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Increasing of Amylopectin in Tetraploid Potato Desiree Cultivar *(Solanum tuberosum* L.*) Via* CRISPR/Cas9

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Abstract: Potato tuber starch characteristic is influenced by the ratio of amylose to amylopectin, which is affected by the granule-bound starch synthase (GBSS) gene. GBSS gene expression was decreased in the leaves of the Desiree potato variety utilizing genome editing using CRISPR-Cas9. Constructs encoding Cas9 gene and sgRNAs targeting GBSS gene were inserted into plant leaves using *Agrobacterium*-mediated transformation delivery. The results obtained lines with mutations in GBSS genes accounting for 21% of regenerated shoots. The identification of mutations within one base pair of the used guide sequence provided further evidence of the considerable similarity between the target region around the protospacer adjacent motif (PAM) position and the used guide sequence. Transforming DNA into potato leaves produced mutants that lacked the Cas9 gene. Using microscopic inspection of iodine-stained starch granules, the increase of amylopectin in the starch granules of editing potato tubers was evaluated.

1 Introduction

The *Solanaceae* family includes tobacco, tomato, eggplant, and pepper. The potato subgenus of the genus *Solanum* contains over 200 species of tuber-linked potatoes (Sevestre et al 2020). Potato is the third-most important crop in terms of food produced for human use. The total production of potatoes worldwide in 2018 was 368.17 million tons (Tiwari et al 2022). Over the past 30 years, potato output has rapidly increased and it is currently one of the top three crops grown for staple foods worldwide. Potato is one of the few crops that is used for starch production, a renewable bulk material in a variety of culinary and non-food applications. One of our most significant starch crops and a common staple diet across the world is the potato (Reddy et al 2018). Plants combine amylose (AM) and amylopectin (AP) to store starch, whose ratio affects the characteristics of the starch. In general, the ratio of AM to AP and their structural variability depends on the botanical origin (Schirmer et al 2013). Rice mutants with a waxy phenotype lack the granule-bound starch synthase (GBSS) gene (Itoh et al 2003). Other plant species, like sorghum, barley, and maize (Liu et al 2007, Morell et al

A preliminary version of the article was deposited as a preprint in Research Square https://www.researchsquare.com/article/rs-2563820/latest

2003), have also been identified to harbor similar mutations (Kawahigashi et al 2013).

Producing new tetraploid cultivars by traditional breeding techniques requires time and effort. On the other hand, crop improvement through insertion/deletion mutagenesis using genome editing is a cutting-edge genomics technology (Hameed et al 2018). Double-stranded breaks (DSBs) are allowed at specific genomic loci, and they are repaired utilizing naturally occurring DNA repair mechanisms, such as nonhomologous end joining (NHEJ) or homologous recombination (HR). Previously, this system could be possible by protein-guided nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). However, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), CRISPR associated (Cas), a new RNA-guided nuclease, has recently attracted attention (Nadakuduti et al 2018).

The biological technology CRISPR/Cas, which makes it straightforward to design and generate gene-specific single guide RNA (sgRNA), is only effective for targeted genome editing. The sgRNA vectors direct *Streptococcus pyogenes* Cas9 (SpCas9) to construct the endogenous DSB repairs, which take place through the error-prone NHEJ or HR pathways. The main objective of this study is to increase the proportion of amylopectin starch in potato micro tubers by inhibiting the function of GBSS gene using the CRISPR/Cas9 technique.

2 Materials and Methods

2.1 Plant materials and expression vectors

Desiree cultivar (*Solanum tuberosum* L.) potatoes (2n=4x=48) were provided by virus-free germplasm bank, Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza-Egypt. All of the tests in this study used explants of leaves.

pChimera cloning vector was produced by Gene Art (Life Technologies Inc., Carlsbad, CA, USA), which contains the sgRNA expression system surrounded by *Avr* II restriction sites **(Fig 1)**.

The binary expression vector is a pCAS9-TPC vector containing the Cas9 expression cassette for conventional cloning used for *Agrobacterium*-mediated transformation **(Fig 2)**.

Both the *Escherichia coli* DH10β strain and the *Agrobacterium tumefaciens* LBA4404 strain bacteria were obtained from the Gene Silencing and Insect (GSIC) laboratory at AGERI.

2.2 Bioinformatics analysis

The fasta format sequence of exon 9 on the granule-bound starch synthase (GBSS) gene was identified in potato plants and a suitable sequence on the NCBI website was found (Andersson et al 2017). Afterward, the CRISPR-P web tool (https://github.com/hao-liu1213/CRISPR-P-2.0) was applied to design an appropriate sgRNA sequence for the GBSS gene. The designed sequences of detection primers for cloning and transformation are shown in **Table 1**.

2.3 Gene cloning

The forward (Fn1) and reverse (Rn1) oligonucleotide primers for sgRNA were combined and annealed, then cloned into the pChimera vector. After that digested with the *Bbs*I restriction enzyme as shown in **Fig 1**. A 1 µg of the oligonucleotides was mixed with a 2 µl of the restriction enzyme buffer; a 1 µl of *Bbs* I, and ddH_2O were added up to 20 µl then incubated at 37 °C for 2 hours. Once the sgRNA vector was digested, a 2 µl was mixed with a 3 µl of annealed oligos, a 1 µl of T4 ligase buffer, a 3 µl of ddH2O, and a 1 µl of T4 ligase. The mixture was then incubated for 1 h at room temperature and then for 4°C overnight (Chauvin et al 2021).

After performing a heat shock procedure to transform the DNA vector into *E. coli* (DH 10 β), the cells were grown on LB media that contained ampicillin (100 mg/l). The positive colonies were detected by PCR analysis using M13 reverse and forward protospacer oligos (sgRNA primer).

Cloning in pCAS9-TPC vector that was digested and miniprepped for the positive colony by *Avr* II reaction enzyme required 1-2 µg of plasmid DNA, a 2 µl of restriction enzyme buffer, a 1 µl of *Avr* II, and a 1 µl of ddH2O to be added to a total volume of 20 µl. This mixture was then incubated for at least 2 hours at 37°C before spending the night at 4°C **(Fig 2)**.

Purified digested pCAS9-TPC construct was directly prepared and eluted in a 30µl of ddH_2O. pCAS9-TPC vector was dephosphorylated by mixing a 3.5µl of phosphatase buffer and a 1.5µl of alkaline phosphatase. Heating was done to inactivate the enzyme in the mixture for 20 minutes at 37°C and ligation was prepared by mixing a 5µl of sgRNA insert with a 5µl of dephosphorylated vector, a 2µl of T4 ligase buffer, a 7µl of ddH_2O and a 1µl of T4 ligase, and incubation for at least 3 hours. A 10 µl of the reaction was converted into *E. coli* (DH10 β) and then plated on LB media with 100 mg/l of spectinomycin. The SS42 forward and SS43 reverse primers at an annealing temperature

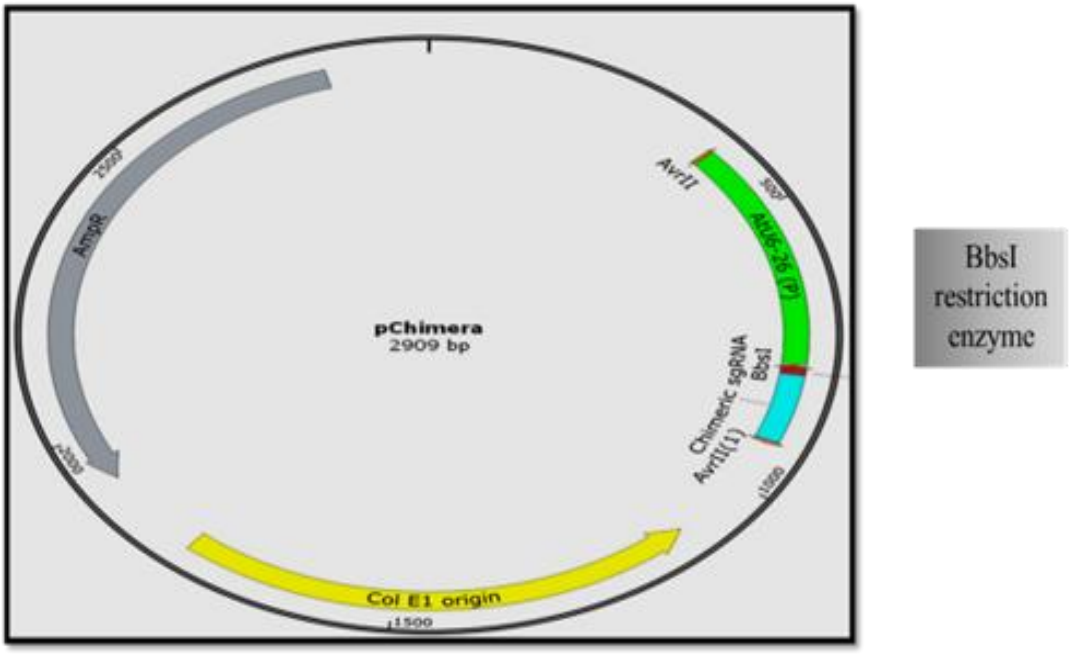

Fig 1. Schematic diagram of the pChimera cloning vector used for classical cloning

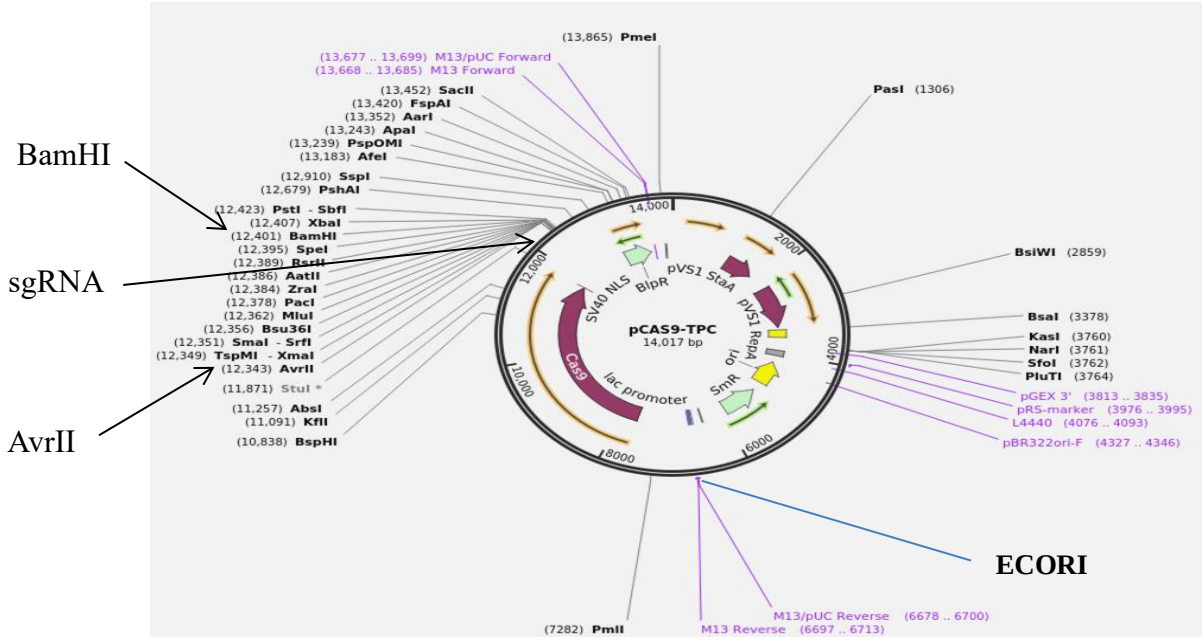

Fig 2. A pCAS9-TPC binary expression vector and restriction sites that were used for *Agrobacterium*-mediated transformation

Table 1. Synthetic primers designed for GBSS gene cloning. M13: a reverse primer of pChimera vector, SS42 forward & SS43 reverse: detected primers of pCAS9-TPC vector and Fn1 forward & Rn2 reverse: primers of oligonucleotides sequence (sg RNA)

Primers	Nucleotide Sequences 5′ → 3′	Expected product (bp)
M13 Reverse	**5′**CACAGGAAACAGCTATGAC**3′**	270
SS42 Forward	**5′**TCCCAGGATTAGAATGATTAGG**3′**	1000
SS43 Reverse	**5′**CGACTAAGGGTTTCTTATATGC**3′**	1000
Fn1 Forward	**5′**ATTGGACAAGAAGATCCCTTTGAT**3′**	20 bp
Rn1 Reverse	**5′**AAACATCAAAGGGATCTTCTTGTC**3′**	20 bp

of 60°C were used to check the colonies using colony-PCR. pCAS9-TPC vector which obtained sgRNA sequence was transformed into *Agrobacterium* cells and the transformed colonies were detected using ss42 forward and ss43 reverse primers.

2.3 Transformation in Desiree cultivar by *Agrobacterium tumefaciens*

Using the nodal cutting technique, the Desiree cultivar was *in vitro* micropropagated as described by Roca et al (1978). Every three to four weeks, nodal cuttings were routinely subcultured on a new media where 3% sucrose was added to the MS salts medium (Murashige and Skoog 1962) and the pH was raised to 5.6.

In a 50 ml LB liquid medium with 50 µl of spectinomycin and 50 µl of streptomycin, a single colony of *Agrobacterium tumefaciens* with pCAS9-TPC plasmid was produced. The *Agrobacterium* cultures were cultivated until $O.D_{600}$ reached 0.8 at 28°C under 200 rpm (Stiekema et al 1988).

Harvested *in vitro* leaves from plantlets were incubated for 5 mins with gentle shaking overnight in a medium containing 30 ml of an *A. tumefaciens* culture. The extra bacteria were then removed using sterile filter paper. The leaves were then spread out over the solid callus induction medium {MS base salt + a 50 g/l of sucrose + a 5 ml/L of 2,4-Dichlorophenoxyacetic acid (1 mg/ml) and a 1 ml/L of cefotaxime (200 mg/l)} for 6 days at 20 ± 2°C in the dark. They were then put into a regeneration medium that contained a 1 ml/L of cefotaxime (200 mg/l), a 50 g/L of sucrose, a 1 mg/L of BA, a 1 mg/L of IAA, a 10 mg/L of GA_3 and a 1 ml/L of MS basal salt combination. Plantlets were subcultured once more after three weeks at 20 ± 2°C. The antibiotics were added to the autoclaved medium under sterile circumstances.

2.4 Genomic DNA Isolation

Samples were collected from 30-40 days old of editing potato Desiree cultivar plants and their control (non-edited plants) from *in vitro* cultures. DNA isolation of samples was conducted using QIAGEN (cat. Nos. 69104 and 69106). Plasmids mini-prep for sgRNA vector and pCAS9-TPC plasmid were extracted from bacterial cells using the QIA prep® Spin Miniprep kit (50) (QIAGEN, Germany). The QIAquick® PCR Purification Kit (QIAGEN Cat. No. 28104) was obtained for PCR purification in accordance with the manufacturer's instructions.

2.5 PCR (polymerase chain reaction)

To confirm the presence or absence of the *bar* gene, the polymerase chain reaction (PCR) was carried out. For both modified and unedited plants, the DNA amplification was achieved a 1 µl of DNA template, a 1 µl of *bar* primers, a 0.5 µl of dNTPs, a 1 µl of MgCl2, a 5 µl of 1X buffer, and a 0.25 µl of Taq polymerase enzyme (Promega, Madison, USA) with a total volume 25 µl. For molecular analysis and bioinformatic analysis, identical reaction conditions with nd1 forward and nd2 reverse primers were employed. Amplification was scheduled at 94°C for 3 min, at 94°C for 30 sec, at 58°C for 40 sec, at 72°C for 1 min during 35 cycles, and a final cycle at 72°C for 7 min as shown in **Table 2**.

2.6 Nucleotide sequencing analysis

Complete nucleotide sequence of the edited and non-edited plants was carried out using the automated DNA sequencer using the private services of Macrogen, Inc. (South Korea).

Table 2. The primer sequence for detection.nd1 forward & nd2 reverse: primers of partial sequence of GBSS gene containing sgRNA sequence with pam site, *bar* forward & reverse: detecting primers (primers of selectable markers) that in pCAS9-TPC vector

Primers	Nucleotide Sequences	Expected product (bp)
nd1 Forward	**5′**AGAATCACATAGGGTGGTTACAG**3′**	590
nd2 Reverse	**5′**GCATAGGATGAGTAGCAGGTC**3′**	590
bar Forward	**5′**TACATCGAGACAAGCACGGT**3′**	400
bar Reverse	**5′**ACGTCATGCCAGTTCCCGTG**3′**	400

2.7 Staining Potato micro tubers with iodine solution

Each individual event's microtubers were examined using the histochemical Lugol-Iodine staining method, which utilized 10% potassium iodide (KI) and 5% iodine (I) in distilled water. The expected knockout, reduced amylose phenotype was visible as a red-brown colored starch staining instead of the usual blue color obtained from wild-type tubers that also contained amylose starch (Andersson et al 2003).

3 Results and Discussions

3.1 Gene cloning

A method, recently developed, called genome editing allows for the change of a particular gene using an artificial nuclease that recognizes certain sequences (Osakabe and Osakabe 2015).

The two primers Fn1 of the sgRNA and M13 reverse primers of the pChimera vector were used in colony PCR analysis. The results showed three positive colonies (3,4 and 5), while the negative control lane did not display any fragment marker at 270 bp size **(Fig 3).**

*Avr*II restriction enzyme was used to digest the sgRNA vector (pChimera) and pCAS9-TPC vector; the two vectors were then ligated together with T4 DNA ligase and the effective cloning of the pCAS9-TPC vector was demonstrated. The *E. coli* (DH10 β) competent cells were afterward exposed to the ligation reaction. To look for positive (transformed) colonies, a colony PCR analysis was performed. The used primers were SS42 forward and SS43 reverse and the expected fragment marker was at 1000 bp **(Fig 4)**.

pCAS9-TPC vector (lane no. 2-9) carrying sgRNA sequence was successfully transformed into *Agrobacterium* cells, and the transformed colonies were identified using the ss42 forward and ss43 reverse primers as shown in **Fig 5.**

3.2 Transformation into potato plants

The potato Desiree cultivar was transformed using the *Agrobacterium*-mediated transformation technique using leaves as an explant. As nodal cutting procedures, leaves from *in vitro* micropropagation were used. After the transformation procedures described above, leaves were chosen and incubated on callus medium (MS basal salt with 5 mg of 2,4-dichlorophenoxyacetic acid/L concentration) in the dark for 6 days at 20 ± 2°C. This was done by LBA4404 strain of *Agrobacterium tumefaciens*, which included the pCAS9-TPC vector to induce callus. On the regeneration medium, Calli (100%) generated from leaf explants were positioned. Through callus induction, leaves generated shoots (75% of them) after 12 weeks **(Fig 6)**. Regeneration and elongation processes are demonstrated as shown in **Fig 7**.

Using *Agrobacterium*-mediated transformation in tetra-allelic mutations with reduced amylose synthesis was performed. Tang et al (2018) demonstrated that the ongoing utilization of CRISPR-Cas9 technologies in rice doesn't result in off-target mutations provided sgRNAs are rigorously generated. The gene-editing technique may speed up the development of a suitable mutant with a particular characteristic, while traditional breeding processes take much longer to create a strain with the target phenotype (Xiong et al 2015). According to Ménoret et al (2013), CRISPR/Cas9 is an effective system for producing a mutation in a specific location with a rise in editing frequency.

3.3 PCR reaction for edited plants

Extracted DNA from edited shoots of Desiree cultivar were analyzed by PCR using two sets of primers for *bar,* nd1 and nd2 genes. The *bar* gene amplified fragment segment was at 400 bp and seen in the amplified DNA of plants obtained from *Agrobacterium* transformation compared to the control as presented in **Fig 8**. For more detection and bioinformatics analysis, the positive and negative edited shoots were analyzed using nd1 and nd2 primers, and the results showed that the DNA amplified fragment was at 590 bp as shown in **Fig 9**.

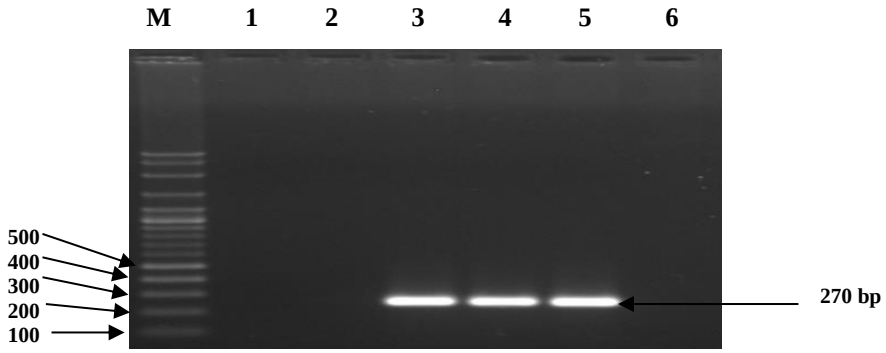

Fig 3. Screening of the converted colonies after pChimera transformation in *E.coli*
Lane M: 100 bp ladder
Lane 1: Negative control
Lane 2 and 6: Negative coloni
Lane 3, 4 and 5: Positive colonies

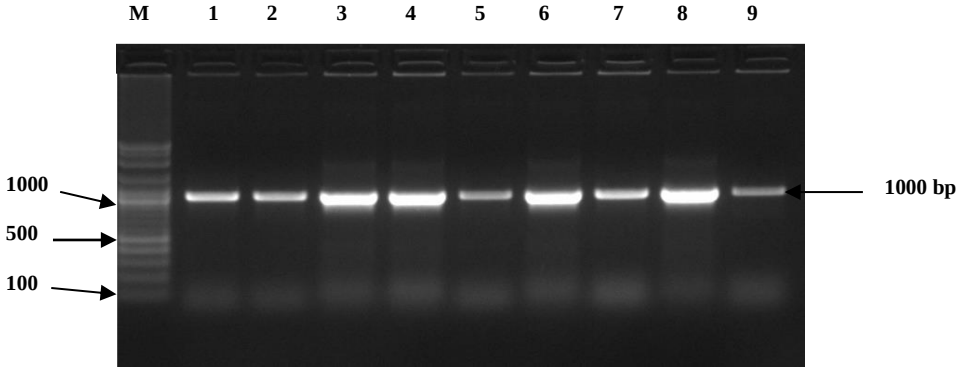

Fig 4. Verification of transformed colonies in *E. coli* following pCAS9-TPC transformation
Lane M: 100 bp ladder
Lane 1: Positive control
Lane 2 to 9: Positive colonies

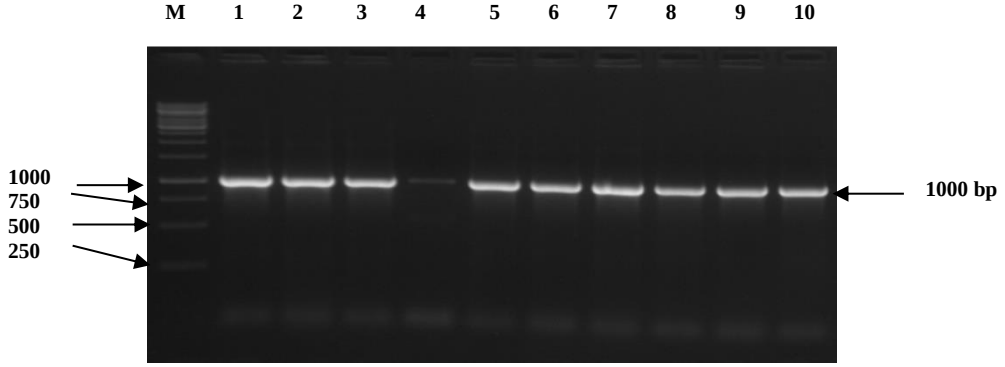

Fig 5. Verification of transformed colonies in *Agrobacterium* following pCAS9-TPC transformation
Lane M: 1 kp ladder
Lane 1: Positive control
Lane 2, 3 and from 5 to 10: Positive colonies

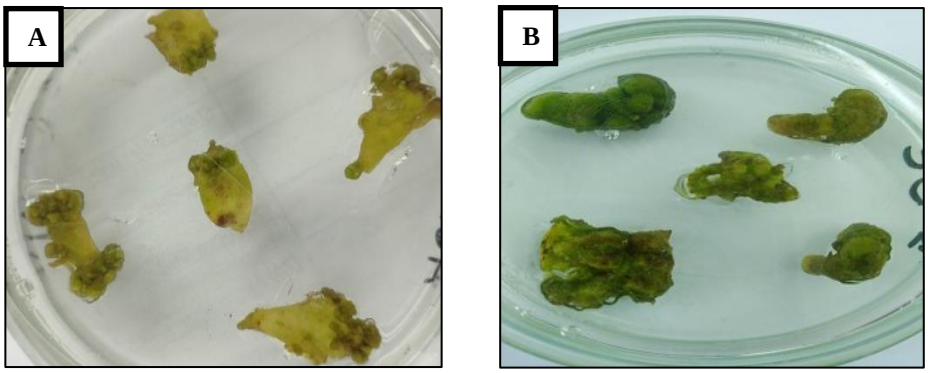

Fig 6. A and B steps of callus formation on callus induction media from leaf explants of Desiree cultivar

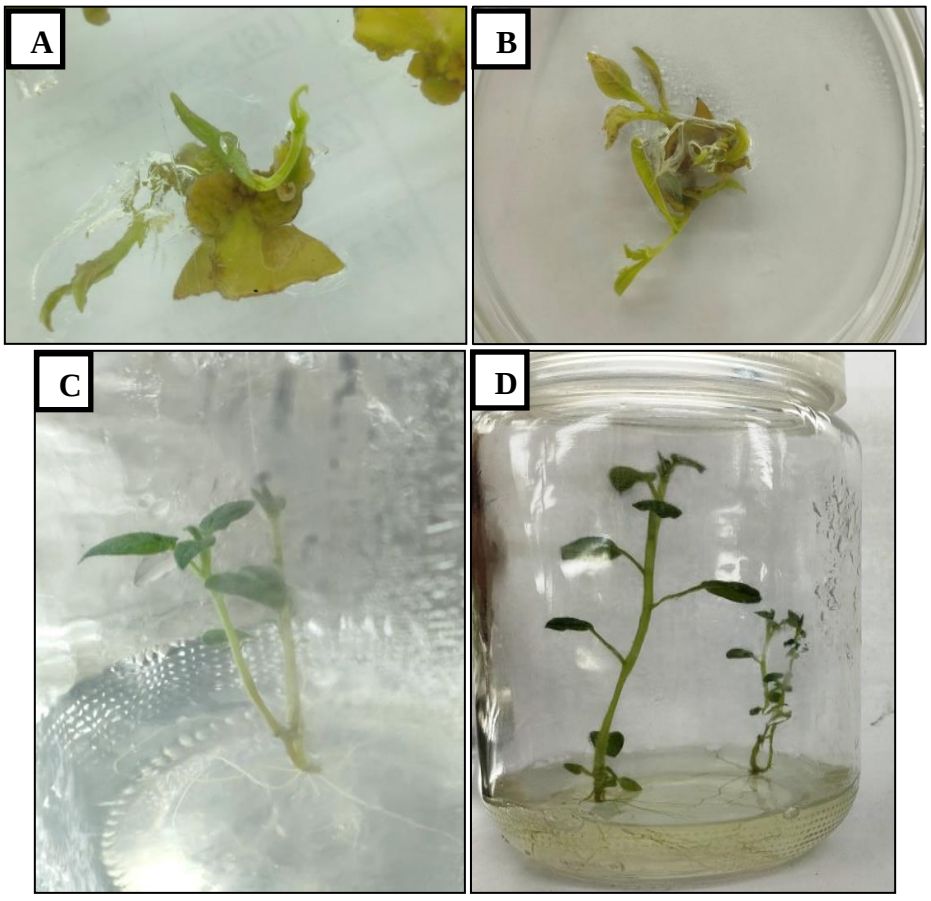

Fig 7. A, B, C and D stages of shoot formation on regeneration media from callus of Desiree cultivar

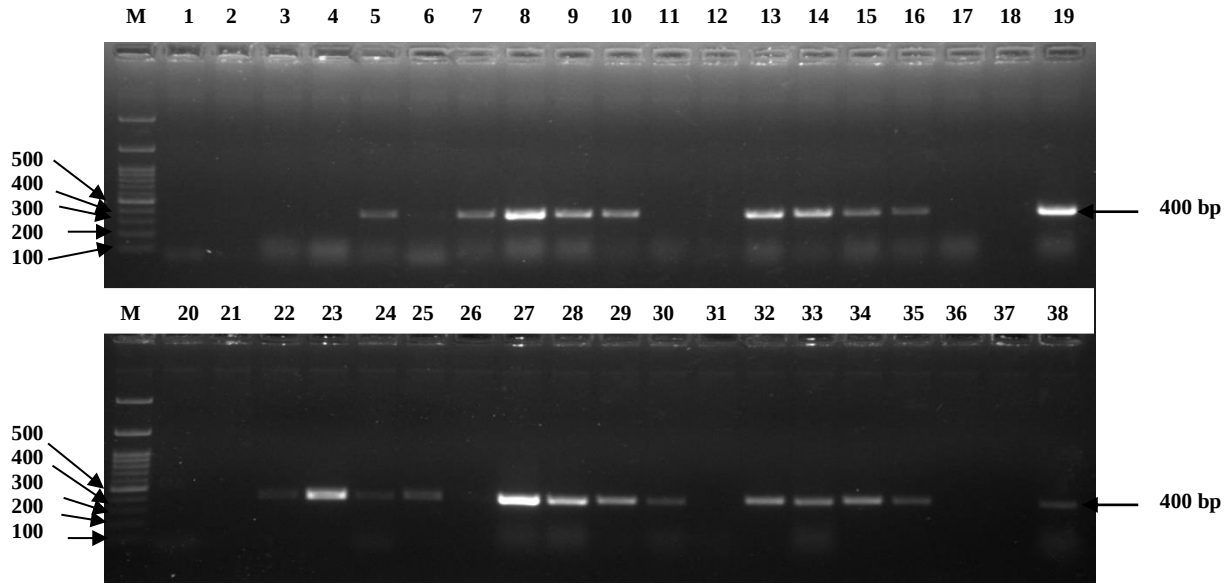

Fig 8. Screening of *bar* gene in edited potato Desiree cultivar
Lane M: 100 bp ladder
Lane 1and 20: Negative control
Lane 2 and 21: Desiree control
Lane 5,7,8,9,10,13,14,15,16,19,22,23,24,25,27,28,29,30,32,33,34,35 and 38: Contained the *bar* gene

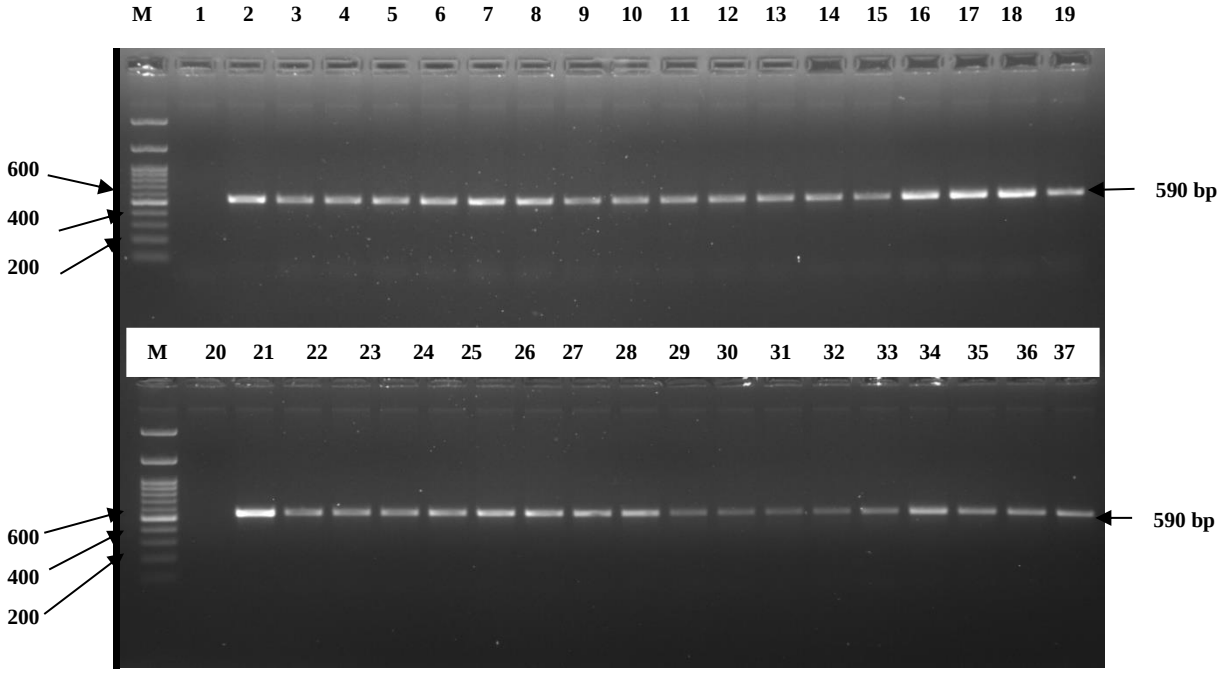

Fig 9. Confirmation of edited plants by using nd1 forward and nd2 reverse primers
Lane M: 100 bp ladder
Lane 1 and 20: Negative control
Lane 2 and 21: Positive control
Lane 3 to 19: Edited plants
Lane 22 to 38: Edited plants

When CRISPR-Cas9 ingredients are transmitted by plasmids, damaged DNA fragments can be integrated into the target site in addition to random locations during the transfection process (Salomon and Puchta 1998). As a result, the insertions in the region of interest were presented in a sizable proportion of mutants. These insertions may have originated from the host genome or plasmid DNA (Kim et al 2017, Liang et al 2017). Such findings were consistent with previous studies on transient expression in potatoes, which showed a significant increase in DNA insertions (Clasen et al 2016, Andersson et al 2018). Additionally, the unexpected insertion could make it harder to recognize the foreign DNA, eventually leading to an underestimation of their frequencies. Total genomic sequences of a mutant line(s) might be a comprehensive and expensive method, as accomplished in previous studies for tomato (Nekrasov et al 2017) and rice (Tang et al 2018).

3.4 Bioinformatics analysis

The most recent detection's PCR products were purified using the QIAquick® PCR Purification Kit (Qiagene), as directed by the manufacturer, and then were forwarded for nucleotide sequencing analysis. However, the remaining plants exhibited mutations at alternative positions (off-targeted mutations) in exon number 9 in the GBSS gene up to 32%. The sequencing data showed that edited plants contained mutations in the target sgRNA sequence up to 21% of regenerated shoots. Since only a 20-nucleotide gRNA sequence controls the CRISPR-Cas9 DSB activity, it can tolerate some base pair mismatches between the gRNA and the target DNA sequence. The genomic DNA areas that were complimentary to the gRNA sequence might only be partly cut by it. Due to the various target DNA locations' nucleotide compositions and genomic contexts, these off-target activities may vary among them (Zhang et al 2016).

To identify mutant alleles and their locus, reads from a sequence were compared with a wild-type sequence (**Table 3)**. Three mutant lines had insertion and deletion mutations that ranged in size from a single bp insertion (+1 to a 3 bp) to one deletion (-3). Additionally, the predominance of off-targeting in the GBSS gene using the gRNA product, also with mismatches in the PAM recognition sequence, shows the importance of evaluating several sgRNAs just before using CRISPR/Cas for efficient genome editing.

GBSS gene had indel mutations in these mutants (**Table 3)**. Four of them, mutant lines #19, #28, #34, and #35, had no wild-type sequences and were four mutant sequences **(Table 3)**. These findings supported the hypothesis that they were four-allele mutants. A four-allele mutant of the GBSS gene was created (GBSS mutant), which developed a normally, generated adequate number of potatoes with a morphological identical to the wild type (**Fig 10)**.

3.5 Starch phenotyping

The starch phenotypes of mutant potato 19, 28, 34, and 35 lines were examined. Light microscopy research on starch was conducted using *in vitro* microtubers. Decreased GBSS activity of the enzyme resulted in starch with a decrease in amylose production and an increase in amylopectin/amylose proportion. Iodine staining produces reddish-brown starch granules that are indicative of an amylopectin starch as opposed to an amylose starch, which is colored blue (Toinga-Villafuerte et al 2022). The red-brown staining with iodine in this study verified four mutant alleles indicating that the starch was of amylopectin quality (**Fig 10)**, showing that the GBSS gene and enzyme activity were knocked out.

This study showed that CRISPRCas9 may effectively silence a GBSS gene expression in a potato plant in a single transduction without the necessity for stable DNA insertion into its backbone. Since one or few features can be introduced to commercially appealing potato types, targeted created mutations using *in vivo* techniques were crucial for future potato breeding. This can then be achieved by avoiding changing the essential total heterozygous genomic environment by prohibiting additional sexual crosses. The GBSS gene's targeted area was used in this investigation to drive the guide sequences. All of these studies showed a rise in mutation levels at the same order of magnitude, demonstrating the reliability of the CRISPR-Cas9 technique in potato breeding and research. Likewise, it was as well demonstrated how to use this cutting-edge technology to produce amylopectin potato starch with uses in both food and technology (Andersson et al 2017).

In potatoes, desirable plant phenotypes and enhanced tuber starch quality features are crucial. Tetraploid potato primary issues are scarcity of trustworthy CRISPR/Cas configuration, target gene selection and efficient plant transformation methods. It is common knowledge that, particularly in potatoes, polyploidy and vegetative multiplication made it much more challenging to improve multigenic qualities than monogenic ones (Tiwari et al 2022).

Table 3. Alignments sequence to a conformable wild-type (WT) sequence showing mutations in a sgRNA in four plant lines. Target sequences of sgRNA were shown in bold letters. Nucleotide deletions were indicated by dashes, '_'; Transition mutations were shown in underline; and single nucleotide insertions were shown in red color. Pam (protospacer adjacent motif) sequences were in a blue color. d have 3 mutated alleles in the target region

			sgRNA		Indels
	WT	**5´**	TTGGCTTGCCTGTT**GACAAGAAGATCCCTTTGATTGG**CTTCATCGG	**3´**	**0**
	19-1	**5´**	TTGGCTTGCCTGTT**GACAAGAAGGTCCCTTTGATTGG**CTTCATCGG	**3´**	**0**
(a)	**19-2**	**5´**	TTGGCTTGCCTGTT**GACAAGAAGGTCCCTTTGATTGG**CTTCATCGG	**3´**	**0**
	19-3	**5´**	TTGGCTTGCCTGTT**GACAAGAAGGTCCCTTTGATTGG**CTTCATCGG	**3´**	**0**
	19-4	**5´**	TTGGCTTGCCTGTT**GACAAGAAGGTCCCTTTGATTGG**CTTCATCGG	**3´**	**0**
	WT	**5´**	TTGGCTTGCCTGTT**GACAAGAAGATCCCTTTGATTGG**CTTCATCGG	**3´**	**0**
	28-1	**5´**	TTGGCTTGCCTGTT**GACAAGAAAGATCCCTTTTGATTCGG**CTTCATCGG	**3´**	**+3**
(b)	**28-2**	**5´**	TTGGCTTGCCTGTT**GACAAGAAAGATCCCTTTTGATTCGG**CTTCATCGG	**3´**	**+3**
	28-3	**5´**	TTGGCTTGCCTGTT**GACAAGAAAGATCCCTTTTGATTGG**CTTCATCGG	**3´**	**+2**
	28-4	**5´**	TTGGCTTGCCTGTT**GACAAGAAAGATCCCTTTGATTGG**CTTCATCGG	**3´**	**+1**
	WT	**5´**	TTGGCTTGCCTGTT**GACAAGAAGATCCCTTTGATTGG**CTTCATCGG	**3´**	**0**
	34-1	**5´**	TTGGCTTGCCTGTT**GAC–AGAA–ATCCCTTTGAT–GG**CTTCATCGG	**3´**	**-3**
(c)	**34-2**	**5´**	TTGGCTTGCCTGTT**GAC–AGAA–ATCCCTTTGAT–GG**CTTCATCGG	**3´**	**-3**
	34-3	**5´**	TTGGCTTGCCTGTT**GAC–AGAA–ATCCCTTTGAT–GG**CTTCATCGG	**3´**	**-3**
	34-4	**5´**	TTGGCTTGCCTGTT**GAC–AGAA–ATCCCTTTGAT–GG**CTTCATCGG	**3´**	**-3**
	WT	**5´**	TTGGCTTGCCTGTT**GACAAGAAGATCCCTTTGATTGG**CTTCATCGG	**3´**	**0**
	35-1	**5´**	TTGGCTTGCCTGTT**GACAAGAAGATCCCTTTTGATTGG**CTTCATCGG	**3´**	**+1**
(d)	**35-2**	**5´**	TTGGCTTGCCTGTT**GACAAGAAGATCCCTTTTGATTGG**CTTCATCGG	**3´**	**+1**
	35-3	**5´**	TTGGCTTGCCTGTT**GACAAGAAGATCCCTTTTGATTGG**CTTCATCGG	**3´**	**+1**
	35-4	**5´**	TTGGCTTGCCTGTT**GACAAGAAGATCCCTTTGATTGG**CTTCATCGG	**3´**	**0**

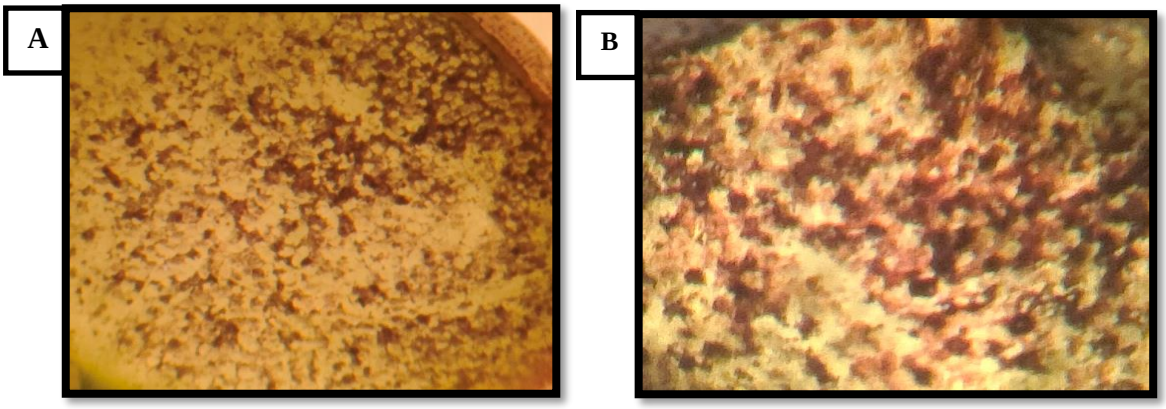

Fig 10. Sections of histochemical iodine staining for *in vitro* microtubers that visualized under light microscopy and showed two different phenotypes. (A) wild-type (Desiree cv.) microtuber section was stained to blue from normal amylase: amylopectin ratio. (B) mutated line microtuber section was stained to red-brown that lacked amylase and was rich in amylopectin

4 Conclusion

This study presented a successful application of CRISPRCas9 to fully knock out a GBSS gene function in a tetraploid potato plant in one round of transfection, without a stable introduction of DNA into the genome. Targeted mutations *via in vivo* methods are of significant importance for future potato breeding since one or a few traits can be modified to commercially interesting potato varieties. This can then be done without disrupting the valuable overall heterozygous genetic context by avoiding additional sexual crosses. In this study, the region of the GBSS gene was targeted, for driving the guide sequences. All the different experiments showed high mutation frequencies, in the same order of magnitude, demonstrating the robustness of the CRISPR-Cas9 technique for potato research and breeding. Here, also the use of this new technology was demonstrated to develop a trait of commercial interest, an amylopectin potato starch, with uses in both food and technical applications.

Desirable plant phenotypes and improved tuber starch quality traits play key roles in potatoes. The availability of robust CRISPR/Cas arrays, target gene selection, efficient plant transformation protocols and minimum off-target mutants are the major issues in tetraploid potatoes. It is a fact that the improvement of multigenic traits is more difficult than that of the monogenic traits, particularly in potatoes, due to polyploidy and clonal propagation.

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