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September 2, 2021

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

Dear Ms. Bernadette Juarez:

Several months ago, we had requested a confirmation process from the Biotechnology Regulatory Services (BRS) for an exemption of the genome-edited potatoes that we had developed (a version of 20-324-01cr). However, the request was rejected by the BRS because potato is a tetraploid crop, and our genome-edited potatoes had two gene edits, even though we had explicitly used only one type of guide RNA (gRNA). Since our potatoes did not meet the criteria to attain the exemption status, particularly the presence of a single gene edit, the BRS suggested that we should submit our request for a regulatory status review (RSR).

Therefore, we request the BRS to evaluate the potato lines that we have developed using genome-editing technology for a regulatory status review. We are providing information in this letter, which may assist the BRS to make a decision regarding our genome-edited potato lines. Essential information that validates our request includes the following:

We used the Cas9-ribonucleoprotein (RNP) delivery system to transfect potato protoplasts using a targeted single gRNA (sgRNA) to edit the *StPPO2* gene (encoding polyphenol oxidase, PPO), which is associated with the enzymatic browning of potatoes. Out of nearly 110 independent genome-edited potato lines that were obtained, we selected 2 browning-suppressed potato (BSP) lines (#38 and #165) based on the levels of PPO activity and the decrease in the extent of browning.

1. The method used to develop the BSP lines did not include the introduction of any foreign DNA sequences into the plant genome. Instead, the genetic modification resulted from base pair (bp) deletions during the natural cellular repair of a targeted DNA break without an externally provided DNA repair template.

2. We present two BSP lines, #38 and #165, both of which are tetraploid with certain deletions in the four alleles of the *StPPO2* gene at the target site, namely a two bp deletion in line #38 and a four bp deletion in line #165.

3. We designed the sgRNAs to specifically target the *StPPO2* gene; thereafter, we performed protoplast transfection for validation of the sgRNAs. Although *StPPO* belongs to a multi-gene family, gene editing occurred only in *StPPO2*. None of the other iso-genes were modified in the target sequences.

4. The obtained BSP lines did not differ from the non-edited potatoes in terms of phenotypic traits, except for the suppressed browning. We did not find any evidence that the BSP lines were more susceptible to pest attack than the control lines.

We are providing all the information related to our work to support our application for a regulatory status review of the genome-edited BSP lines. However, if you need any additional information to assist the review procedure, please let me know.

Sincerely yours,

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Taxonomic description of the comparator plant, potato

The potato that we used for our gene-modification experiment is *Solanum tuberosum* L. (cultivar: Desiree). Desiree potatoes were developed in the mid-20th century in the Netherlands. The Desiree tuber has an elongated oval shape, its skin is bright red, and the flesh is yellow. Moreover, the Desiree potatoes have a starchy and tender texture upon cooking. Therefore, this potato variety has become commercially successful in Europe as well as other continents because of its wide range of daily culinary applications.

Desiree potatoes are known for their resistance to diseases, such as potato warts and skin spots. Additionally, this cultivar is resistant to viruses like potato virus Y (PVY), potato virus A (PVA), and potato virus X (PVX), but it is susceptible to diseases like leaf late blight and scab. Over the years, a large number of potato varieties have been bred and marketed; hence, Desiree is no longer a global favorite potato cultivar. However, people still prefer this potato variety for cooking purposes.

Biological properties of potato

Potato is the world's fourth-largest food crop. It is a staple food item worldwide, and it contains 79% water, 17% carbohydrates, 2% proteins, and a negligible amount of fat. A 100 gm portion of raw potato provides 77 kCal of food energy, and it is a rich source of vitamin B6 and vitamin C.

The potatoes that are cultivated worldwide have evolved from varieties that originated in south-central Chile. The main potato species that is grown all over the world for commercial purposes is *Solanum tuberosum*. Genetically, it is a tetraploid with 48 chromosomes, i.e. it has 2 chromosome sets (2n + 2n, autopolyploid).

Potatoes are mostly cross-pollinated by insects, but a substantial amount of self-pollination occurs as well. On the contrary, in farms, potatoes are propagated asexually by planting cut tubers that include at least one or two eyes (sprout). In a large nursery, potatoes are generally grown from seed potatoes, and the tubers are specifically monitored for the occurrence of diseases so that they can consistently provide healthy plants.

During the manufacturing or cooking process, potatoes readily undergo enzymatic browning upon exposure to air after they are peeled; this shortens their shelf life and reduces their marketability. The *StPPO2 gene* encodes polyphenol oxidase (PPO) that oxidizes polyphenol compounds present in cells to produce L-3, 4-dihydroxyphenylalanine (L-DOPA; Matias et al., 2020). Thereafter, the L-DOPA is converted to DOPA quinone that undergoes oxidative polymerization, leading to the formation of a dark brown pigment and the consequent browning of the potatoes.

A new trait of the plant

The main objective of our experiment was to suppress browning of the potatoes (BSP, browning suppressed potato) after cutting or peeling. We were able to obtain Desiree potato cultivars that exhibited much less browning than the control potatoes (non-edited). Furthermore, except for the suppressed browning trait, no other phenotypic differences were observed between the genome-edited and non-edited potatoes.

Mechanism of action: Development of the *StPPO2* knock-out potato (detailed description)

The StPPO2 gene was knocked out using CRISPR/Cas9 technology (Puchta, 2017; Gao, 2018; Chen et al., 2019). An RNP containing an sgRNA along with the Cas9 protein was introduced into potato protoplasts using polyethylene glycol (PEG)-mediated transfection (Liang et al., 2017). Gene editing was performed by knocking out the nucleotides of the *StPPO2* gene sequence using the sgRNA that had been designed to target the complementary DNA sequence of the *StPPO2* gene. We obtained 110 genome-edited potatoes, each of which had some form of *StPPO2* gene modification, such as deletion or insertion of nucleotides in *the StPPO2 sequence*. However, we selected only those potatoes that had undergone small deletions at the target site of *StPPO2*. The modified potatoes exhibited a lower PPO activity and a consequent reduction in the enzymatic browning process than the control potatoes (non-edited).

1. Designing the sgRNA for targeting the *StPPO2* gene

The *StPPO2* gene belongs to *the StPPO* gene family that consists of nine genes. Interestingly, the sequences of eight of these nine genes (*StPPO1 – 8*) have been reported, and they are located in a cluster on chromosome #8. Four of the *StPPO* genes (*StPPO1 – 4*) are involved in the browning of potatoes (González et al., 2020), and the *StPPO2* gene plays a particularly significant role in the process. Therefore, we selected *StPPO2* as our target sequence for developing genome-edited potatoes.

First, we selected four different target sgRNAs (**Table 1**) based upon the *StPPO2* gene sequence using the CRISPR RGEN Tools website (www.rgenome.net; Cho et al., 2013). Primers designed for synthesizing these four sgRNAs as well as performing the deep sequencing (Deep-Seq) analysis are shown in **Table 2.** We performed sgRNA validation using RNP-transfected protoplasts (Woo et al., 2015); **Figure 1** shows the validation results for the four sgRNAs. Transfection using the *StPPO2-4* sgRNA generated a 2.1% in-del frequency with several deletion patterns, whereas transfections using *StPPO2-1, -2,* and *-3* sgRNAs did not generate an in-del frequency. Therefore, we selected the *StPPO2-4* sgRNA sequence to design the final sgRNA that would be used to edit the *StPPO2* gene.

2. No modifications observed in the target sites of *StPPO1, StPPO3,* and *StPPO4* iso-genes

We compared the sequence homology of the *StPPO2-4* sgRNA with the sgRNA 20 sequence in the *StPPO1, StPPO3,* and *StPPO4* genes and found a maximum of 15 bp sequence homology (excluding the NGG sequence) in the *StPPO1* gene (**Figure 2**). Furthermore, with respect to the other two genes, we obtained sequence homologies of 14 bp and 8 bp in *StPPO3* and *StPPO4*, respectively. We also analyzed the whole nucleotide sequences of the four genes, namely *StPPO1, 2, 3,* and *4*, and the nucleotide sequence homology with *StPPO2* was approximately 60% (data not included).

As *the StPPO2* gene belongs to a multi-gene family, a question may arise whether the *StPPO2-4* sgRNA could target a gene sequence at the same site of *StPPO1, StPPO3,* or *StPPO4*. Hence, we isolated DNA from the genome-edited potato lines (#38 and #165), performed PCR amplification of fragments containing the sgRNA target site, and analyzed the sequencing data. We did not observe any genetic modifications in the target sequences of *StPPO1, StPPO3,* and *StPPO4* (**Figure 3**). Therefore, the gene-edits showing the deletions that had occurred in the two potato lines took place only in the *StPPO2* gene but not in the other iso-genes (*StPPO1, 3,* and *4*).

3. Potato protoplast isolation

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4. CRISPR/Cas9 RNP transfection and tissue culture

The number of isolated protoplasts was counted under a microscope using a hemocytometer (NanoEnTek, USA), and there were approximately 100,000 protoplasts. These protoplasts were then selected for CRISPR/Cas9 RNP transfection for each experiment (Malnoy et al., 2016; Andersson et al., 2018).

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5. Deep-Seq analysis of *StPPO2* edited line

We obtained 590 individual potato plants (referred to as potato lines) and analyzed them using *the StPPO2* gene Deep-Seq analysis (Illumina HiSeq 2000). Genomic DNA was extracted from the leaves of regenerated potato plants using a FavorPrep Plant Genomic DNA Extraction Mini Kit (FAVORGEN, Taiwan) according to the manufacturer's instructions. Indels in the *StPPO2* gene were identified using Deep-Seq analysis; the primers used for this process are listed in **Table 2.**

The analysis revealed that 110 of the 590 regenerated potato lines had modified *StPPO2* genes, thereby showing a gene-editing efficiency of approximately 18.64% (data not shown). For the initial screening, we selected two potato lines, #38 and #165, both of which showed a 100% deletion frequency (**Figure 11**). Any given gene in a tetraploid potato is represented by four different alleles per locus per genotype. *The StPPO2* gene in the genome of line #38 showed a two bp deletion in the target site of the four alleles (-2/-2/-2/-2), while the genome of line of #165 showed a four bp deletion in the target site of the four alleles (-4/-4/-4/-4). Apart from this, we obtained many different deletion profiles or patterns in the 110 genome-edited potato lines (data not shown).

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7. Measurement of PPO enzyme activity among genome-edited (GE) potato lines

Regenerated potato seedlings were planted and cultivated in pots for 12 weeks. The tubers of the GE lines (#38 and #165) as well as the wild-type (WT) plants were harvested (**Figure 15A**) and sampled for PPO enzyme activity measurement. The PPO activity was measured based on the protocol described by Chi et al. (2014). The two GE lines (#38 and #165) exhibited a significantly lower PPO activity than the WT potato plants—the PPO activities of the potato lines #38 and #165 were reduced by 67.3% and 93.4%, respectively **(Figure 15B)**. Based on the data from other GE lines (not shown), we observed an apparent tendency of the genome-edited potatoes to exhibit a reduced PPO activity to a certain extent. Therefore, deleting nucleotides of the *StPPO2* gene using CRISPR/Cas9 technology has successfully manipulated the PPO activity in the potatoes. [CBI-deleted

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8. Determination of enzymatic browning in GE lines

To evaluate the level of potato browning, we selected tubers of a similar size and peeled off their skins. The browning levels were monitored at 0, 8, and 24 h after exposure to air. The relative browning levels at the tuber surface were significantly lower in the GE lines, #38 and #165, than in the WT tubers (**Figure 16).** This observation confirmed that the browning of potatoes was suppressed to a certain extent in the GE lines as compared to that in the WT

potatoes. The lower browning levels were maintained up to 72 h after exposure to air (data not shown), after which, the skin was completely dried out, and the test was concluded.

No difference between control potatoes and genome-edited potatoes other than browning suppression

1. Phenotypic traits

In Korea, potatoes are mainly grown in fields, and a disease-free seed system efficiently manages the growth of healthy seed potatoes in the nursery. Since the Korean government does not have a regulatory system for genome-edited crops to date, we could not conduct a large-scale analysis to detect the phenotypic changes in the BSP lines in the field. However, we cultivated the genome-edited lines (#38 and #165) and non-edited potato (WT) in the potato nursery farm, which has a vinyl plastic house (GMO isolated) and obtained a satisfactory yield. The potatoes were phenotypically identical, and we did not detect any distinct phenotypic differences, including the structural morphologies of leaf and stem, between them (**Figure 17A**). In addition, all the tubers harvested from the 4-month-old potatoes grown in the nursery were similar (**Figure 17B**).

2. No possibility of pest genome insertion and no damage from pests

The BSP lines described here were constructed by RNP transfection into protoplasts, followed by tissue culture and regeneration. After that, we selected genome-edited potatoes based upon confirmation by NGS analysis. We did not use *the Agrobacterium*-mediated transformation method for genome editing; instead, sgRNA and Cas9 protein (RNP) were used for protoplast transfection. As foreign DNA was not introduced during genome editing and genetic transfection, the BSP lines did not have an increased risk for plant pest or pathogen infection during tissue culture and regeneration.

In the potato nursery, we cultivated the genome-edited and non-edited (control) potatoes simultaneously for three generations (third seed-potato harvesting by vegetative reproduction). There was no specific pest damage in the genome-edited potatoes as compared to the control potatoes. Therefore, we did not find any evidence that pests may cause more damage to the genome-edited potatoes than the non-edited (control) ones. Previous studies have reported the browning suppression of potatoes by knocking out the same gene, *PPO* (Chi et al., 2014; González et al., 2020). Furthermore, those manuscripts did not report any other differences in traits or pest-related symptoms during the growth period.

Globally, potatoes have been adapted and managed to plant pest risk in farms because of their long growing history. In addition, GM potatoes have already been marketed following the appropriate safety risk assessments. Therefore, the potential risks of pest attack in potatoes have been well recognized and controlled.

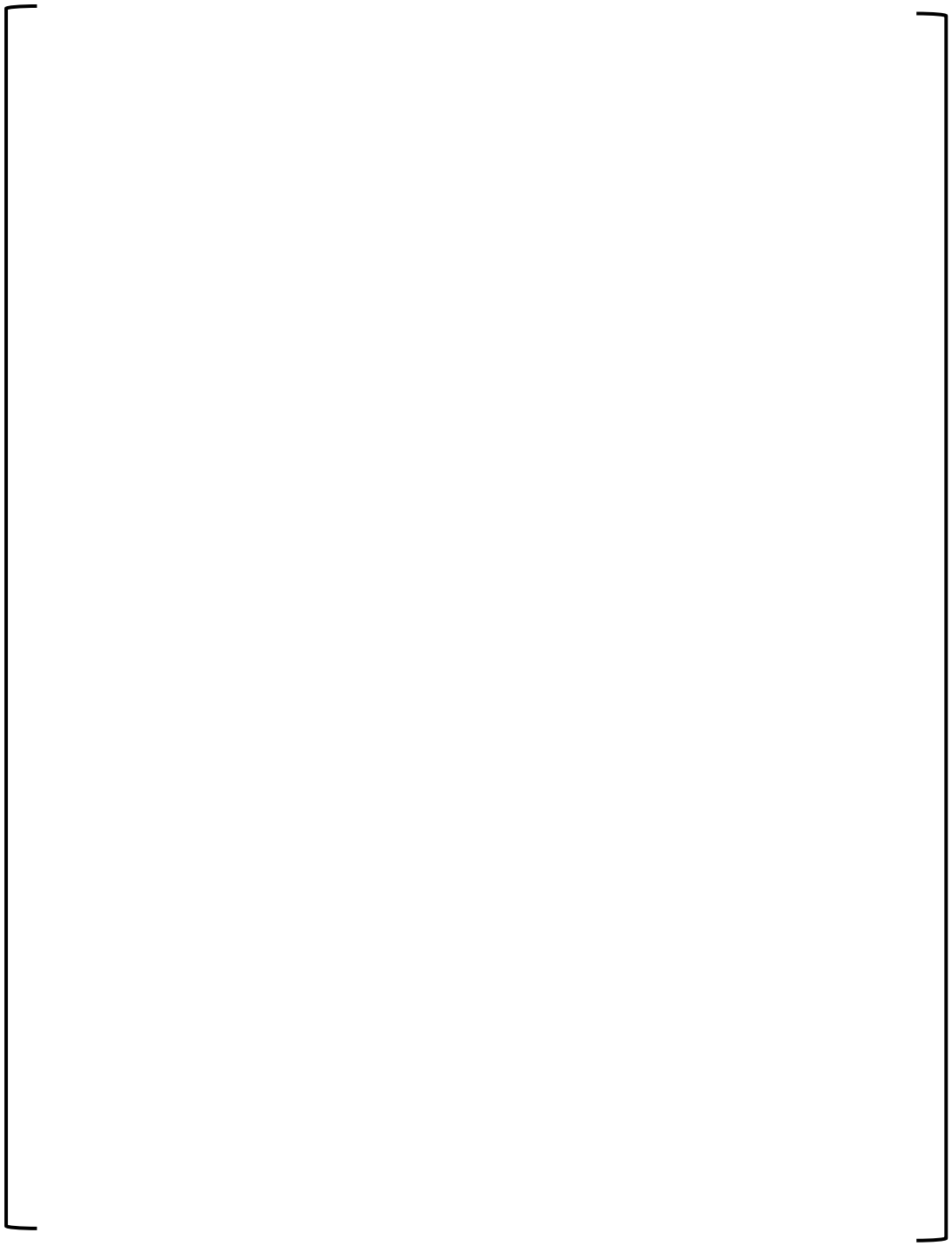
Conclusion

We used the CRISPR/Cas9 RNP system to induce mutagenesis in the *StPPO2* gene in a tetraploid potato cultivar (Desiree). By transfecting *StPPO2-4* sgRNA along with Cas9 protein into potato protoplasts, we obtained genome-edited (GE) potato lines. We selected two genome-edited potato lines (#38 and #165). The tubers of GE lines showed lower PPO activity than the non-edited Desiree cultivar (control or WT), with a consequent reduction in enzymatic browning. Hence, this RNP transfection method provided a feasible means for creating novel potato lines.

Interestingly, potato lines #38 and #165 have a two bp deletion and a four bp deletion in the target gene sequence, respectively. Still, they are phenotypically identical to the non-edited (control) potatoes, except for the browning suppression trait. The GE potato lines have been

cultivated in potato nursery farms for the last two years, and there has been no evidence of increased risk of plant pest attack in the GE lines compared to that in the control potatoes.

Figure 1. Validation of transfection of potato protoplasts using four different *StPPO2* sgRNAs: (A) *StPPO2-1*, (B) *StPPO2-2*, (C) *StPPO2-3*, and (D) *StPPO2-4*. *StPPO2-4* was chosen for gene editing based on the observed indel frequency (2.1%). sgRNA, single guide RNA; indel, insertion/deletion. Sample, edited protoplasts; control, non-edited protoplasts



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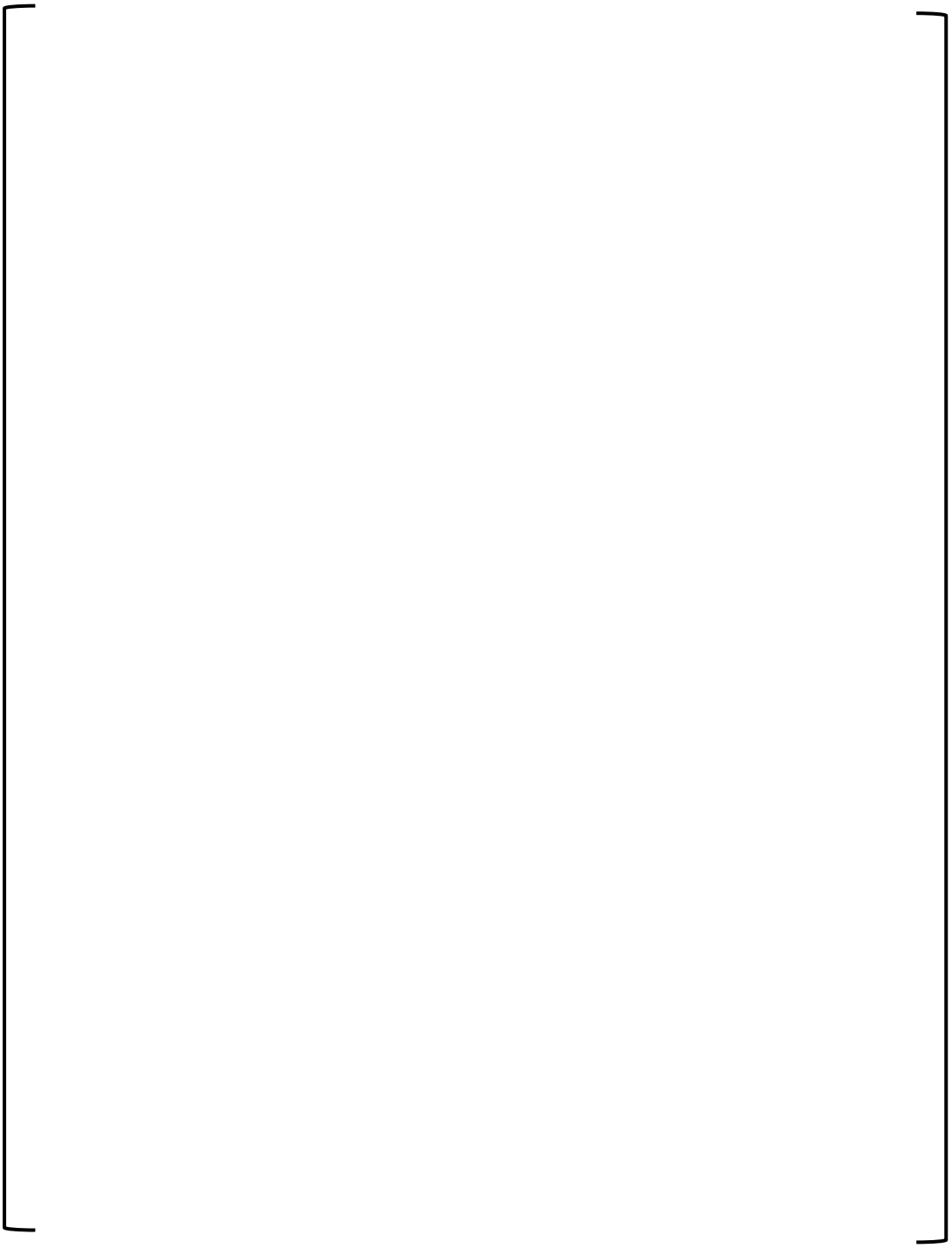

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Figure 2. Selection of StPPO2-4 sgRNA of *StPPO2* gene. Homology search of sgRNA sequence of *StPPO* genes involved in potato browning.

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Figure 3. Comparison of target sequence homology among *StPPO* genes (1, 2, 3, and 4) in potatoes. WT: control Desiree; #38 and #165 are genome-edited (GE) potatoes.

Figure 4. Isolation of protoplasts from the leaves of potato seedlings. (A) VCP treatment of the leaves, (B) Layer of intact protoplasts in the middle region after sucrose gradient separation (red arrow area), (C) Protoplasts observed under a microscope at 40x using a hemocytometer. VCP, Viscozyme, Celluclast, and PectinEX

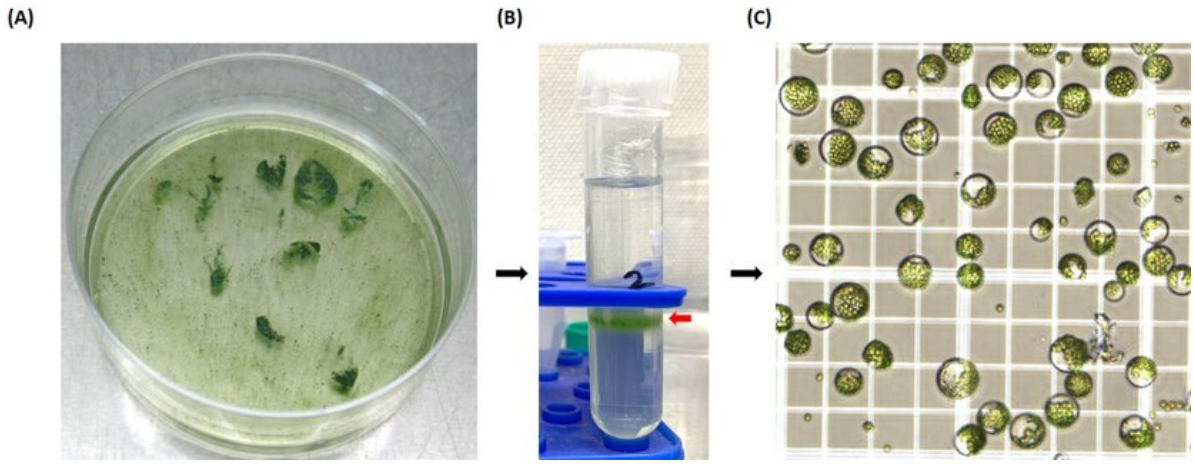


Figure 5. Potato protoplast culture after protoplast transfection with CRISPR/Cas9 RNP. Cell division was observed at (A) 1 week and (B) 2 weeks after transfection. CRISPR, clustered regularly interspaced short palindromic repeats; RNP, ribonucleoprotein.

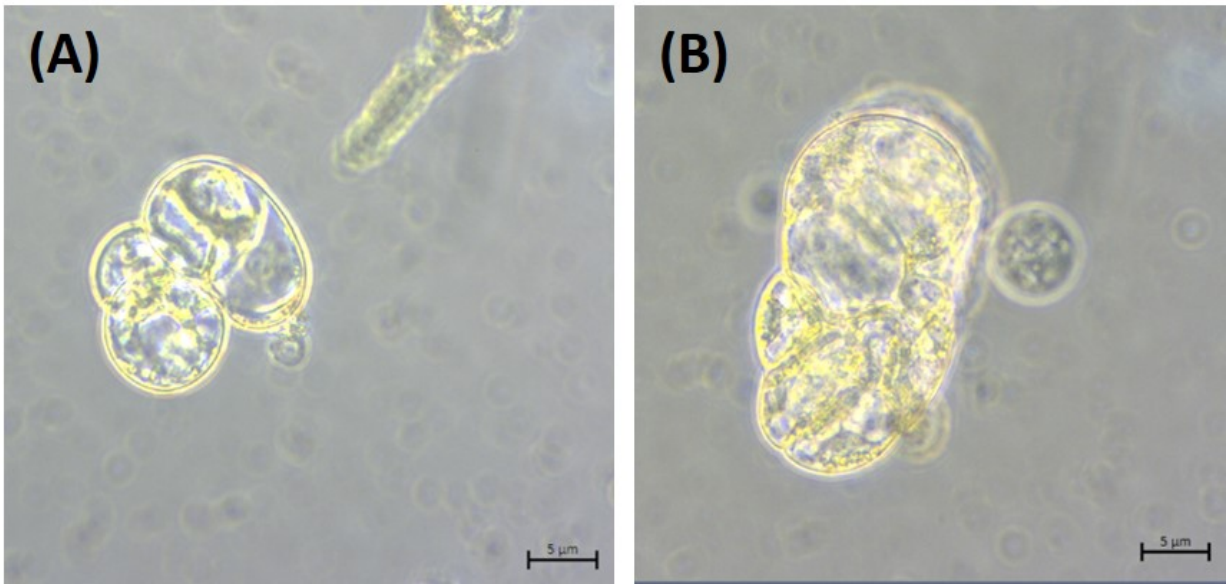
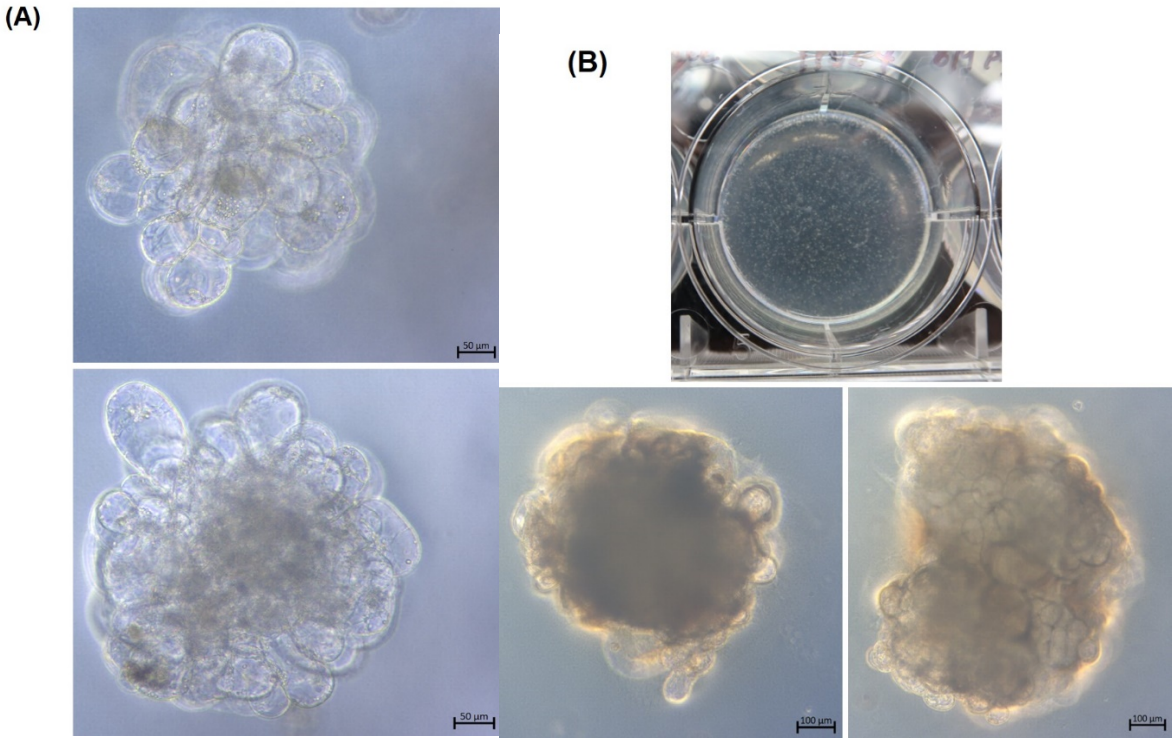


Figure 6. Formation of microcalli and callus. (A) Cell division observed in PIM at 3 weeks after transfection. (B) Early stage of microcalli formation in CFM at 4 weeks after transfection. (C) Callus development in CFM at 5 weeks after transfection. PIM, protoplast inducing media; CFM, callus formation media.



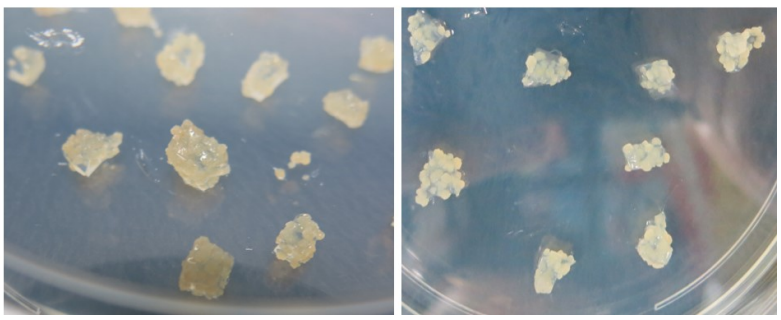


Figure 7. Calli cultured in SIM after being transferred from CFM. Calli growth after transfection at (A) 6 weeks and (B) 7 weeks, and (C) magnification at 9 weeks. SIM, shoot inducing media; CFM, callus formation media.

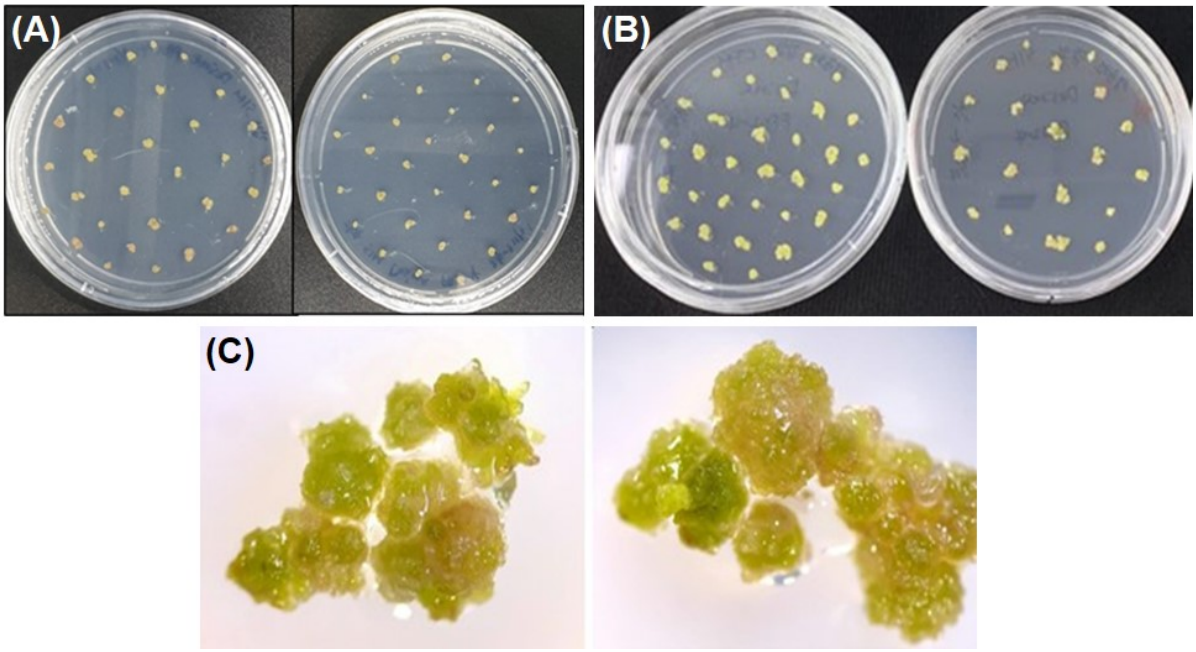


Figure 8. Shoot formation observed in the callus cultured in shoot induction media at (A) 11 weeks and (B) 12 weeks after transfection.

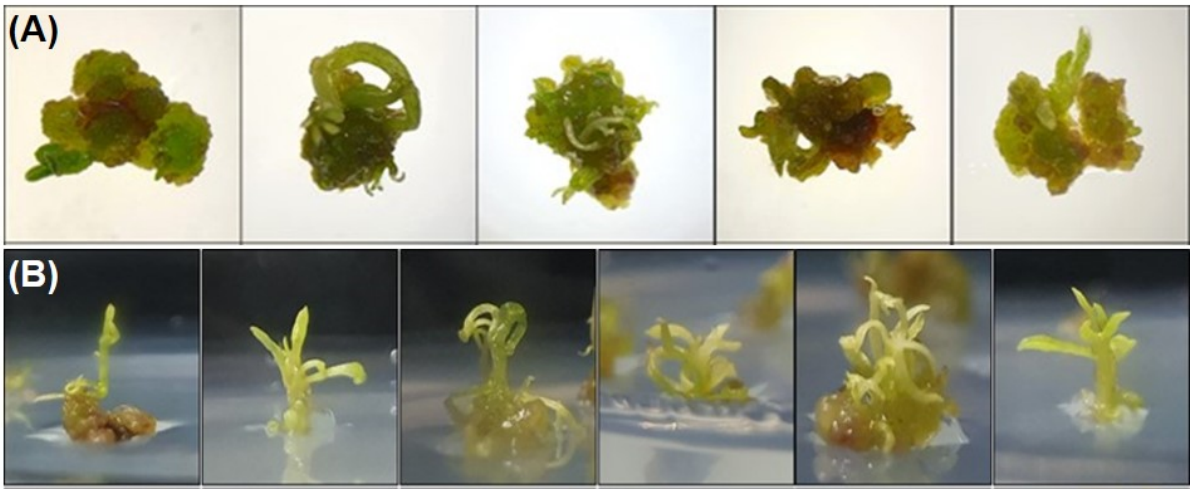


Figure 9. Shoots of potato plants showing root formation in root induction media after 14 weeks.

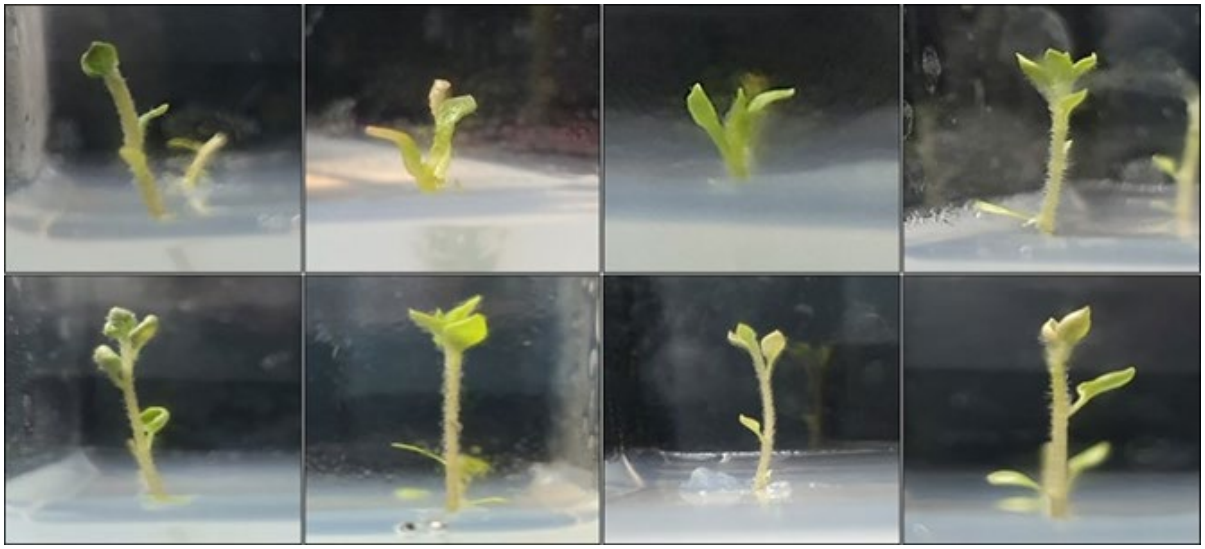


Figure 10. Regenerated potato seedlings cultured in vitro for 16 weeks after protoplast transfection

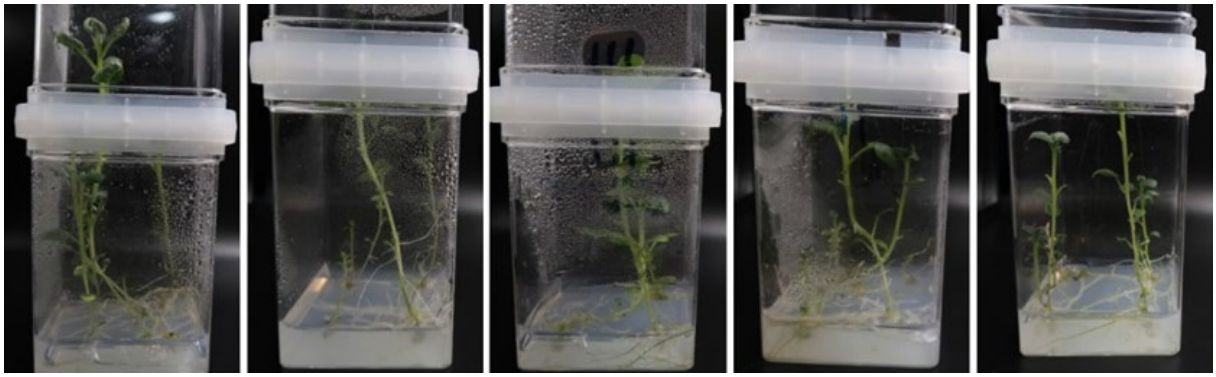


Figure 11. Deep sequencing analysis of *StPPO2-4* edited lines. Each line showed different deletion pattern with nearly identical reads. Line #38: 2 bp deletion pattern in four alleles; #165: 4-bp deletion pattern in four alleles. sgRNA, single guide RNA; WT, wild type; in-del, insertion/deletion. [ ] CBI-deleted

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Figure 12. PCR primers designed for reading DNA sequences of *StPPO2* gene.

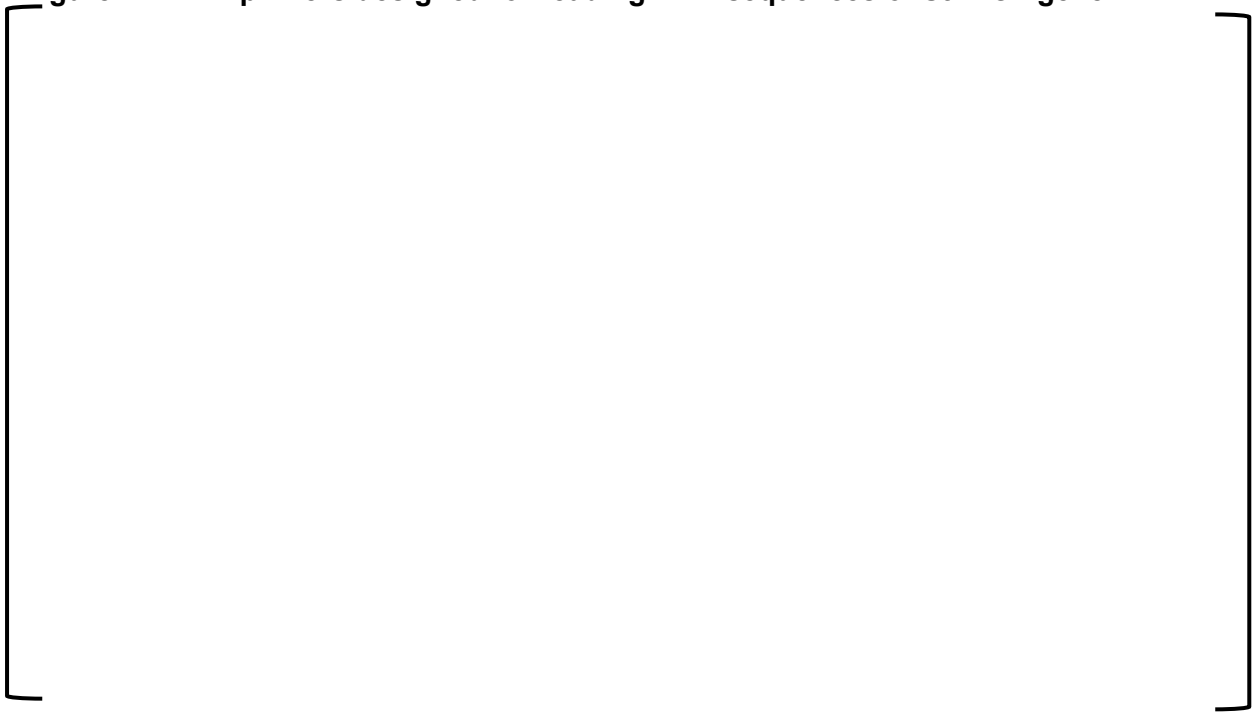

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Figure 13. DNA sequences of *StPPO2* gene from Desiree cultivar (WT1 to 4) and the edited lines, #38 and #165. Red letters are target sequences of Desiree cultivar, and yellow letters are target sequences of edited lines, #38 and #165.

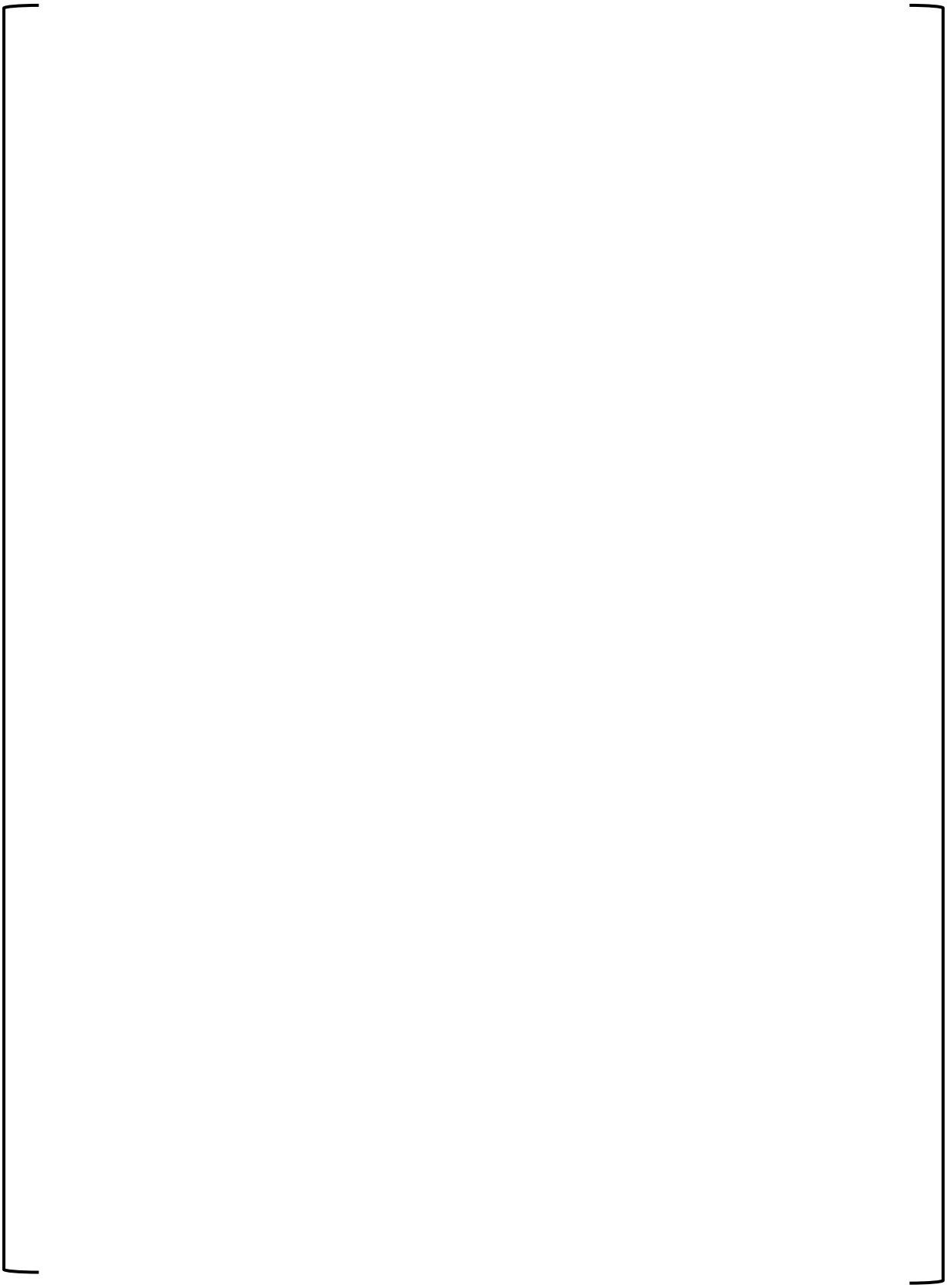

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Figure 14. DNA sequence comparison of *StPPO2* gene from four DNA samples of Desiree cultivar and edited lines, #38 and #165. Line #38 shows 2 bp deletion and #165 line shows 4 bp deletion on the target site.

Figure 15. Genome-edited potato tubers harvested from pot. A: 3-month-old GE (genome-edited) potatoes. B: PPO activity of GE potatoes. PPO, polyphenol oxidase; WT, wild type

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Figure 16. Comparative analysis of browning levels at different time points. Potato tubers peeled completely were exposed to air at room temperature (25°C), and the browning change was monitored for 24 h. WT, wild type; GE, genome-edited.

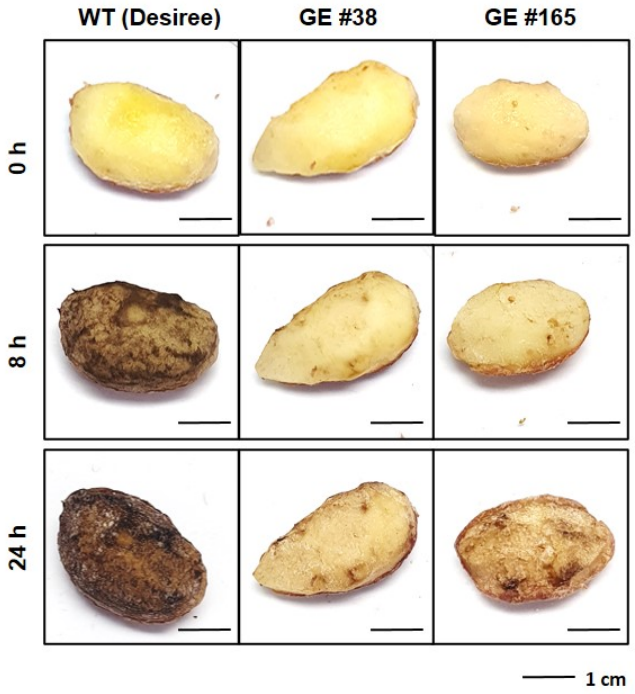

Figure 17. No distinct difference in phenotypes. Matured leaf and stem among three cultivars (WT, GE #38, GE #165) are identical (A). Tubers harvested from 4 months old potatoes grown in the nursery are alike (B). WT, wild type; GE, genome-edited.

A.

WT (Desiree) **GE #38** **GE #165**

B.

Table 1. Target sequences of *StPPO2* gene

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Table 2. Primers used in synthesis of single guide RNAs and deep sequencing analysis

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Table 3. CPW and VCP solution used to isolate potato protoplasts

CPW (Cell and Protoplast Washing) solution			
	Components	**Stock name**	**1 X CPW concentration (Final Conc.)**
CPW salts	**$CaCl_2$-$2H_2O$**	**Stock B**	**1480 mg/L**
	KH_2PO_4	**Stock A**	**27.2 mg/L**
	KNO_3		**101 mg/L**
	$MgSO_4$-$7H_2O$		**246 mg/L**
	KI		**0.16 mg/L**
	$CuSO_4$-$5H_2O$		**0.025 mg/L**
			pH 5.7
Stock A solution was prepared at a 100× concentration, and Stock B solution was prepared at a 10× concentration.			
VCP (Viscozyme, Celluclast, and PectinEX) solution			
component	**content (to make 0.5 mL)**		
CPW stock A (X 100)	**5 mL**		
CPW stock B (X 10)	**50 mL**		
Mannitol	**45 g**		
MES buffer	**533 mg**		
Viscozyme	**5 mL**		
Celluclast	**2.5 mL**		
PectinEX	**2.5 mL**		
	pH 5.7		
The prepared solution was filtered via sterilization by using a filter containing a 0.22 μm pore size, and then stored in a refrigerator.			

Table 4. W5 solution used to isolate protoplasts from potato plants

W5 solution (1 liter, pH adjusted to 5.7 with KOH)		
2 mM MES (pH 5.7)	**0.3904 g**	**Filter sterilized and stored at 4℃**
154 mM NaCl	**8.998 g**	
125 mM $CaCl_2$	**13.8726 g**	
5 mM KCl	**0.3728 g**	

Table 5. Polyethylene glycol solution used for the transfection protocol

Preparation method of PEG transfection solution	
40% PEG Solution	**To make 2.5 mL**
PEG 4000	**1 g**
1M Mannitol	**0.5 mL**
1M $CaCl_2$	**0.25 mL**
ddH_20	**Add 1 mL to be 2.5 mL**

Table 6. Culture media used for plant growth

PIM	1 L (pH 5.7)	CFM	1 L (pH 5.7)
1/2 B5 medium	1.58 g	MS salts	
Sucrose	103 g	Mannitol	4%
2,4-D	0.2 mg	sucrose	2.5g/L
BAP	0.3 mg	B5 vitamin	
MES	0.1 g	zeatin	3mg/L
CaCl2·2H2O	375 mg	IAA	2 mg/L
NaFe-EDTA	18.35 mg	GA3	1 mg/L
Sodium succinate	270 mg		
SIM	**1 L (pH 5.7)**	**RIM**	**1 L (pH 5.7)**
MS with vitamin	4.4 g	MS with vitamin	4.4 g
sucrose	10g/L	sucrose	20g/L
zeatin	2mg/L	GA3	0.1 mg/L
NAA	0.1 mg/L		
GA3	0.1 mg/L		

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CBI Justification

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By kldiggs for BRS Document Control Officer at 2:51 pm, Sep 02, 2021

September 2, 2021

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

Dear Ms. Bernadette Juarez:

ToolGen, Inc. has developed a modified potato cultivar by editing *StPPO2* gene using the CRISPR/Cas9 RNP technology. Recently, we have filed a patent with research data of this cultivar to protect intellectual property. Please be aware that some of the information disclosed in the RSR request files attached is confidential and should not be shared. Thus, we want to claim the specific information as CBI.

Sincerely yours,

Chee Hark Harn, Ph. D.
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