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RE: Inquiry regarding APHIS position on non-segregant CRISPR/Cas9 mutagenized *Glycine max* (soybean) line as a non-regulated article

Dr. Michael,

With this letter, we are asking the Biotechnology Regulatory Services to de-regulate a null-segregant CRISPR/Cas9 mutagenized soybean line 590-4-28-5 containing no transgenic sequences that was derived from a transgenic parent line 590-4. We plan to field test seedlings at the Sand Plain Research Farm at Becker, Minnesota for tolerance to drought and salt stress.

The transformation was carried out on Soybean (*Glycine max*) cultivar Bert using a disarmed *Agrobacterium rhizogenes* strain transformed with a CRISPR associated protein 9 (Cas9) reagent described below. When integrated into the plant genome, the expressed CRISPR reagent was guided to two loci by expressed guide RNAs to the coding region of two *Glycine max* DOUBLE-STRANDED RNA-binding protein2 (*Drb2a* and *Drb2b*). This action induced a double-stranded break at both target sites that was repaired by the plant's DNA repair mechanism resulting in frame-shift mutations that deactivated these genes.

Null-segregant mutant lines were generated by self-pollinating the original transformed line and the screening of parent and progeny lines by 20X whole genome sequencing (WGS). PCR assays based on WGS data was used to confirm null segregant progeny. The segregation of integrated transgenic material was possible since the reagent transgene was not linked to either *Drb2a* or *Drb2b* loci.

The construct used to deliver the mutagenesis reagent is comprised of the following components: A *Glycine max* Ubiquitin promoter (Gmubi) driving strong constitutive expression of the *Arabidopsis* codon-optimized CRISPR associated protein 9 (Cas9). The Cas9 (CRISPR associated protein 9) is a RNA-guided DNA endonuclease enzyme used to mutate gene(s) of interest. The octopine synthase terminator (OCS) from *Agrobacterium tumefaciens* was used to end transcription of the Cas9 cassette. To drive expression of guide RNA sequences, the U6 and At7sL polymerase III promoters from *Arabidopsis thaliana* were used. The guide RNAs *drb2ab* and *drb2abcd* targeted the second exon of *Drb2a*, Glyma.12g075700; *Drb2b*, Glyma.11g145900). The guide RNA cassette was terminated by the NOS terminator from *Agrobacterium tumefaciens*. The *bar* (bialaphos resistance) gene from *Streptomyces hygroscopicus* was used as a selectable marker for transgenic selection and was constitutively expressed by a 35S promoter from Cauliflower

mosaic caulimovirus (CaMV). The *bar* gene encodes a phosphinothricin acetyl transferase (PAT) enzyme that detoxifies applied phosphinothricin herbicide. The 35ST_polyA from Cauliflower mosaic caulimovirus was used to terminate *bar* transcription. Left (LB) and Right (RB) T-DNA border sequence from *Agrobacterium tumefaciens* span the T-DNA sequence for integration into the plant genome.

We have introduced two mutations into loci at Glyma.12g075700 and Glyma.11g145900 that have disrupted the expression of two *Drb2* genes, *Drb2a* and *Drb2b* in the recipient organism *Glycine max* cultivar Bert. These mutants will be used to study a putative drought and salt tolerance trait. Although the original transgenic line was generated using a construct containing plant pest sequences, the resulting null segregant does not contain such sequences. Therefore we are writing to obtain confirmation that this line is not a regulated article under 7 CFR Part 340. Please confirm that this understanding is correct.

Sincerely,

Shaun Curtin

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