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Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737

September 30, 2019

Dear Ms. Juarez,

Per instructions on the APHIS web site, I am submitting a letter of inquiry regarding whether a genome edited tomato variety is regulated under CFR part 340. I believe that all of the relevant information is provided in this letter. Please note that this request covers the knockout of a gene, *JOINTLESS2*, in three inbred tomato lines. The construct used to generate these materials and the methods used to characterize the products are identical to materials that were previously deemed to not be regulated in a letter from your office dated 05/14/2018. The current request thus seeks a similar determination on new breeding lines.

Sincerely,

Harry J Klee
Professor

Developer name: Harry J Klee, University of Florida, Horticultural Sciences, PO Box 110690, Gainesville FL 32611
Contact information: (Ph) 352 3928249 (email) hjklee@ufl.edu
Organism: Tomato (*Solanum lycopersicum*)
Intended activity: Release
Genetic change: Loss of function of JOINTLESS2 (J2) due to CRISPR-Cas9 induced mutation
Vector: *Agrobacterium tumefaciens* delivery of CRISPR-Cas9 protein and guide RNAs
Name of construct: pAGM4723

Summary

- Loss of J2 function is a highly valuable trait that will result in significant labor cost reduction
- The natural *j2* mutant results in a loss of *J2* transcript and protein but has undesirable effects on fruit shape
- We have produced genome edited mutations in *J2*, using a CRISPR-Cas9 system, that no longer produce functional J2 protein.
- Plants have been backcrossed to wild type plants and progeny containing the mutations but lacking the Agrobacterium T-DNA used to deliver the CRISPR-Cas9 protein have been identified. These plants contain the altered *j2* but no longer contain any pathogen-derived DNA elements.
- The genome-edited j2 loss-of-function mutant has the desired phenotype with none of the undesired phenotypes
- The precise editing of the J2 gene is more suited to agricultural production, equivalent to the natural mutant but without the associated linkage drag effects on fruit morphology

Background.

J2 is a gene that is essential for formation of the abscission zone that is normally present in the pedicel of a tomato plant. In a wild type plant, the ripe fruit falls off of the plant upon maturity,

breaking off precisely and cleanly at the pedicel abscission zone. In commercial practice, this process results in a portion of the stem (pedicel) remaining attached to the fruit. This stem piece is woody and if not removed, can puncture the skins of other fruits in a container, making them unsalable. As a consequence, hand harvesting ripe fruit in the field is a two step process of removing the fruit from the plant and then removing the attached pedicel from the fruit. By comparison, processing tomatoes grown for paste production, have a natural mutation, *j2*, that does not express a functional J2 protein. That mutation is derived from the wild relative of cultivated tomatoes, *Solanum cheesmaniae.* The *j2* mutant fails to develop the pedicel abscission zone and when harvested, the fruit comes cleanly off of the plant. Breeders of fresh market tomatoes have evaluated the j2 mutant but it has not been deployed in commercial germplasm because introduction of the mutant has been associated with misshaped fruit that were deemed less desirable. The major unanswered question is whether those misshaped *j2* fruits are the direct effect of loss of J2 function or whether another gene close to *J2* on the chromosome is responsible for the fruit shape effect. Breeders refer to a trait caused by a gene nearby the gene of interest as linkage drag. To date, breeders have not physically separated the fruit shape effect from the abscission zone effect and have largely abandoned efforts to deploy it in commercial fresh market varieties despite the obvious financial reward. Recently, the *J2* gene was identified (Soyk, et al., 2017, Cell 169: 1142-1155). The gene maps very close to the centromere on chromosome 12 in an area with suppressed recombination, explaining the difficulty of separating the desired from the undesired effects of the locus. *J2* encodes a MADS-Box transcription factor (Solyc12g038510), a class of genes involved in developmental determination of floral organ identity. The *S. cheesmaniae* j2 mutation contains a retrotransposon insertion in the first intron of the gene that results in a failure of the natural *J2* transcript to accumulate.

Labor cost and availability is a major factor that is threatening the viability of the Florida fresh market tomato industry. This cost drove us to reconsider the value of *j2* as a tool to reduce labor cost. In theory, varieties lacking the pedicel abscission zone could reduce labor costs up to 50% since a *j2* mutant would cleanly detach from the plant. We used genome editing, specifically CRISPR-Cas9-induced DNA misrepair, to produce non-functional J2 protein. By specifically inactivating only J2, we showed that the fruit shape effects are due to loss of J2 function.

Methods.

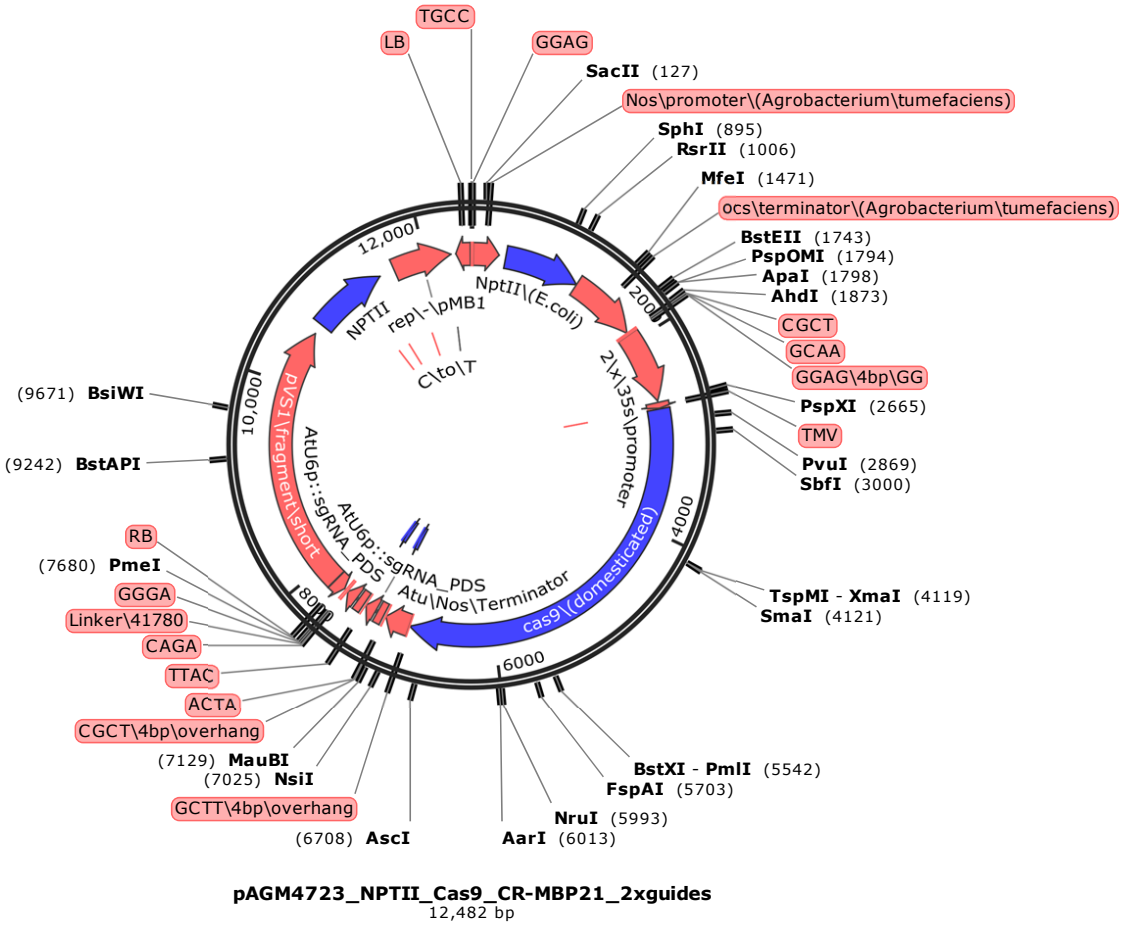
A vector was built for the purpose of CRISPR-Cas9 and guide RNA delivery (Figure 1). That vector was introduced into *Agrobacterium tumefaciens* strain EHA105 and used to produce transgenic tomato plants (McCormick S. et al., 1986, Plant Cell Rep. 5, 81–84) in the following inbred breeding lines: Fla 7907B, Fla 7781Ty1 and Fla 8872B. The presence of transferred DNA (T-DNA) was validated by PCR analysis using primers designed to amplify the NPTII selectable marker present in the plant (Figure 2). Transgenic plants were grown to maturity in an enclosed greenhouse following protocols approved by the University of Florida Institutional Biosafety office. One transgenic event was selected for further analysis in each genetic background. DNA from regenerated plants for each inbred line was isolated and the genomic regions covering the *J2* gene was amplified using PCR and subjected to DNA sequence analysis. Sequence analysis indicated altered sequences in the *J2* gene in each case:

- Fla 7907B event 18254 contains a 1 base pair (bp) insertion that results in a truncated j2 protein (Figures 3-5).
- Fla 7781Ty1 event 18362 contains a 6 bp deletion that removes two amino acids from the j2 protein (Figures 6-8).
- Fla 8872B event 18341 contains a 16 bp deletion that results in a truncated j2 protein (Figures 9-11).

When the first generation transgenic events (T0) reached the flowering stage, pollen from wild type, non-transgenic parental lines (Fla 7907B, Fla 7781Ty1 and Fla 8872B) was used to pollinate emasculated flowers of each transgenic plant. Seeds from the resulting cross-pollinated fruits were used to produce the T1 generation plants. These T1 plants were screened by PCR analysis to identify progeny that had inherited the mutated *j2* event but not the T-DNA. T-DNA absence was initially scored based on failure to amplify a portion of the NPTII gene with the same primer set used to identify the T-DNA in the T0 generation (PCR primers 1F and 1R, Figure 2). Absence of the T-DNA was confirmed with three additional sets of PCR primers designed to amplify different parts of the T-DNA (primers 2F/2R, 3F/3R and 4F/4R, Figure 2). Plants that inherited the T-DNA were identified, validating that the markers detected the T-DNA. Plants that did not inherit the T-DNA did amplify the altered j2 gene, validating that high quality DNA was extracted from each plant. Plants that contained the altered *j2* and were azygous for the T-DNA were grown to maturity and allowed to self-pollinate. Seeds from the subsequent T1/F1 generation were then screened for individuals that were homozygous for the *j2* altered (*j2/j2*). These homozygous lines were again screened with T-DNA PCR markers to confirm absence of the T-DNA. These T2 plants did not have pedicel abscission zones, as predicted, indicating a lack of functional J2 protein. As with the previously submitted 2018 lines, the new genome edited lines completely lack the abscission zone. Fruits from each line are completely indistinguishable from those of the parental lines except for the lack of pedicel. Field trials of each genome edited line, conducted under USDA APHIS permit (Notification No. 19-071-101n) in Live Oak FL indicated that there was no difference in fruit shape, plant performance or yield relative to the parental controls.

We conclude that the *J2* gene in the genome edited lines is non-functional. The mutation is equivalent to the natural *j2* mutant widely deployed in processing tomato varieties and widely grown in the U.S. We expect that the genome edited *j2/j2* trait will be highly desirable for introduction into commercial fresh market varieties.

Definition:
Abscission zone. A layer of cells in the pedicel that controls shedding of the flower/fruit. The point of natural separation between the plant and the organ. The process of shedding is called abscission.
Linkage drag. Refers to two or more genes in close proximity on a chromosome. The genes are close enough that genetic linkage is difficult or impossible to break. Gene A has the desired trait. Gene B, genetically linked confers an undesirable phenotype that cannot be eliminated.
Pedicel. The stalk attaching a flower/fruit to the main stem of a plant.



pAGM4723_NPTII_Cas9_CR-MBP21_2xguides
12,482 bp

Figure 1. Map of the plasmid, pAGM4723, used to introduce Cas9 and guide RNAs into tomato varieties Fla 7907B, Fla 7781Ty1 and Fla 8872B.

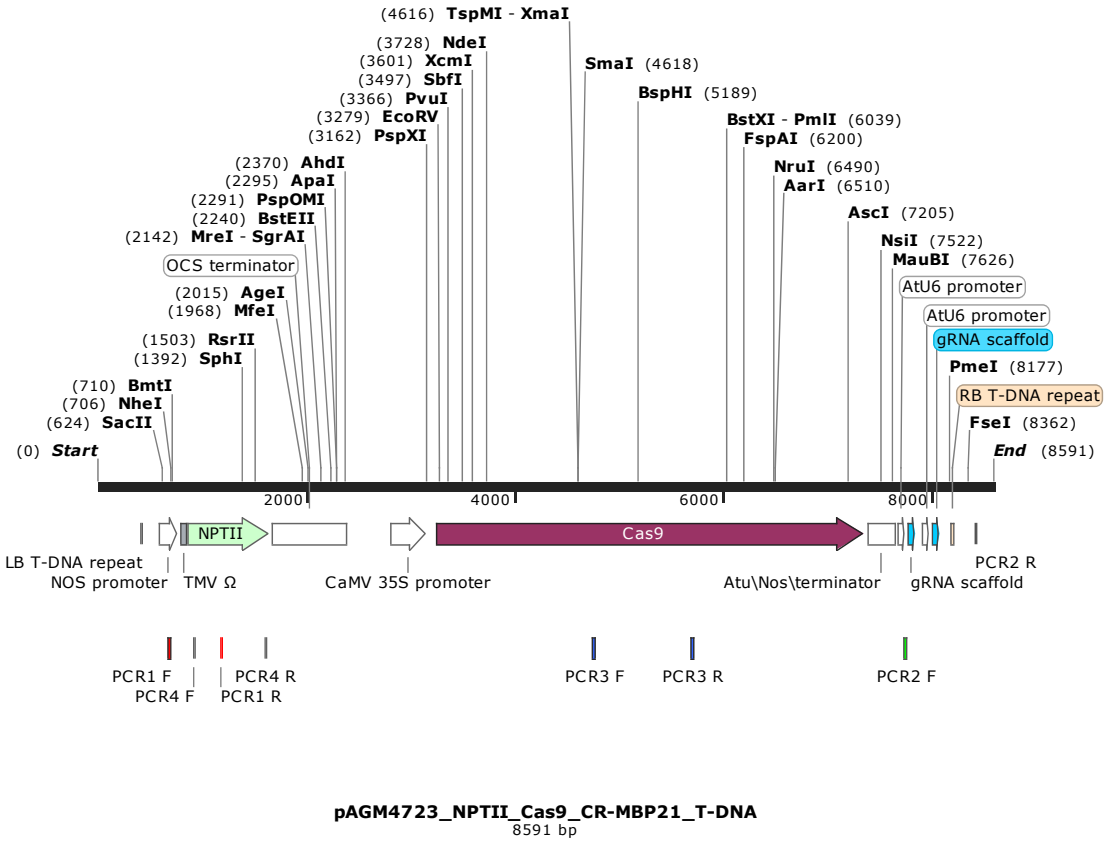


Figure 2. Region of pAGM4723 encoding the T-DNA. NPTII is the plant selectable marker conferring kanamycin resistance on transformed plants. Cas9 expression is driven by the cauliflower mosaic virus 35S transcriptional promoter. Positions of PCR primer pairs used to detect the presence of T-DNA are indicated.

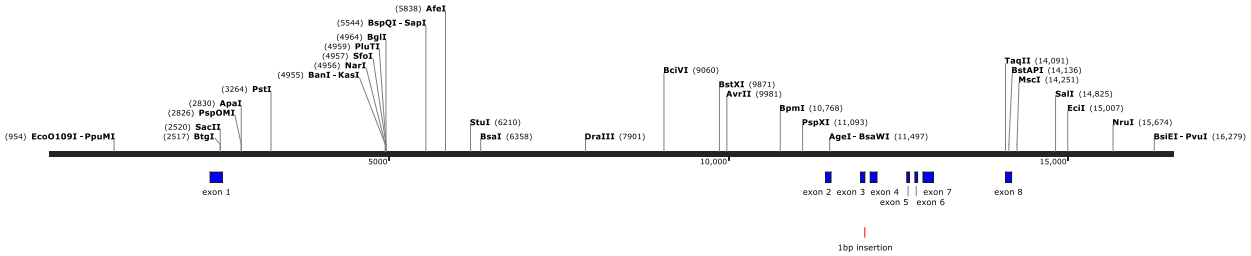


Figure 3. Schematic showing the 1 bp insertion in event 18254. Structure of the J2 gene from the transcriptional start site through to the stop site. Exons, encoding the J2 protein are indicated in dark blue. The locations of the Cas9-induced insertion is indicated in red.

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Figure 4. Alignments of a portion of the wild type and genome-edited event 18254 *J2* gene.

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Figure 5. Sequences of the wild type genome-edited event 18254 J2 peptides. The genome-edited line encodes a truncated protein.

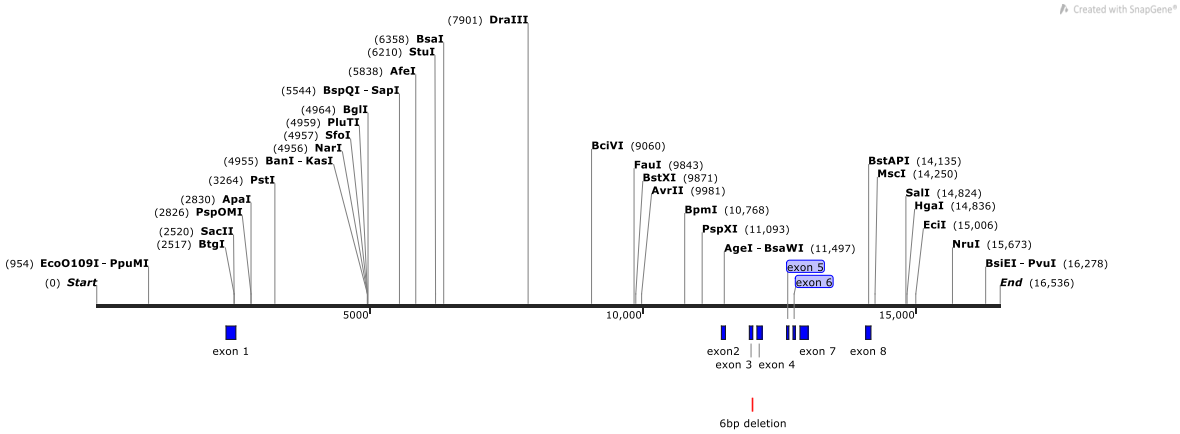

Figure 6. Schematic showing the 6 bp deletion in event 18362. Structure of the J2 gene from the transcriptional start site through to the stop site. Exons, encoding the J2 protein are indicated in dark blue. The locations of the Cas9-induced deletion is indicated in red.

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Figure 7. Alignments of a portion of the wild type and genome-edited event 18362 J2 gene.

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Figure 8. Sequences of the wild type genome-edited event 18362 J2 peptides.

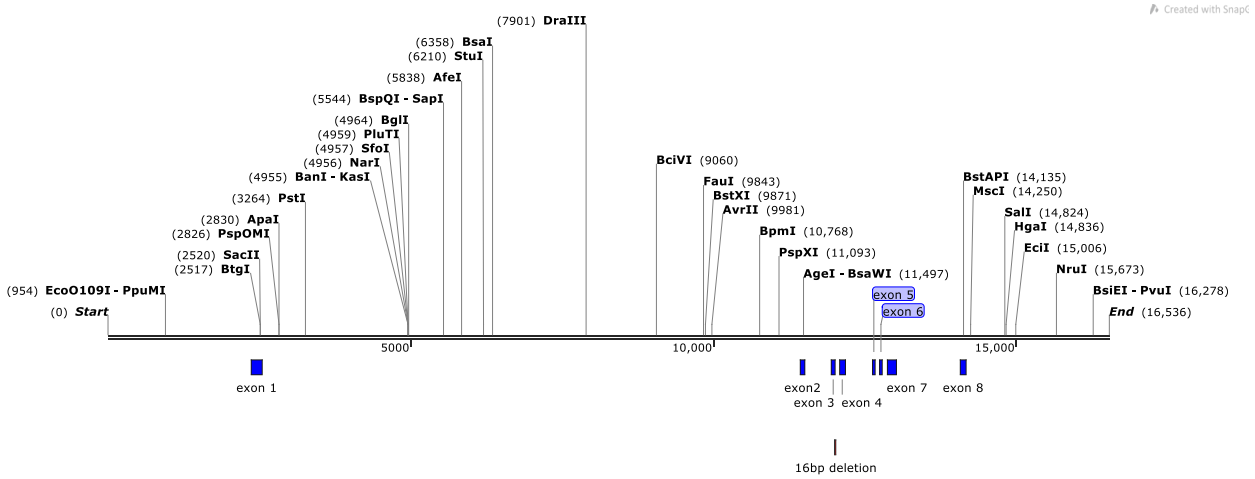

Figure 9. Schematic showing the 16 bp deletion in event 18341. Structure of the J2 gene from the transcriptional start site through to the stop site. Exons, encoding the J2 protein are indicated in dark blue. The locations of the Cas9-induced deletion is indicated in red.

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Figure 10. Alignments of a portion of the wild type and genome-edited event 18341 J2 gene.

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Figure 11. Sequences of the wild type genome-edited event 18341 J2 peptides. The genome-edited line encodes a truncated protein.