CoverCress

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By llightle at 3:16 pm, Jan 18, 2024

1/18/2024
Bernadette Juarez
U.S. Department of Agriculture
APHIS Deputy Administrator
Biotechnology Regulatory Services

Dear Deputy Administrator Juarez,

With this letter we respectfully request a Regulatory Status Review from USDA-APHIS's Biotechnology Regulatory Services (BRS) for the following Plant Trait Mechanism of Action: *Thlaspi arvense* L. (pennycress; field pennycress) CRISPR/Cas9 generated mutant lines, featuring disruption of any combination of: [ ] to produce a lower glucosinolate phenotype. This PTMOA is described in previously submitted RSR's 22-241-01rsr, 22-292-01rsr, and 23-264-01rsr. This RSR is a resubmission of the recently withdrawn 22-241-01rsr, reducing the number of gene edits within this PTMOA pathway. CBI-Deleted

USDA has previously evaluated pennycress lines with the same phenotype as the PTMO in this RSR via the AIR process. Lines containing disruption in genes (*AOP2*, [ ]) that are in the same biosynthesis pathway and that exhibit the same phenotype as our additional edits [ ], were evaluated in some of these and other AIR requests[1]. *AOP2*, [ ] are examples of several genes that, when disrupted, reduce glucosinolate levels in pennycress. This RSR requests evaluation of pennycress with mutations in additional genes that result in the same low glucosinolate phenotype, [ ]. In response to each of several previously submitted AIR letters, BRS deregulated pennycress lines harboring disruptions in *AOP2,* [ ], and other genes, concluding that: "…your genome edited pennycress lines are not themselves plant pests". USDA additionally stated in several AIR response letters that pennycress is not listed as a Federal noxious weed pursuant to 7 CFR part 360 and USDA has no reason to believe that the intended phenotypes of the pennycress lines would increase the weediness of pennycress. CBI-Deleted CBI-Deleted (2) CBI-Deleted CBI-Deleted CBI-Deleted

Further, via the confirmation of exemption process, single edits in the [ ] (23-107-01cr), [ ] (22-336-02cr), and [ ] (22-336-01cr) genes have been deemed "achievable by conventional breeding and unlikely to pose an increased plant pest risk relative to their conventionally bred counterparts". CBI-Deleted CBI-Deleted (2)

1. Information about Requestor

First Name: Marcia

Last Name: Weldon

Position: Regulatory & Stewardship Manager

[1] Cover Cress, Inc., USDA response January 29, 2020; CoverCress, Inc., USDA response May 7, 2020; CoverCress, Inc., USDA response August 31, 2020; Illinois State University, USDA response August 24, 2020

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Organization Name (if applicable): CoverCress, Inc.

Contact information (choose one or both)

Telephone: [ ] CBI-Deleted

Email address: mweldon@covercress.com

2. Does the request contain Confidential Business Information (CBI)?

Yes, this RSR request contains CBI.

This RSR for gene-edited pennycress contains confidential business information that could harm CoverCress, Inc. if publicly disclosed prior to publication of patent applications or other disclosures with that information. The specific information that could cause competitive harm are the names and sequences of the low glucosinolate target genes, as well as the specific modifications and the mechanism of action. Premature disclosure of that information could allow competitors to develop competitive products much more quickly than if the information was kept as CBI and/or trade secret. CoverCress, Inc. treats this information as private both customarily and actually and provides this information to the government under an assurance of privacy. The information is confidential within the meaning of 5 U. S. C. §552(b)(4), the Freedom of Information Act's Exemption 4.

3. Description of the comparator plant:

Scientific name (genus, species): *Thlaspi arvense*

Common Name: pennycress; field pennycress; CoverCress™

Subspecies / Cultivar / Breeding Line:

Field pennycress is an oilseed crop undergoing domestication for use as an alternative source of biofuel and feedstock. Besides the economic benefit(s), the crop will provide ecosystem services as a cover crop in a two-year corn-soybean rotation. Although a prolific seed producer, interspecific hybridization is uncommon in the genus *Thlaspi* and there are no reports of interspecific hybrids with *Thlaspi arvense* (Al-Shehbaz, 1986; Best & McIntyre, 1975; Warwick et al., 2002).

CoverCress, Inc. has developed, through selection and breeding, several elite germplasm pennycress lines that have optimized agronomic characteristics. The improved germplasm are referred to as CoverCress™ lines. It is envisioned that current and future lines from the breeding program, optimized for commercial production, will undergo late stage editing to produce the lower glucosinolate phenotypes.

4. Genotype of the modified plant (if genetic material is not inserted into the genome):

Nature of modification(s):

Mutations will be introduced into pennycress cultivars using a CRISPR/SpCas9 DNA construct designed to target genomic edits to the [ ] genes. This CBI-Deleted transgene construct will be delivered to the plant using a disarmed *Agrobacterium tumefaciens* strain (GV3101) and a standard floral dip transformation method. When integrated into the plant genome, the expressed *Streptococcus pyogenes* CRISPR-associated protein 9 (SpCas9) endonuclease will be guided to three unique, targeted loci (the [ ] genes). At these locations, the SpCas9 endonuclease will CBI-Deleted catalyze double-stranded DNA breaks, which would then be repaired by the plant's error-prone endogenous non-homologous end joining (NHEJ DNA) repair mechanisms, resulting in heritable mutations at the targeted loci.

The design pipeline has two steps to ensure the gRNA is specific to the gene and to reduce off-targeting. First, an online gRNA design tool that has an inbuilt pennycress genome is used to identify gRNA target sites and provide a list of putative off-target sites. Furthermore, a blast search is performed with a candidate protospacer sequence against the pennycress genome to avoid the use of a protospacer with potential off targets.

DsRED fluorescent protein from Discosoma will be included in the plasmid to confer red fluorescence in plants that successfully take up the plasmid introduced by *A. tumefacians*. The presence of the edits in T_1 plants will be confirmed through visualization of red fluorescent protein under a light system and confirmatory PCR screening of a fragment of the T-DNA. Seed from the progeny T_2 generation will then be screened for segregants that do not have the transgene as indicated by lack of red fluorescence. Resulting seedlings in the T_2 generation will be screened again for negative presence of DsRED and Cas, as well as homozygous edits to [ ]. CBI-Deleted

Examples of these edits are listed in the following section.

Sequence and Comparison of each Modification

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The top line of the following sequence comparison is the unmodified sequence of the [ ] CBI-Deleted gene. One representative modified gene sequence of [ ] is included for comparison. CBI-Deleted One site in the gene is edited and includes a 2 bp deletion, highlighted in red. The full gene sequences and sequence comparison are included in Appendix 1.

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The top line of the following sequence comparison is the unmodified sequence of [ ]. CBI-Deleted One representative modified gene sequence of [ ] is included for comparison. One site CBI-Deleted in the gene is edited and includes a single T insertion, highlighted in red. The full gene sequences and sequence comparison are included in Appendix 1.

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The top line of the following sequence comparison is the unmodified sequence of the [ ] CBI-Deleted gene. One representative modified gene sequence of [ ] is included for comparison. One CBI-Deleted site in the gene is edited and includes a single base pair insertion, highlighted in red.

[

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5. Description of new trait

Intended trait: reduced glucosinolate seeds

Intended phenotype: low accumulation of total glucosinolates in seeds; reduction of total glucosinolate levels to below 30 µmol/g of total seed weight

Pennycress contains high levels of oil (~25-35%) that makes it a desirable ultra-low carbon fuel feedstock (Altendorf et al. 2019; McGinn et al. 2019; Moser et al. 2009). In addition to this primary value for fuel, the seed could provide an energy source for animal feeds such as chicken feed. Field pennycress or CoverCress™ also contains 80-110µmol/g glucosinolates derived through the aliphatic glucosinolate pathway (Chopra et al., 2018b; Chopra et al., 2020; Sedbrook et al., 2014). Glucosinolates are biologically active compounds found in the Brassicaceae family of plants, including broccoli, cabbage, cauliflower, rapeseed, mustard, and horseradish, and provide defense mechanisms for plants. Over 200 types of glucosinolates are found in brassicas (Prieto et al., 2019); pennycress contains the glucosinolate sinigrin (Chopra et al., 2020). Sinigrin and its metabolite allyl isothiocyanate can reduce palatability of food and feed at lower levels and result in toxic effects at higher levels. Therefore, presence of glucosinolates (which is almost all sinigrin) in field pennycress or CoverCress™ impacts the ability to include the seed or meal in feed applications above limited inclusion rates (Chopra et al., 2020).

Description of each MOA:

Low total glucosinolate MOA

Glucosinolates are secondary metabolites important for plant defense during development and exposure to biotic and abiotic environmental conditions, as well as helpful in the suppression of weeds. Sønderby et al. (2010) gives an overview of the glucosinolate biosynthesis pathway. Glucosinolate biosynthesis in brassicas requires three steps, outlined

in Figure 3: 1) amino acid chain elongation, 2) oxidation and sulfation to generate the core glucosinolate structure and 3) secondary side chain modifications to generate unique forms of glucosinolates. Levels of glucosinolate in the seed are regulated by transporters. There are more than 200 glucosinolates identified and classified in brassicas. Field pennycress or CoverCress™ contains 80-100µmol/g glucosinolates in the form of sinigrin (>99% of the glucosinolate found in pennycress) derived through the aliphatic glucosinolate pathway (Chopra et al., 2018b; Chopra et al., 2020; Sedbrook et al., 2014).

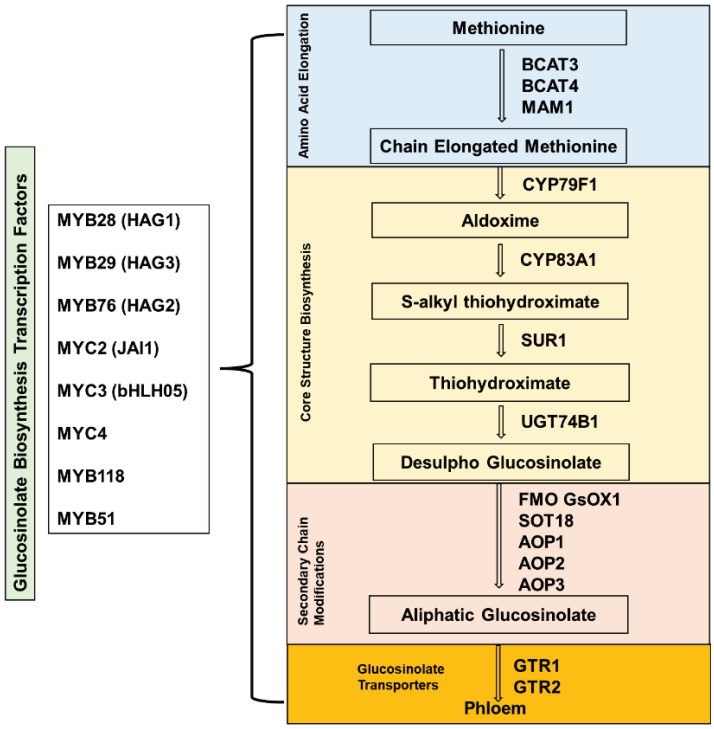

Figure 3: Major steps in glucosinolate biosynthesis

The rationale for gene target selection was to identify those genes that result in reduced glucosinolate levels in the seed through a combination of reduced production and mobilization (each contributing ~10-30% reduction), but do not impact other biosynthetic pathways or cause accumulation of glucosinolates in other parts of the plant. To reach the desired 70-80% reduction in glucosinolate levels will require loss of function mutations in a combination of gene targets in this RSR request.

These three targets include transcription factors ([ ]), and transporter ([ ]). The genes and their respective contribution to achieve reduced glucosinolate levels are described below. **CBI-Deleted (2)**

[ ] is a MYB protein that directly regulates the expression of aliphatic glucosinolates biosynthesis in brassicas [ ]. The representative edited allele for [ ] has a 2-bp deletion that causes a frameshift which results in a premature **CBI-Deleted** **CBI-Deleted** **CBI-Deleted**

stop codon and a truncated, non-functional protein. This results in a reduction in sinigrin levels compared to wild type. Evidence from greenhouse and field studies demonstrates that plants grow normally throughout their lifecycle and no apparent reduction in plant height or changes in leaf morphology have been observed.

[ ] is a bHLH protein that is known to interact directly with glucosinolate-related transcription factors, and it helps coordinate expression of several biosynthesis genes required to accumulate glucosinolate [ ]. We generated alleles in pennycress to test this hypothesis and found via RT-PCR that genes involved in glucosinolate biosynthesis were reduced significantly in various stages of plant development. The representative edited allele for [ ] has a 1-bp insertion that causes a frameshift which results in a premature stop codon and a truncated, non-functional protein. [ ] pennycress edits will have a reduction in sinigrin levels compared to its co-grown wild type. Evidence from both greenhouse and field studies demonstrates that plants grow normally throughout their lifecycle and no apparent reduction in plant height or changes in leaf morphology have been observed.

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[ ] encodes a high-affinity, proton-dependent glucosinolate-specific transporter that is crucial for the transport of both methionine- and tryptophan-derived glucosinolates to seeds [ ]. Gene edited alleles for loss of function of [ ] in pennycress resulted in a reduction in sinigrin levels compared to wild type, with no impact on other biosynthetic pathways or observed accumulation of glucosinolates in other parts of the plant. The representative edited allele for [ ] provided has a 1-bp insertion that causes a frameshift which results in a premature stop codon and a truncated, non-functional protein. Evidence from both greenhouse and field studies demonstrates that plants with this loss of function grow normally throughout their lifecycle unlike observations in *Brassica juncea* (Tan et al. 2022).

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To achieve meaningful reduction in glucosinolate levels, knockouts in multiple gene targets across the various stages of production and transport are needed to reach the phenotype goal of below 30 µmol/g of total seed weight.

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Appendix 1 – Full Sequences and Sequence Comparisons

Unmodified and Modified Sequences of [ ] CBI-Deleted

Protospacer Sequence Highlighted Yellow, Edit Highlighted Red

[ ] Wild Type, Unmodified Sequence CBI-Deleted

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[ ] Modified Sequence, Representative Sequence CBI-Deleted

Ta [ ] Mutant CDS: CBI-Deleted

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[ ] Wild Type, Unmodified Sequence CBI-Deleted

Ta [ ] WT CDS: CBI-Deleted

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[ ] Modified Sequence, Representative Sequence CBI-Deleted

Ta [ ] Mutant CDS: CBI-Deleted

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[ ] Wild Type, Unmodified Sequence CBI-Deleted

Ta [ ] WT CDS: CBI-Deleted

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[ ] Modified Sequence, Representative Sequence CBI-Deleted

Ta [ ] Mutant CDS: CBI-Deleted

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Sequence Comparisons of the unmodified and modified genes

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The top lines of the following sequence comparison are the unmodified sequence of the [CBI-Deleted] gene. One representative modified gene sequence of [ ] is CBI-Deleted (2) included for comparison. One site in the gene is edited; the modified sequence includes a 2 bp deletion. The edit is in red.

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The top lines of the following sequence comparison are the unmodified sequence of the [CBI-Deleted

] gene. One representative modified gene sequence of [ ] is CBI-Deleted (2)

included for comparison. One site in the gene is edited; the modified sequence includes a single T insertion. The edit is in red.

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The top lines of the following sequence comparison are the unmodified sequence of the [CBI-Deleted
] gene. One representative modified gene sequence of [ ] is CBI-Deleted (2)
included for comparison. One site in the gene is edited; the modified sequence includes a single T insertion. The edit is in red.

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