco* I and probed with the *gm-fad*2-1 gene probe. Approximately 2 µg of genomic DNA was digested and loaded per lane. Approximately two gene copies of plasmid PHP19340 (Lane 1) or plasmid PHP17752 (Lane 20) was spiked into 2 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	2 copies PHP19340 + control (Jack)
2	DNA molecular weight markers DIG VII
3	control (Jack)
4	305423 soybean/ plant 8 (T5)
5	305423 soybean/ plant 9 (T5)
6	305423 soybean/ plant 10 (T5)
7	305423 soybean/ plant 11 (T5)
8	305423 soybean/ plant 12 (T5)
9	305423 soybean/ plant 13 (T5)
10	305423 soybean/ plant 14 (T5)

Lane	Sample
11	305423 soybean/ plant 38 (T4)
12	305423 soybean/ plant 39 (T4)
13	305423 soybean/ plant 40 (T4)
14	305423 soybean/ plant 41 (T4)
15	305423 soybean/ plant 42 (T4)
16	305423 soybean/ plant 43 (T4)
17	305423 soybean/ plant 44 (T4)
18	control (Jack)
19	DNA molecular weight markers DIG VII
20	2 copies PHP17752 + control (Jack)

Appendix 2, Figure 22. Southern Blot Analysis of 305423 Soybean: *gm-hra* Probe and *Nco* I Digest

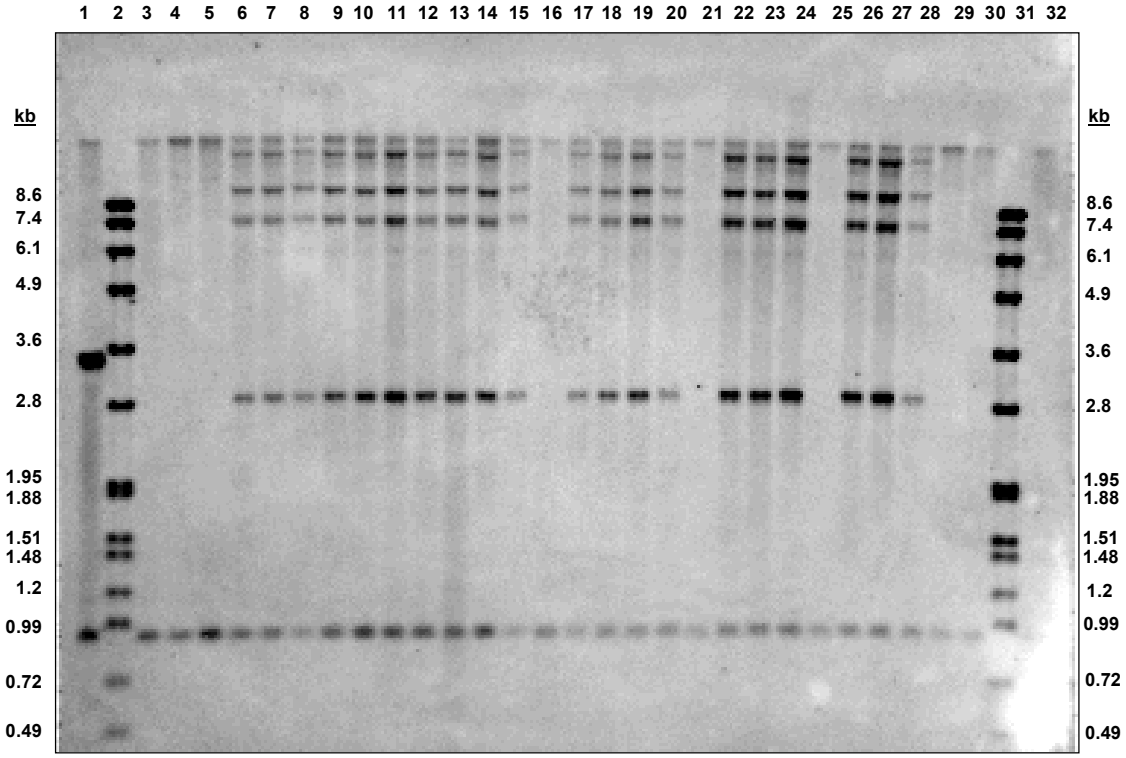


Genomic DNA isolated from leaf tissue of individual plants of 305423 soybean (T5 and T4 generation) and of unmodified control soybean (Jack) was digested with *Nco* I and probed with the *gm-hra* gene probe. Approximately 2 µg of genomic DNA was digested and loaded per lane. Approximately two gene copies of plasmid PHP19340 (Lane 1) or plasmid PHP17752 (Lane 20) was spiked into 2 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	2 copies PHP19340 + control (Jack)
2	DNA molecular weight markers DIG VII
3	control (Jack)
4	305423 soybean/ plant 8 (T5)
5	305423 soybean/ plant 9 (T5)
6	305423 soybean/ plant 10 (T5)
7	305423 soybean/ plant 11 (T5)
8	305423 soybean/ plant 12 (T5)
9	305423 soybean/ plant 13 (T5)
10	305423 soybean/ plant 14 (T5)

Lane	Sample
11	305423 soybean/ plant 38 (T4)
12	305423 soybean/ plant 39 (T4)
13	305423 soybean/ plant 40 (T4)
14	305423 soybean/ plant 41 (T4)
15	305423 soybean/ plant 42 (T4)
16	305423 soybean/ plant 43 (T4)
17	305423 soybean/ plant 44 (T4)
18	control (Jack)
19	DNA molecular weight markers DIG VII
20	2 copies PHP17752 + control (Jack)

Appendix 2, Figure 23. Southern Blot Analysis of 305423 Soybean F2 Generation: *gm-fad2-1* Probe and *Nco* I Digest

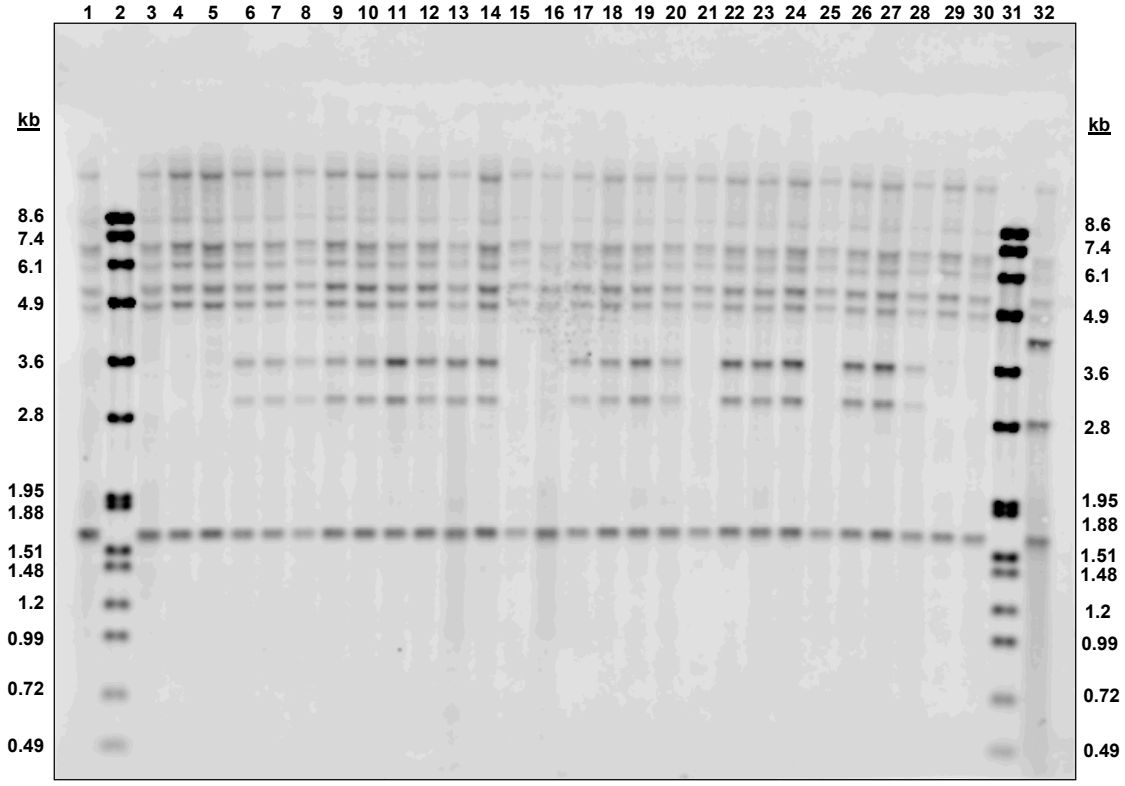


Genomic DNA isolated from leaf tissue of individual plants of 305423 soybean (F2 generation) and of unmodified control soybean (Jack and elite variety) was digested with *Nco* I and probed with the *gm-fad2-1* gene probe. Approximately 2 µg of genomic DNA was digested and loaded per lane. Approximately one gene copy of plasmid PHP19340 (Lane 1) or plasmid PHP17752 (Lane 32) was spiked into 2 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP19340 + control (Jack)
2	DNA molecular weight markers DIG VII
3	control (Jack)
4	305423 soybean / plant 53
5	305423 soybean / plant 54
6	305423 soybean / plant 55
7	305423 soybean / plant 56
8	305423 soybean / plant 57
9	305423 soybean / plant 58
10	305423 soybean / plant 59
11	305423 soybean / plant 60
12	305423 soybean / plant 61
13	305423 soybean / plant 62
14	305423 soybean / plant 63
15	305423 soybean / plant 64
16	305423 soybean / plant 65

Lane	Sample
17	305423 soybean / plant 66
18	305423 soybean / plant 67
19	305423 soybean / plant 68
20	305423 soybean / plant 69
21	305423 soybean / plant 70
22	305423 soybean / plant 71
23	305423 soybean / plant 72
24	305423 soybean / plant 73
25	305423 soybean / plant 74
26	305423 soybean / plant 75
27	305423 soybean / plant 76
28	305423 soybean / plant 77
29	305423 soybean / plant 78
30	control (Elite variety)
31	DNA molecular weight markers DIG VII
32	1 copy PHP17752 + control (Jack)

Appendix 2, Figure 24. Southern Blot Analysis of 305423 Soybean F2 Generation: *gm-hra* Probe and *Nco* I Digest



Genomic DNA isolated from leaf tissue of individual plants of 305423 soybean (F2 generation) and of unmodified control soybean (Jack and elite variety) was digested with *Nco* I and probed with the *gm-hra* gene probe. Approximately 2 µg of genomic DNA was digested and loaded per lane. Approximately one gene copy of plasmid PHP19340 (Lane 1) or plasmid PHP17752 (Lane 32) was spiked into 2 µg of control soybean (Jack) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP19340 + control (Jack)
2	DNA molecular weight markers DIG VII
3	control (Jack)
4	305423 soybean / plant 53
5	305423 soybean / plant 54
6	305423 soybean / plant 55
7	305423 soybean / plant 56
8	305423 soybean / plant 57
9	305423 soybean / plant 58
10	305423 soybean / plant 59
11	305423 soybean / plant 60
12	305423 soybean / plant 61
13	305423 soybean / plant 62
14	305423 soybean / plant 63
15	305423 soybean / plant 64
16	305423 soybean / plant 65

Lane	Sample
17	305423 soybean / plant 66
18	305423 soybean / plant 67
19	305423 soybean / plant 68
20	305423 soybean / plant 69
21	305423 soybean / plant 70
22	305423 soybean / plant 71
23	305423 soybean / plant 72
24	305423 soybean / plant 73
25	305423 soybean / plant 74
26	305423 soybean / plant 75
27	305423 soybean / plant 76
28	305423 soybean / plant 77
29	305423 soybean / plant 78
30	control (Elite variety)
31	DNA molecular weight markers DIG VII
32	1 copy PHP17752 + control (Jack)

Appendix 3. Description of Statistical Analyses

3.1. Trait Inheritance Data (Section V-F)

Based on Mendel's segregation law, the expected segregation ratios listed in Table 3 were tested by the statistic:

$\chi^2 = \Sigma [(|o - e| - 0.5)^2 / e]$

For a two-genotype case, the statistic can be expressed as:

$\chi^2 = (| n_{obs(pos)} - n_{exp(pos)} | - 0.5)^2 / n_{exp(pos)} + (| n_{obs(neg)} - n_{exp(neg)} | - 0.5)^2 / n_{exp(neg)}$

where o = observed frequency of the genotype; e= expected frequency of the genotype; and 0.5 = Yates correction for continuity (Yates, 1934). χ^2 follows a chi-squared distribution with one degree of freedom (df) for two-genotype cases (Agresti, 2002).

3.2. Agronomic Data from Experiments A and C (Section VIII-B)

Data presented in Tables 8 and 10 were statistically analyzed using the following linear mixed model:

$y_{ijk} = \mu_i + r_k + \delta_{ik} + \varepsilon_{ijk}$

$r_k \sim iid\ N(0,\sigma^2_{loc}),\ \delta_{ik} \sim iid\ N(0,\sigma^2_{loc\times genotype})$, and $\varepsilon_{ik} \sim iid\ N(0, \sigma^2_{resid})$

Where μ_i denotes the mean of the i[th] genotype (pos or neg, fixed effect), r_k denotes the effect of the k[th] location (random effect), δ_{ik} denotes the interaction between the i[th] genotype and the k[th] location (random effect), and ε_{ijk} denotes the effect of the j[th] plot assigned the i[th] genotype in the k[th] location (random effect or residual). Notation ~iid $N(0, \sigma_a^2)$ denotes random variables that are identically and independently distributed (iid) as normal with zero mean and variance σ_a^2.

3.3. Agronomic Data from Experiments B (Section VIII-B)

Data were analyzed using a linear mixed model designed to account for the design effects of location and blocks within location. The linear mixed model assumed the entries were a fixed effect while the locations, blocks within locations and the entry by location interaction were random effects. A significant difference between the mean of 305423 soybean and the means of a set of comparable control lines was established with a P-value <0.05.

Early population, seedling vigor, disease incidence, insect damage, days to maturity, lodging, shattering score, final population and yield data (presented in Table 9) were analyzed using the following linear mixed model:

$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$

$\ell_j \sim iid\ N(0,\sigma^2_{Loc}),\ r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep}),\ (\mu\ell)_{ij} \sim iid\ N(0,\sigma^2_{Loc\times Ent}),$ and $\varepsilon_{ijk} \sim iid\ N(0,\sigma^2_{plot})$

Where μ_i denotes the mean of the i[th] entry (fixed effect), ℓj denotes the effect of the j[th] location (random effect), $r_{k(j)}$ denotes the effect of the k[th] block within the j[th] location (random effect), $(\mu\ell)_{ij}$

denotes the interaction between the entries and locations (random effect) and ε_{ijk} denotes the effect of the plot assigned the i^{th} entry in the k^{th} block of the j^{th} location (random effect or residual). Notation ~iid $N(0,\sigma^2_a)$ indicates random variables that are identically and independently distributed (iid) as normal with zero mean and variance σ^2_a.

Plant height data (Table 9) were analyzed using the following linear mixed model:

$$y_{ijkl} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk} + \delta_{ijkl}$$

$\ell_j \sim iid\ N(0,\sigma^2_{Loc})$, $r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep})$, $(\mu\ell)_{ij} \sim iid\ N(0,\sigma^2_{Loc\times Ent})$, $\varepsilon_{ijk} \sim iid\ N(0,\sigma^2_{plot})$, and $\delta_{ijkl} \sim iid\ N(0,\sigma^2_{observations})$

Where μ_i denotes the mean of the i^{th} entry (fixed effect), ℓj denotes the effect of the j^{th} location (random effect), $r_{k(j)}$ denotes the effect of the k^{th} block within the j^{th} location (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the i^{th} entries and j^{th} locations (random effect), ε_{ijk} denotes the effect of the plot assigned the i^{th} entry in the k^{th} block of the j^{th} location (random plot), and δ_{ijkl} denotes the effect of the plant assigned the l^{th} plant in the i^{th} entry in the k^{th} block of the j^{th} location (observational error).

3.4. Nutrient Composition Data from Experiment B (Section IX)

Data were analyzed using a linear mixed model designed to account for the design effects of location and blocks within location. The linear mixed model assumes the entries are a fixed effect while the locations, blocks within locations and the entry by location interaction are random effects. A significant difference between the mean of 305423 soybean and the means of a set of comparable control lines was established with an FDR-adjusted P-value <0.05 for each analyte.

Composition data presented in Section IX, Tables 13-18, were analyzed using the following linear mixed model:

$$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$$

$\ell_j \sim iid\ N(0,\sigma^2_{Loc})$, $r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep})$, $(\mu\ell)_{ij} \sim iid\ N(0,\sigma^2_{Loc\times Ent})$, and $\varepsilon_{ijk} \sim iid\ N(0,\sigma^2_{plot})$

Where μ_i denotes the mean of the i^{th} entry (fixed effect), ℓj denotes the effect of the j^{th} location (random effect), $r_{k(j)}$ denotes the effect of the k^{th} block within the j^{th} location (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the entries and locations (random effect) and ε_{ijk} denotes the effect of the plot assigned the i^{th} entry in the k^{th} block of the j^{th} location (random effect or residual). Notation ~iid $N(0,\sigma^2_a)$ indicates random variables that are identically and independently distributed (iid) as normal with zero mean and variance σ^2_a.

Appendix 4. USDA Field Trials of 305423 Soybean

Appendix 4, Table 1. USDA Field Trials of 305423 Soybean

Year of planting	Permit number	Permit valid date	305423 soybean plantings			
			State	Number of counties	Acreage	Purpose of release
2002	02-023-06R	4/10/2002	HI	1	0.016	breeding
	02-023-05R	4/10/2002	IA	1	0.0004	breeding
2003	03-022-04R	5/1/2003	HI	1	0.205	breeding
	03-022-03R	4/15/2003	IA	1	0.078	breeding
2004	04-020-01R	4/13/2004	IA	3	1.527	yield testing
	04-020-02R	4/15/2004	HI	1	0.457	breeding
2005	05-024-01R	5/12/2005	IA	4	5.950	breeding, yield testing, regulatory trial
			IL	2		
			HI	1		
			MN	1		
			NE	1		
	05-024-02R	5/25/2005	HI	1	1.549	breeding
2006	06-019-01R*	5/1/2006	IA	7	49.341	yield testing
			IL	5		
			IN	1		
			MN	1		
			OH	1		

Note: in USDA field data reports, 305423 soybean (event DP-3Ø5423-1) is called EAFS 3054.2.3.
* Final field test report not yet submitted to USDA.

Appendix 4, Table 2. Experiment A, 2005: Insect and Disease Stressor Incidence Comparison of 305423 and Control Soybean Lines

State/ Location	Acreage of 305423 soybean	Planting date/ Harvest and/or destruction date	Stressor	Observation 1		Observation 2		Observation 3	
				Range of severity in 305423 Soybean[1]	Difference with control	Range of severity in 305423 soybean	Difference with control	Range of severity in 305423 soybean	Difference with control
IA/ Stuart	0.017	06/06/2005/ 10/11/2005	**Insect**						
			Bean leaf beetle (*Certoma trifurcata*)	ND[2]	no	mild	no	mild	no
			Soybean aphid (*Aphis glycines*)	ND	no	mild	no	ND	no
			Disease						
			Bacterial blight (*Pseudomonas savastanoi* pv. *glycinea*)	ND	no	mild	no	ND	no
			Brown spot (*Septoria glycines*)	ND	no	mild	no	moderate	no
IA/ Johnston	0.017	06/05/2005/ 10/26/2005	**Insect**						
			Bean leaf beetle (*Certoma trifurcata*)	mild	no	moderate	no	mild	no
			Soybean aphid (*Aphis glycines*)	ND	no	moderate	no	ND	no
			Disease						
			Bacterial blight (*Pseudomonas savastanoi* pv. *glycinea*)	ND	no	ND	no	mild	no
			Brown spot (*Septoria glycines*)	mild	no	mild	no	mild	no
IA/ Cedar Falls	0.017	06/06/2005/ 10/24/2005	**Insect**						
			Bean leaf beetle (*Certoma trifurcata*)	ND	no	mild	no	mild	no
			Soybean aphid (*Aphis glycines*)	ND	no	mild	no	ND	no
			Disease						
			Bacterial blight (*Pseudomonas savastanoi* pv. *glycinea*)	ND	no	ND	no	mild	no
			Brown spot (*Septoria glycines*)	ND	no	ND	no	mild	no

Note: In USDA field data reports, event DP-3Ø5423-1 is called EAFS 3054.2.3.

[1] Scale: Mild – very little insect injury (<10%) visible;
Moderate –noticeable plant tissue damage (10% to 30%);
Severe – significant plant tissue damage (>30%).

[2] ND: not detected

Appendix 4, Table 3. Experiment B, 2005: Insect and Disease Stressor Incidence Comparison of 305423 and Control Soybean Lines

State/ Location	Acreage of 305423 soybean	Planting date/ Harvest and/or destruction date	Stressor	Observation 1		Observation 2		Observation 3		Observations 4 and 5	
				Range of severity in 305423 Soybean[1]	Difference with control	Range of severity in 305423 soybean	Difference with control	Range of severity in 305423 soybean	Difference with control	Range of severity in 305423 soybean	Difference with control
IL/ Wyoming	0.009	06/23/2005/ 11/01/2005	**Insect**								
			Bean leaf beetle (*Certoma trifurcata*)	mild	no	mild	no	mild	no	mild[4]	no[4]
			Grasshopper (Orthoptera)	ND[2]	no	ND	no	ND	no	mild[4]	no[4]
IA/ Richland	0.009	06/22/2005/ 11/10/2005	**Insect**								
			Bean leaf beetle (*Certoma trifurcata*)	mild	no	mild	no	moderate	no	-[3,4]	-[4]
			Soybean aphid (*Aphis glycines*)	ND	no	ND	no	moderate	no	-[4]	-[4]
MN/ Paynesville	0.009	06/23/2005/ 11/05/2005	**Insect**								
			Grasshopper (Orthoptera)	ND	no	ND	no	mild	no	mild[4]	mild[4]
			Soybean aphid (*Aphis glycines*)	ND	no	mild	no	mild	no	mild[4]	no[4]
			Disease								
			Downey mildew (*Peronospora manshurica*)	ND	no	ND	no	ND	no	mild[4]	no[4]
			Powdery mildew (*Microsphaera diffusa*)	ND	no	ND	no	ND	no	mild[4]	no[4]
NE/ York	0.009	06/20/2005/ 10/19/2005	**Insect**								
			Bean leaf beetle (*Certoma trifurcata*)	mild	no	no	no	no	no	-[4]	-[4]
			Grasshopper (Orthoptera)	mild	no	mild	no	mild	no	-[4]	-[4]
			Disease								
			Brown spot (*Septoria glycines*)	ND	no	mild	no	mild	no	-[4]	-[4]
ON, Canada/ Thorndale	0.009	6/30/2005/ 12/7/2005	**Disease**								
			White mold (*Sclerotinia sclerotiorum*)	ND	no	ND	no	mild	no	ND	no
ON, Canada/ Branchton	0.009	6/30/2005/ 11/22/2005	**Insect**								
			Soybean aphid (*Aphis glycines*)	mild	no	ND	no	-	-	-[4]	-[4]

Note: In USDA field data reports, event DP-3Ø5423-1 is called EAFS 3054.2.3.

[1] Scale: Mild – very little insect injury (<10%) visible;
Moderate –noticeable plant tissue damage (10% to 30%);
Severe – significant plant tissue damage (>30%).

[2] ND: not detected

[3] -: Observation not taken

[4] -: Observation 5 not taken

Appendix 4, Table 4. Experiment C, 2006: Insect and Disease Stressor Incidence Comparison of 305423 and Control Soybean Lines

State/ Location	Acreage of 305423 soybean	Planting date/ Harvest and/or destruction date	Stressor	Observation 1		Observation 2		Observation 3		Observation 4	
				Range of severity in 305423 soybean[1]	Difference with control	Range of severity in 305423 soybean	Difference with control	Range of severity in 305423 soybean	Difference with control	Range of severity in 305423 soybean	Difference with control
IA/ Stuart	0.0155	05/20/2006/ 11/09/2006	**Insect**								
			Bean leaf beetle (*Certoma trifurcata*)	mild	no	mild	no	mild	no	ND	no
			Soybean aphid (*Aphis glycines*)	ND[2]	no	mild	no	ND	no	ND	no
			Disease								
			Bacterial blight (*Pseudomonas savastanoi* pv. *glycinea*)	ND	no	ND	no	mild	no	mild	no
			Bean pod mottle virus	ND	no	ND	no	ND	no	mild	no
			Brown spot (*Septoria glycines*)	ND	no	ND	no	mild	no	mild	no
IA/ Johnston	0.0155	05/19/2006/ 11/10/2006	**Insect**								
			Bean leaf beetle (*Certoma trifurcata*)	mild	no	mild	no	mild	no	ND	no
			Soybean aphid (*Aphis glycines*)	ND	no	mild	no	mild	no	ND	no
			Disease								
			Bacterial blight (*Pseudomonas savastanoi* pv. *glycinea*)	ND	no	ND	no	mild	no	mild	no
			Bean pod mottle virus	ND	no	ND	no	ND	no	mild	no
			Brown spot (*Septoria glycines*)	ND	no	mild	no	mild	no	mild	no
IA/ Cedar Falls	0.0155	06/01/2006/ 11/15/2006	**Insect**								
			Bean leaf beetle (*Certoma trifurcata*)	mild	no	mild	no	mild	no	ND	no
			Soybean aphid (*Aphis glycines*)	ND	no	mild	no	ND	no	ND	no
			Disease								
			Bacterial blight (*Pseudomonas savastanoi* pv. *glycinea*)	ND	no	ND	no	mild	no	mild	no
			Bean pod mottle virus	ND	no	ND	no	ND	no	mild	no
			Brown spot (*Septoria glycines*)	ND	no	ND	no	ND	no	mild	no
IL/ Princeton	0.0155	05/20/2006/ 10/27/2006	**Insect**								
			Bean leaf beetle (*Certoma trifurcata*)	ND	no	ND	no	mild	no	mild	no
			Japanese beetle (*Popillia japonica*)	ND	no	ND	no	mild	no	mild	no
			Disease								
			Anthracnose (*Colletotrichum* spp.)	ND	no	ND	no	ND	no	mild	no
			Brown stem rot (*Phialophora gregata*)	ND	no	ND	no	ND	no	mild	no

Note: In USDA field data reports, event DP-3Ø5423-1 is called EAFS 3054.2.3.

[1] Scale: Mild – very little insect injury (<10%) visible;
Moderate –noticeable plant tissue damage (10% to 30%);
Severe – significant plant tissue damage (>30%).

[2] ND: not detected

Appendix 5. Methods and Results for Characterization of the GM-HRA Protein

5.1. Protein Purification from 305423 Soybean Plant Tissue

For final purification, the GM-HRA protein was extracted from approximately 80 grams of 305423 soybean tissue in 250 milliliters (ml) of homogenization buffer consisting of 50 mM Tris-HCl, pH 7.5, 5 mM sodium pyruvate, 10 µM flavin adenine dinucleotide, 1 mM EDTA, 5% glycerol, 5 mM magnesium chloride, 50 mM sodium chloride, 5% (w/w) polyvinylpolypyrrolidone, and EDTA-free Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA). Particulate leaf material in the resulting slurry was removed by filtering through four layers of cheese cloth. The filtrate was further clarified by centrifugation for 20 minutes at 30,500 g.

The GM-HRA protein was partially purified from the extract using two immunoaffinity columns that had been prepared by coupling mouse monoclonal antibodies recognizing both endogenous GM-ALS and transgenic GM-HRA proteins to AminoLink Plus Coupling Gel (Pierce Biotechnology, Inc., Rockford, USA) according to the manufacturer's instructions. The clarified leaf extract was loaded onto the affinity column, which had been pre-equilibrated with the homogenization buffer. Unbound material was removed by washing with the homogenization buffer. Bound GM-HRA protein was eluted using ImmunoPure IgG Elution Buffer (Pierce Biotechnology, Inc.). Collected fractions (2 ml each) were analyzed by SDS-PAGE and western blot. The fractions containing the GM-HRA protein were pooled and concentrated using a Nanosep 10K concentrator (Pall Corporation, NY, USA).

5.2. Protein Expression in *E. coli* and Purification

The mature form of the GM-HRA protein (excluding the chloroplast transit peptide sequence) was produced. The GM-HRA protein was expressed in *E coli* strain BL21 (DE3) RIPL as a fusion protein containing a His-T7 tag and was purified using a immobilized metal affinity column (Ni-NTA His Bind resin, Novagen, EMD Biosciences, Inc., San Diego, CA) followed by cleavage of the fusion tag with thrombin (Calbiochem, # 605195, EMD Biosciences, Inc.), which was used in-solution at 10 units per mg of protein, after it was eluted from the Ni-NTA column. The cleaved tag and the thrombin were removed from the purified protein by diafiltration. The thrombin cleavage of the tag resulted in one extra N-terminal glycine residue as the N-terminal amino acid, which is not found in the mature GM-HRA protein sequence. The material was then dialyzed into 0.1 M ammonium bicarbonate pH 7.5 and then lyophilized.

5.3. Method for Determination of Protein Concentration

Total protein concentration was determined using Coomassie Plus Protein Assay Reagent (Pierce Biotechnology, Inc.). Bovine serum albumin (BSA) was used as the protein standard. After incubation for approximately five minutes, absorbance was measured at 595 nm.

5.4. SDS-PAGE and Western Blot Methods

5.4a. SDS-PAGE Method

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by first mixing GM-HRA protein samples from both sources with Laemmli sample buffer (Gradipore Limited, Australia) containing 100 mM dithiothreitol and heating the solution at 100°C for approximately five minutes. The prepared protein samples were loaded into a 10-20% gradient Ready Gel Tris-HCl gel (Bio-Rad Laboratories, Inc., Hercules, CA). PageRuler pre-stained Protein Ladder (Fermentas, Inc., Hanover, MD) molecular weight markers were loaded on the gel

to provide a visual estimate of molecular weight. Electrophoresis was conducted using the Ready Gel Cell system (Bio-Rad Laboratories, Inc) with Tris-glycine running buffer (Gradipore Limited) and a constant 150 volts (V) for approximately 60 minutes or until the dye front neared the bottom of the gel. Upon completion of electrophoresis, the gels were either removed from the gel cassette and prepared for western blot analysis or were stained with Coomassie Blue. Prior to staining, the gels were washed three times with deionized water for approximately five minutes each. The gels were then stained for approximately 60 minutes with GelCode Coomassie Blue stain reagent (Pierce Biotechnology, Inc.), and rinsed with deionized water at least four times for approximately ten minutes each.

Protein purity was determined using densitometry on Coomassie Blue stained gels with Kodak 1D Image Analysis software (Eastman Kodak Company, Rochester, NY), and the relative contribution of the individual protein bands within a lane were determined to provide an estimation of purity.

5.4b. Western Blot Method

Following SDS-PAGE electrophoresis, the resulting gel was soaked in transfer buffer (48 mM Tris-HCl at pH 8.6, 39 mM glycine, 0.0375% sodium dodecyl sulfate, and 20% methanol) for approximately 20 minutes. The polyvinylidene difluoride (PVDF) membrane, (Bio-Rad Laboratories, Inc.) was placed in 100% methanol briefly, followed by immersion in transfer buffer for 10-15 minutes. The Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell system (Bio-Rad Laboratories, Inc.) was used to transfer the proteins from the gel to the membrane at 11 V for approximately 60 minutes.

Following protein transfer, the membrane was washed four times for approximately five minutes each in Classic buffer (50 mM Tris-HCl, pH 7.0, 500 mM sodium chloride, and 0.5% Tween-20), and then blocked by incubating in phosphate-buffered saline solution with Tween-20 (PBST; 8.1 mM phosphate buffer, pH 7.4, 137 mM sodium chloride, 2.7 mM potassium chloride, and 0.05% Tween-20) containing 5% non-fat dry milk for approximately 60 minutes.

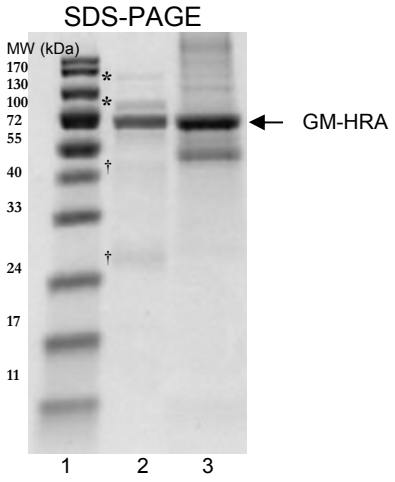
The blocked membrane was washed four times for approximately five minutes each in Classic buffer, and then incubated for approximately 60 minutes with a GM-HRA-specific primary rabbit polyclonal antibody diluted 1:2,500 in PBST containing 5% non-fat dry milk. The unbound antibodies were rinsed from the membrane with four washes of Classic buffer for approximately five minutes each, and then incubated for approximately 60 minutes with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate, Promega Corporation, Madison, WI) diluted 1:10,000 in Classic buffer. The membrane was then washed four times with Classic buffer for approximately five minutes each. The blot was then soaked in phosphate buffered saline solution (PBS; 8.1 mM phosphate buffer, pH 7.4, 137 mM sodium chloride, and 2.7 mM potassium chloride) for approximately five minutes and developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc.) according to the manufacturer's instructions. The molecular weight of the recognized protein band was estimated using PageRuler Prestained Protein Ladder (SM0671, Fermentas, Inc.)

5.5. Results of SDS-PAGE and Western Analyses

SDS-PAGE analysis of the microbial and 305423 soybean plant-derived GM-HRA proteins revealed both proteins migrating as expected at approximately 65 kDa (Appendix 5, Figure 1). Microbial GM-HRA protein has greater than 75% purity following densitometry analysis. Upon western blot analysis, both the microbial and 305423 soybean-derived GM-HRA proteins were recognized by GM-HRA-specific antibodies, and confirmed the presence of GM-HRA protein by detection of a single band recognized at approximately 65 kDa (Appendix 5, Figure 2).

SDS-PAGE and western analysis confirmed that both microbially expressed and 305423 soybean-derived GM-HRA had the expected molecular weight and immunoreactivity and were equivalent to each other in these analyses.

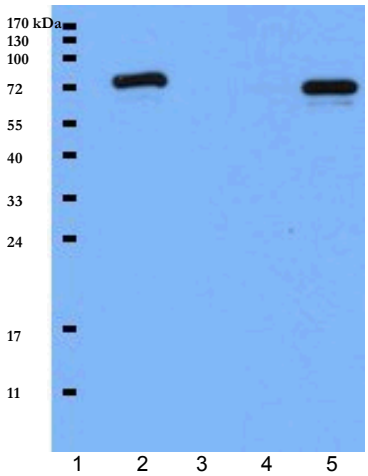
Appendix 5, Figure 1. SDS-PAGE Analysis of Plant-Derived and Microbially Expressed GM-HRA Proteins



* Unidentified protein
† Identified as putative GM-HRA degradation peptide products

Lane	Sample Description
1	Protein Molecular Weight Marker (Fermentas, #SM0671)
2	Microbially Expressed GM-HRA Protein (~1.5 µg)
3	Plant-Derived GM-HRA Protein (~1.5 µg)

Appendix 5, Figure 2. Western Blot Analysis of Plant-Derived and Microbially Expressed GM-HRA Proteins



Lane	Sample Description
1	Protein Molecular Weight Marker (Fermentas, #SM0671)
2	Plant-Derived GM-HRA Protein(~20 ng)
3	Blank
4	GM-ALS Reference Protein* (~20 ng)
5	Microbially Expressed GM-HRA Protein (~20 ng)

* GM-ALS reference protein derived from a microbial expression system, and was expected not to be recognized by GM-HRA-specific antibodies

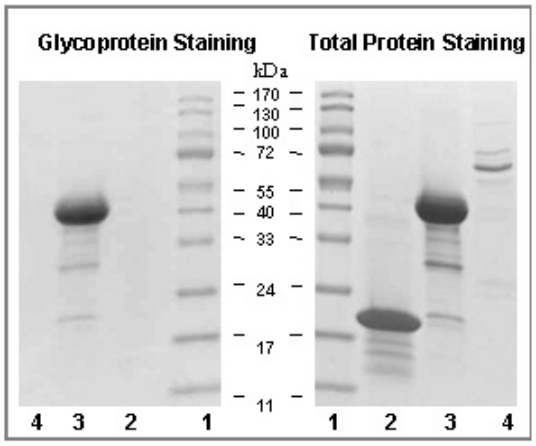
5.6. Method for Detection of Protein Glycosylation

A GelCode Glycoprotein staining kit (Pierce Biotechnology, Inc.) was used according to the manufacturer's instructions to determine whether the microbially expressed and plant-derived GM-HRA proteins were glycosylated. SDS-PAGE was conducted as described previously. Following electrophoresis, the gel was fixed with 50% methanol for approximately 30 minutes, washed with 3% acetic acid, then incubated with an oxidizing solution for approximately 15 minutes and washed three times with 3% acetic acid. The gel was incubated with GelCode glycoprotein staining reagent for 15 minutes, treated with the reducing reagent, and washed extensively with 3% acetic acid and deionized water. Following glycoprotein detection, the gel was scanned and the image was captured electronically. The same gel was then stained with Coomassie Blue to visualize all protein bands.

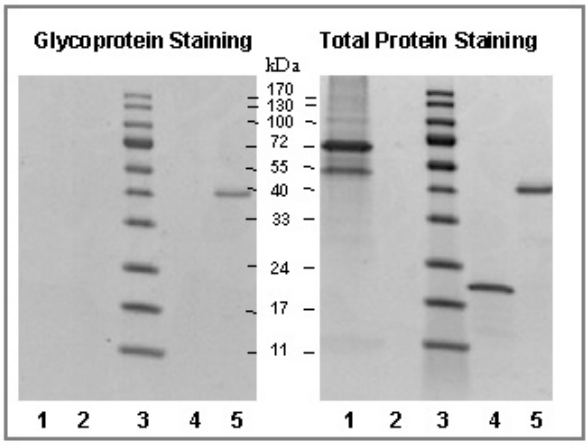
5.7. Results of Protein Glycosylation Analysis

Glycosylation analysis demonstrated that there was no detectable glycosylation of either the microbial expressed or 305423 soybean plant-derived GM-HRA proteins (Appendix 5, Figure 3). The glycoprotein positive control (horseradish peroxidase) was stained and clearly visible as a magenta colored band and the negative control (soybean trypsin inhibitor) did not show any staining. This confirms that the microbially expressed and 305423 soybean plant-derived GM-HRA proteins are non-glycosylated and hence are equivalent with respect to protein glycosylation.

Appendix 5, Figure 3. Glycosylation Analysis of Microbially Expressed (Panel A) and Plant-Derived (Panel B) GM-HRA Proteins



Panel A



Panel B

Lane	Sample Description
1	Protein Molecular Weight Marker (Fermentas, #SM0671)
2	Soybean Trypsin Inhibitor (~20 µg)
3	Horseradish Peroxidase (~20 µg)
4	Microbial GM-HRA Protein (~1 µg)

Lane	Sample Description
1	305423 Soybean-Derived GM-HRA Protein (~1.5 µg)
2	Blank
3	Protein Molecular Weight Marker (Fermentas, #SM0671)
4	Soybean Trypsin Inhibitor (~1 µg)
5	Horseradish Peroxidase (~1 µg)

5.8. Matrix Assisted Laser Desorption Ionization-Mass Spectrometry (MALDI-MS) Method

A gel slice containing a Coomassie Blue stained GM-HRA protein band was excised from a SDS-PAGE gel, placed in a labeled tube and shipped overnight on dry ice to the Keck Biotechnology Resource Laboratory (Yale University, New Haven, CT) for trypsin digestion and MALDI-MS analysis. The protein in the gel slice was digested with trypsin for 18 hours at 37°C, and an aliquot of the digest was analyzed by MALDI-MS on a Waters MALDI-L/R spectrometer (Waters Corporation, Milford, MA, USA) in the reflecton mode of operation. Detected peptide peaks were considered a match if the observed experimental mass of peptides were within 100 parts per million (ppm) of the theoretical mass of the peptides predicted from an *in silico* trypsin cleavage of the GM-HRA protein sequence. Allowances were made for the following modifications to the peptides: oxidation of methionine or tryptophan residues (observed value is 15.995 Da greater than the theoretical value), and modification of cysteine residue by acrylamide free radicals during SDS-PAGE (observed value is 71.037 Da greater than the theoretical value).

5.9. Results of MALDI-MS Analysis

MALDI-MS analysis of the trypsin digests of the microbially expressed and 305423 soybean plant-derived GM-HRA proteins identified 18 and 10 peptides, respectively, that matched with theoretical peptide masses predicted from *in silico* trypsin digestion of the GM-HRA protein (data not shown). This provides 38% and 19.5% coverage, respectively, of the GM-HRA amino acid sequence. These data support the equivalency of the microbially expressed and the 305423 soybean plant-derived GM-HRA proteins.

5.10. N-Terminal Amino Acid Sequencing Method

Protein samples separated by SDS-PAGE and electrophoretically transferred to a PVDF membrane as described earlier were stained with Ponceau S solution (Sigma-Aldrich, St. Louis, MO) to visualize the protein bands. The observed bands were excised and shipped to the Keck Biotechnology Resource Laboratory (Yale University, New Haven, CT) for Edman N-terminal amino acid sequencing using the Procise 494 cLC analyzer (Applied Biosystems, Inc., Foster City, CA) equipped with an online high performance liquid chromatography (HPLC) system.

5.11. Results from N-Terminal Amino Acid Sequencing

The N-terminal sequences of the microbially expressed and plant-derived GM-HRA proteins were consistent with the N-terminal sequence expected for each (data not shown). As expected the microbial GM-HRA protein did possess an N-terminal glycine that was not present in the plant derived GM-HRA. The 305423 plant-derived GM-HRA N-terminal sequence was consistent with the expected sequence of the mature form of the GM-HRA protein following cleavage of the N-terminal chloroplast transit peptide in plant tissues.

5.12. Method for ALS Activity Assay for Microbial GM-HRA Protein

A known amount (0 to 50 ug) of the microbial GM-HRA lyophilized powder was resuspended in 2 mM phosphate buffer pH 7.4, 177.4 mM NaCl, 0.54 mM KCl, 10% glycerol, 0.5 mM thiamine pyrophosphate (TPP), 2 mM flavin adenine dinucleotide (FAD), 0.1 mM pyruvate, 0.5 mM $MgCl_2$ and then diluted in extraction buffer (0.1 M phosphate buffer pH 7.5, 10% glycerol, 0.5 mM TPP, 20 µM FAD, and 2 mM $MgCl_2$). Acetone standard was diluted in 0.1 M phosphate buffer, pH 7.5 and triplicate 100 µl aliquots of protein and standard dilution were dispensed into a 96 well plate. Then 5 µl of either 100 mM phosphate buffer or ALS inhibitor, chlorsulfuron (5µg/ml) was added to wells as applicable. This was followed by the addition of 10 µl of a 1.1 M pyruvate solution to

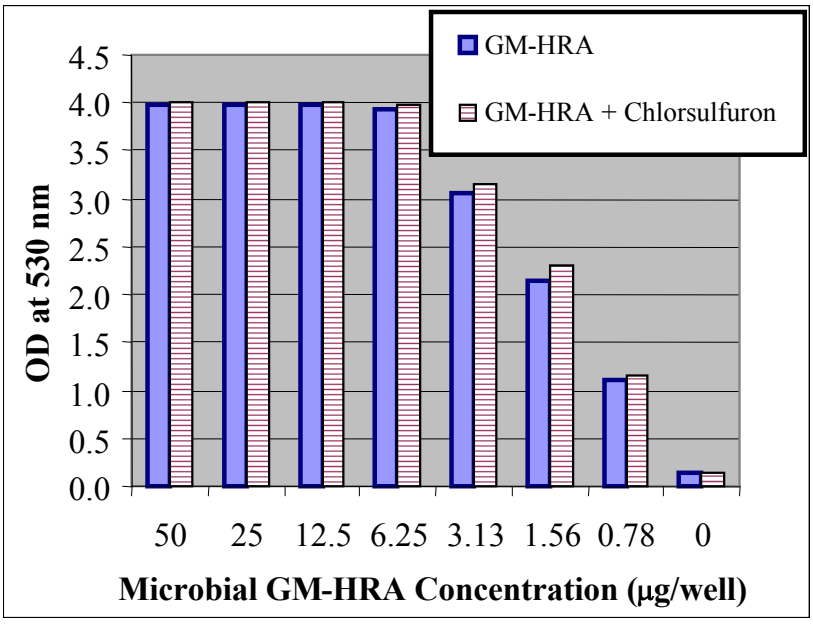
each well to initiate enzymatic reaction, The plates were incubated at 37°C for one hour, at which point 5 µl of 2 N H_2SO_4 was added to stop the reaction. The plates were incubated at 60°C for 15 minutes and then cooled at room temperature for 15 additional minutes prior to the detection step.

Indirect detection of the enzymatic reaction was done by adding 50 µl of creatine/naphtol solution per well, followed by incubation at 60° C for 15 minutes. Plates were allowed to cool at room temperature for an additional 15 minutes and then read at 530 nm using a SpectraMax[1] Model 190 spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA). The relative amount of product produced by the enzyme sample was interpolated from the standard curve.

5.13. Results of the ALS Activity Measurement of Microbial GM-HRA Protein

The activity of the microbial GM-HRA protein was determined by a manual assay for activity measurement of acetolactate synthesis. As expected, results demonstrated that the GM-HRA protein has equivalent ALS biochemical activity (Appendix 5, Figure 4) both in the presence and absence of 100ng/ml chlorosulfuron (an inhibitor of ALS activity).

Appendix 5, Figure 4. Acetolactate Synthase (ALS) Activity Assay on Microbial GM-HRA Protein in the Presence or Absence of Herbicide



5.14. Electrospray Mass Spectroscopy Method for Microbial GM-HRA Protein

The microbially expressed GM-HRA protein was sent to the Keck Biotechnology Resource Laboratory (Yale University, New Haven, CT) and subjected to reverse-phase desalting procedure on a C4 ZipTip (Millipore Corporation, Billerica, MA). The protein was eluted in 50% acetonitrile containing 0.2% formic acid and then analyzed using electrospray ionization mass

[1] Registered trademark of Molecular Devices Corporation.

spectroscopy (ESMS) with a Waters/Micromass Q-tof Micro instrument (Waters Corporation, Milford, MA). The ESMS spectra were recorded in the positive ion mode, and the raw data was transformed using the maximum entropy algorithm that calculates molecular size to charge.

5.15. Results of Electrospray Mass Spectroscopy Analysis of Microbial GM-HRA Protein

Analysis of the microbial GM-HRA protein by electrospray mass spectroscopy identified a major peak at 65,316 Da. This was consistent with the expected molecular mass of 65,312 Da for the mature GM-HRA protein after accounting for the expected extra N-terminal glycine residue (data not shown).

5.16. Method for Amino Acid Composition Analysis of Microbial GM-HRA Protein

The microbial GM-HRA protein was sent to Keck Biotechnology Resource Laboratory (Yale University, New Haven, CT) for amino acid analysis. Both proteins were hydrolyzed at 115°C in 100 µl of 6 N HCl, and 0.2% phenol containing 2 nM norleucine as an internal standard. Amino acid composition analysis was completed in triplicate using a Beckman Model 7300 (Beckman Coulter, Inc., Fullerton, CA) ion-exchange instrument. The instrument was calibrated with a 2 nM mixture of amino acids and was operated according the manufacturer's instructions. Using this type of analysis method glutamine/glutamic acid and asparagine/aspartic acid were not individually quantified and the methionine value is predicted to be less than the theoretical value.

The results from the amino acid composition analysis were used to determine the concentration of the microbial GM-HRA protein in the sample. Four amino acids (asparagine/aspartic acid, leucine, lysine and phenylalanine) were chosen for the quantification based on the closeness of the fit of the observed number of residues to the expected value. The concentration was calculated using the following equation:

$$\text{nmole GM-HRA protein} = \frac{\text{(nmole asparagine/aspartic acid + nmole leucine+ nmole phenylalanine + nmole lysine)}}{\text{(expected \# of residues asparagine/aspartic acid + expected \# of residues leucine + expected \# of residues phenylalanine + expected \# of residues lysine)}}$$

$$= (0.959 + 0.409 + 0.926 + 0.465) / (52 + 54 + 23 + 25)$$

$$= 0.0179 \text{ nmoles of GM-HRA protein}$$

$$\text{concentration} = \frac{\text{nmole GM-HRA protein x theoretical molecular weight}}{\text{sample size x \% loaded onto analyzer}}$$

$$= (0.0179 \text{ nmole x } 65.3159 \text{ µg /nmole}) / (1610 \text{ µg x } 0.0025) = 0.291 \text{ mg in 1 mg of lyophilized powder}$$

The final concentration of microbial GM-HRA protein was a calculated average of the concentrations of each of the three replicates.

5.17. Results of Amino Acid Composition Analysis of Microbial M-HRA Protein

Based on the amino acid composition determination, the calculated concentration of GM-HRA in the lyophilized protein powder was found to be 29.1%, or 0.291 mg in 1 mg of lyophilized powder (data not shown).

Appendix 6. Methods for Determination of the GM-HRA Protein Concentration

Concentrations of the GM-HRA protein in 305423 and control soybean tissues were determined using enzyme linked immunosorbent assay (ELISA) developed at Pioneer Hi-Bred International, Inc.

6.1. Storage and Processing of Tissue Samples

Upon receipt, all plant tissue samples were stored in temperature-monitored freezers at <-10°C.

Forage samples were coarsely homogenized on dry ice using a Stephan VCM 12 (Stephan Machinery Singapore Pte Ltd, Singapore) blender for approximately 2 minutes and sub-sampled.

All samples were lyophilized at <-12°C under vacuum. The lyophilization time varied between 18 to 72 hours depending on the sample size and tissue type.

Leaf, forage and grain tissues were finely ground for approximately 60 seconds using a GenoGrinder (BT&C/OPS Diagnostics, Metuchen, NJ, USA) at room temperature. Root samples were finely ground for approximately 2 minutes using a Harbil 5G High-Speed Mixer (IDEX Corporation, Northbrook, IL, USA).

Between lyophilization and grinding, samples were stored frozen in temperature-monitored freezers at <-10°C.

6.2. Extraction of Processed Soybean Tissues

Processed soybean tissues were weighed into 1.2 ml tubes at the following target weights: 10 mg for leaf and grain, and 20 mg for root and forage tissues. Each sample was extracted with 600 microliters (µl) of chilled (2-8°C) GAT ELISA Buffer (GEB) that is comprised of 25 millimolar (mM) HEPES (Sigma-Aldrich, Inc., St. Louis, MO, USA), 25 mM CAPS (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA), 150 mM NaCl, 0.1% Tween-20 (ICI Americas, Inc., London, UK), 0.5% PVP-40 (Sigma-Aldrich, Inc.), 10% Ethylene Glycol, and 5% StabilZyme Select (SurModics, Inc., Eden Prairie, MN, USA). Two 5/32" steel balls were added to the tube and the samples were homogenized with a single 30 second cycle at 1500 strokes per minute using a SPEX Certiprep GenoGrinder^3 (BT&C/OPS Diagnostics, Metuchen, NJ, USA). Following centrifugation, supernatants were removed, diluted and analyzed for extractable GM-HRA protein concentrations using a specific enzyme linked immunosorbent assay (ELISA).

6.3. Determination of GM-HRA Protein Concentrations

The GM-HRA ELISA method utilized a sequential "sandwich" format for the determination of the presence of GM-HRA protein in soybean plant tissue extracts. In this assay, sample extracts were incubated for one hour in stabilized 96-well plates that were pre-coated with a GM-HRA-specific antibody. Unbound substances were washed from the plate, and a different GM-HRA-antibody that had been conjugated to the enzyme horseradish peroxidase (HRP) was added to the wells. Bound GM-HRA protein was sandwiched between the antibody coated on the plate and the antibody-HRP conjugate. At the end of the one hour incubation, unbound substances were washed from the plate. Detection of the bound GM-HRA protein-antibody complex was accomplished by the addition of a substrate solution, which generated a colored product in the presence of HRP. The reaction was stopped with stop solution (hydrochloric acid) and the optical density of each well was determined using a Molecular Devices (Molecular Devices Corporation, Sunnyvale, CA) plate reader with a wavelength setting of 450 nm minus 650 nm. SoftMax Pro software (Molecular Devices Corporation) was used to perform the calculations that generated the quadratic fit for the standard curve and converted the sample OD (optical density) values to

GM-HRA protein concentration values. The mean concentration from the duplicate wells in ng/ml was used in the calculation of the reported GM-HRA concentration of each sample (ng/mg dry weight).

The quantitative range for the assay was 1.0 ng/ml ± 10% to 20 ng/ml ± 10% which allows a full range of 0.9 to 22 ng/ml. The lower limit of quantitation (LLOQ) in ng/mg dry weight for each tissue was based on extraction volume (µl) to weight ratios, the limit of quantitation for the ELISA in ng/ml, and the dilutions used for analysis. In this study, the sample LLOQ on a ng/mg dry weight basis for GM-HRA was 0.54 ng/mg dry weight for leaf and grain, and 0.27 ng/mg dry weight for root and forage.

6.4. Calculations for Determining Concentrations of GM-HRA Protein

SoftMax Pro software (Molecular Devices Corporation) was used to perform the calculations required to convert the OD values obtained by the microtiter plate reader to concentration values.

A standard calibration curve was included on each ELISA plate. The equation for the standard curve was generated by the software, which used a quadratic fit to relate the mean OD values obtained for the standards to the respective standard concentrations (ng/ml).

The regression equation was applied as follows: $y = Cx^2 + Bx + A$

Where x = known standard concentration and y = respective mean absorbance value (OD).

Interpolation of the sample concentration (ng/ml) was done by solving for x using the values for A, B, and C that were determined for the standard curve.

$$\text{Sample concentration (ng/ml)} = \frac{-B + \sqrt{B^2 - 4C * (A - \text{sample OD})}}{2C}$$

i.e.: given curve parameters of A = 0.143, B = 0.00625, C= -0.00000399 and a sample OD = 0.249

$$\text{Concentration} = \frac{-0.00625 + \sqrt{0.00625^2 - 4\ (-0.00000399)\ (0.143 - 0.249)}}{2\ (-0.00000399)} = 17 \text{ ng/ml}$$

The sample concentration was adjusted for the dilution factor.

Adjusted concentration = concentration * dilution factor

i.e.: given a concentration of 17 ng/ml and a dilution factor of 10

Adjusted result = 17 ng/ml * 10 = 170 ng /ml

Sample concentration values obtained from the SoftMax Pro software were converted from ng/ml to ng/mg tissue dry weight as follows:

ng/mg tissue dry weight = ng/ml *extraction volume (ml)/mg tissue dry weight

i.e.: if the adjusted concentration = 170 ng/ml, extraction volume = 0.60 ml, and a tissue weight = 10.0 mg

ng/mg tissue dry weight = 170 ng/ml * 0.60 ml/10.0 mg = 10 ng/mg tissue dry weight

Appendix 7. Synthesis and Metabolism of 17-Carbon Fatty Acids

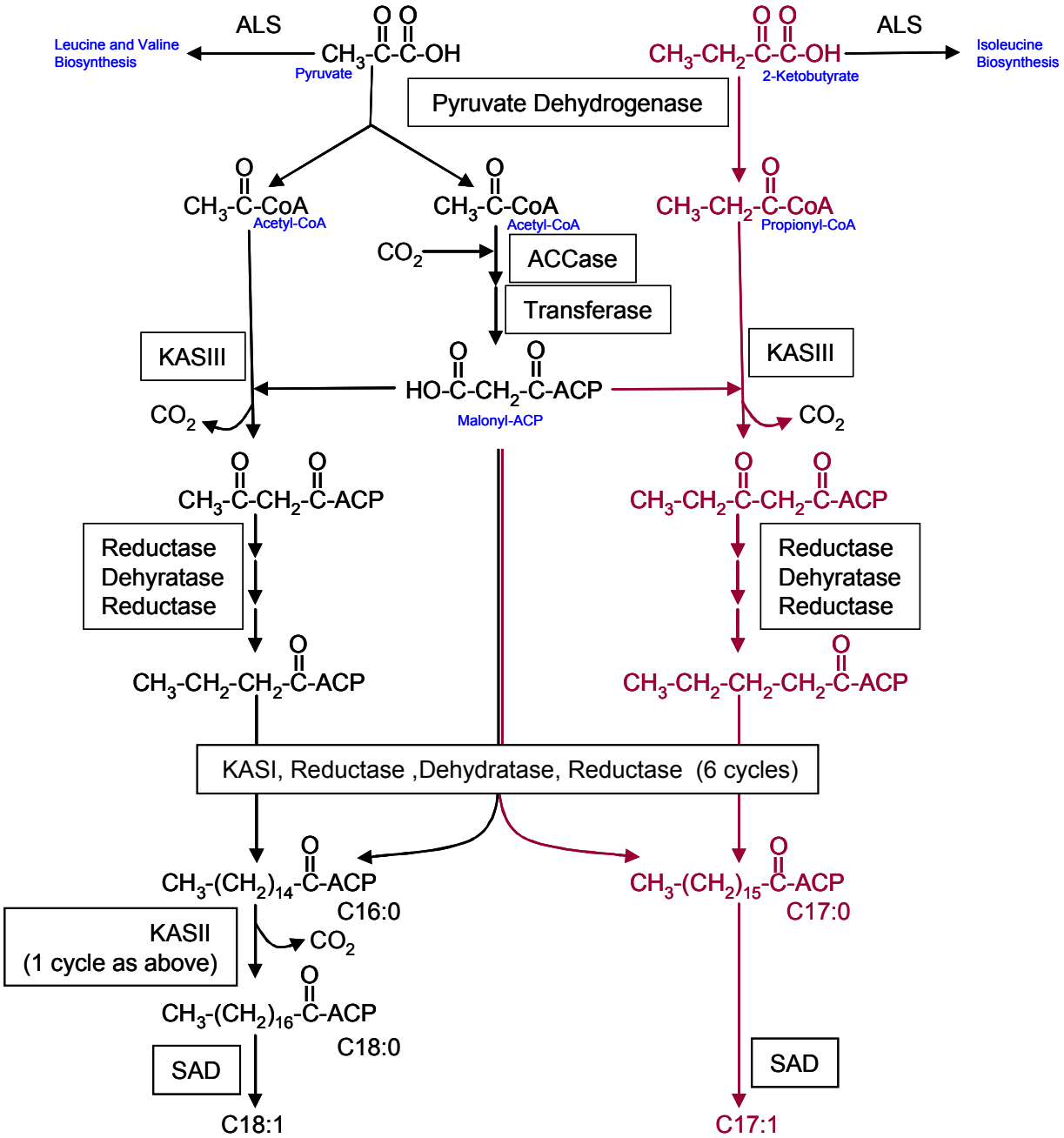
7.1. Synthesis of 17-Carbon Fatty Acids in Soybean

The biosynthetic pathway for fatty acids in plants begins with the conversion of pyruvate to acetyl-CoA. One acetyl-CoA molecule is then converted to malonyl-CoA and joined with a second acetyl-CoA molecule forming a four carbon fatty acid. Subsequent additions of two carbon moieties from malonyl-CoA result in fatty acids with an even number of carbons, such as 16 carbon (C16:0) and 18 carbon (C18:0) fatty acids. Odd numbered fatty acids result from the conversion of 2-ketobutyrate to a three carbon compound, propionyl-CoA, followed by addition of a two carbon moiety from malonyl-CoA forming a five carbon fatty acid. Subsequent additions of two carbon moieties from malonyl-CoA result in fatty acids with an odd number of carbons such as C17:0.

The primary pathway for both even-chain and odd-chain fatty acids begins with the pyruvate dehydrogenase complex (Appendix 7, Figure 1). Pyruvate is converted to acetyl-CoA by the pyruvate dehydrogenase complex and then used in the production of fatty acids with even chain numbers. However it is known that in *E. coli,* for example, 2-ketobutyrate can serve as a substrate for pyruvate dehydrogenase with a K_M that is about ten times higher than that of pyruvate. K_M is the affinity of the enzyme for a substrate or tightness of binding of the substrate to the enzyme. The higher the K_M, the lower the affinity of the enzyme for the substrate. The product of the 2-ketobutyrate reaction with pyruvate dehydrogenase is the three carbon compound propionyl-CoA instead of the two carbon acetyl-CoA from pyruvate. Fatty acid biosynthesis that initiates with propionyl-CoA results in fatty acids with an odd number of carbons.

One of the specific herbicide resistance mutations (tryptophan 560 to leucine) introduced into GM-ALS to form the GM-HRA enzyme may increase the 2-ketobutyrate pool available for C17:0 fatty acid synthesis. In studying the structure of *E. coli* ALS, it was shown that a mutation at amino acid position 464 changing the native tryptophan to leucine resulted in a 50-fold decrease in substrate preference for 2-ketobutyrate versus pyruvate (Ibdah *et al.*, 1996). The mutation also confers a high level of resistance to inhibition by ALS inhibiting herbicides on the *E. coli* ALS enzyme. Tryptophan 464 in the *E. coli* ALS is equivalent to tryptophan 560 in the soybean ALS. The mutation in the GM-HRA allele of GM-ALS is from tryptophan to leucine and occurs at position 560, and thus could have a similar loss in substrate preference. With the loss of substrate preference for 2-ketobutyrate it is reasonable to expect that the concentration of 2-ketobutyrate relative to pyruvate is increased in soybeans expressing GM-HRA. The increased levels of 2-ketobutyrate would become more available for pyruvate dehydrogenase and subsequent formation of odd chain fatty acids. Although fatty acids containing 17 carbons (C17:0, heptadecanoic acid and C17:1, heptadecenoic acid) are commonly found in soybean oil, their levels could be increased by the mutation in GM-HRA.

Appendix 7, Figure 1. Fatty Acid Biosynthesis – Even and Odd Chain Pathway



Even chain fatty acid biosynthesis is depicted in black arrows (left side of diagram) and odd chain fatty acid biosynthesis is depicted in red arrows (right side of diagram).

KAS I = 3-ketoacyl-acyl carrier protein synthase I
KAS II = 3-ketoacyl-acyl carrier protein synthase II
KAS III = 3-ketoacyl-acyl carrier protein synthase III
SAD = stearoyl-acyl carrier protein desaturase
ACCase = acetyl coenzyme A carboxylase

7.2. 17-Carbon Fatty Acid Metabolism in Humans and Animals

The metabolism of even chain fatty acids in humans and animals occurs through a process called β-oxidation. During this process, fatty acids enter the metabolizing cell and are activated with coenzyme A (CoA) by cytoplasmic fatty acyl-CoA synthetase. Oxidation occurs in the mitochondria as a cyclic degradative pathway by which two carbon units (in the form of acetyl-CoA molecule) are cleaved one at a time from the carboxy-terminus. The acetyl-CoA molecules then enter the Krebs metabolic cycle.

The metabolism of odd chain fatty acids proceeds in the same stepwise process of cleavage of acetyl-CoA units from the carboxy terminus as described above for even chain fatty acids. However, the terminal metabolic substrate is propionyl-CoA (a three carbon substance) rather than the two carbon acetyl-CoA which is the terminal metabolic substrate of β-oxidation of even chain fatty acids. Refer to Appendix 7.1. for information regarding biosynthesis of 17-carbon fatty acids in soybean.

Propionyl-CoA is enzymatically carboxylated to methylmalonyl-CoA by propionyl CoA carboxylase using biotin as a cofactor. Methylmalonyl-CoA is further metabolized to succinyl-CoA by methylmalonyl-CoA mutase using vitamin B12 as a cofactor. Succinyl-CoA subsequently enters the Krebs cycle (reviewed in Gropper *et al*., 2005).

Accordingly, odd-chain fatty acids such as C:17 should be readily metabolized to Krebs cycle intermediates that are in turn used in energy production.