Increasing of Amylopectin Starch Content in Tetraploid Potato (Solanum tuberosum L.) Using CRISPR/Cas9 System

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Title Page

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Abstract

Background

The potato tuber starch trait is affected by the composition of amylose and amylopectin. The synthesis of amylose in amyloplasts is catalyzed by granule-bound starch synthase (GBSS). GBSS gene expression was inhibited via CRISPR-Cas9-mediated genome editing in leaves of the Desiree Potato cultivar. Constructs containing the Cas9 gene and sgRNAs targeting the GBSS gene were introduced by *Agrobacterium*-mediated transformation delivery into protoplasts.

Methods and results

Outcomes included lines with mutations in all or only some of the homoeoalleles of GBSS genes and lines in which homoeoalleles carried different mutations and multiple alleles that were up to 21 % of regenerated shoots. Mutations were found in one bp from the used guide sequence, verifying the high homology between a guide sequence and a target region near the protospacer adjacent motif (PAM) site. DNA delivery into protoplast resulted in mutants with no detectable Cas9 gene. Microscopic evaluation of iodine-stained starch granules was shown to be a sensitive system for qualitative and quantitative examination of amylose formation in starch granules of transgenic potato tubers.

Conclusion

This study presented the successful application of CRISPRCas9 to fully knockout a GBSS gene function in a potato tetraploid plant in one round of transfection, without a stable introduction of DNA into the genome. In this study, the region of the GBSS gene was targeted, for driving the guide sequences. all the different experiments yielded 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.

Keywords

CRISPR-Cas9, amylopectin, granule-bound starch synthase, sgRNAs, *Agrobacterium*-mediated transformation.

Introduction

The Solanaceae family includes plants including tobacco, tomato, eggplant, and pepper. The subgenus Potato of the genus Solanum contains over 200 species of tuber-bearing potatoes (Sevestre et al. 2020). After rice (*Oryza sativa* L.) and wheat (*Triticum* spp.), potato (*Solanum tuberosum* L.), a member of the Solanaceae family, is the third-most important crop in terms of food produced for human use. (Tiwari et al. 2022). The total amount of potatoes produced worldwide in 2018 was 368.17 million tonnes (FAOSTAT 2018). 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. One of the few crops used for starch production, a renewable bulk material used in a variety of culinary and non-food applications, is the potato. One of our most significant starch crops and a common staple diet all across the world is the potato (Reddy et al. 2018). Plants combine amylose and amylopectin to store starch, whose ratio affects the characteristics of the starch (Schirmer et al. 2013). The enzyme granule-bound starch synthase (GBSS) is essential for the creation of amylose (Seung et al. 2015). Starch generated in mutants deficient in this enzyme is amylose-free. Rice mutants with a waxy phenotype lack the GBSS gene (Itoh et al. 2003). Other plant species, like sorghum, barley, and maize (Liu and Liu 2007; Morell et al. 2003), have also been discovered to harbour similar mutations (Kawahigashi et al. 2013).

Tetraploid, extremely heterozygous, and vegetatively propagated, the cultivated potato. As a result, creating new cultivars by traditional breeding techniques requires time and effort. crop improvement through gene knockdown and insertion/deletion mutagenesis using genome editing, 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 was made 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, is the most effective for targeted genome editing (sgRNA). 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. By blocking the activity of the GBSS gene, this study aimed to enhance the starch content in the micro tubers.

Materials

Plant material and explants type:

The Agricultural Genetic Engineering Research Institute's stock virus-free germplasm bank provided the Desiree cultivar of *Solanum tuberosum* L. tetraploid potatoes (2n=4x=48) (AGERI). All of the tests in this study used explants of leaves.

Methods

Delivery and expression vectors:

The cloning vector was produced by pChimera Gene Art (Life Technologies Inc., Carlsbad, CA, USA). A construct containing the sgRNA expression system surrounded by *Avr* II restriction sites was used for traditional cloning into the binary Cas9 expression vector pCAS9-TPC. Built on top of pPZP201 is pCAS9-TPC (a binary expression vector was constructed that used for *Agrobacterium*- mediated transformation). Both the *Escherichia coli* DH10 strain and the *Agrobacterium tumefaciens* LBA4404 strain were received from the Gene Silencing and Insect (GSIC) Lab. at AGERI.

Bioinformatics analysis:

We identified the fasta format sequence of exon 9 on the granule-bound starch synthase (GBSS) gene in potato plants and found a suitable sequence on the NCBI website (Andersson et al. 2017). Following that, the CRISPR-P web tool (https://github.com/haoliu1213/CRISPR-P-2.0) was used to design an appropriate sgRNA sequence for the GBSS gene. The designed sequences of detection primers for cloning and transformation are provided in Table (1).

Table (1): Synthetic primers designed for GBSS gene cloning. M13 reverse: 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).

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

Gene cloning:

As annealed oligonucleotides, forward (Fn1) and reverse (Rn1) oligonucleotides were combined, and the annealed oligonucleotides were then cloned into the pChimera vector by digesting the pChimera vector with the *Bbs*I restriction enzyme (Fig 1). A 1 µg of the DNA was combined with a 2 µl of the buffer for restriction enzyme, a 1 µl of *Bbs* I, and a ddH_2O was included in a volume total of a 20 µl and were incubated at a 37 °C for 2 hours. Once the sgRNA vector had been digested, a 2 µl of it was combined 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. (Chauvin et al. 2021).

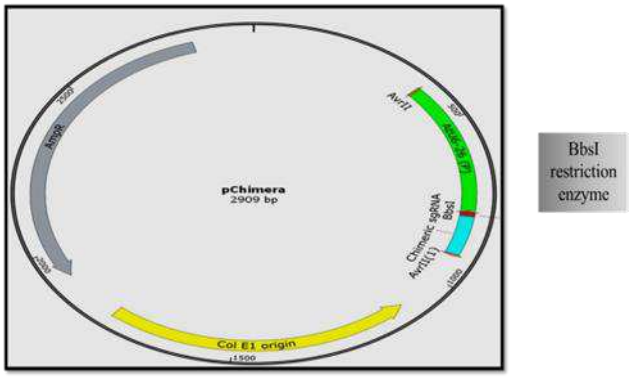

Fig 1. Traditional cloning of the pChimera cloning vector into the binary Cas9 expression vector pCAS9-TPC.

After performing a heat shock procedure to transform the vector DNA into *E. coli* (DH 10 β), the cells were grown on LB media that also contained ampicillin (100 mg/l). Using M13 reverse and forward protospacer oligos, checked colonies were performed by colony-PCR and annealed at 56 °C.

Cloning in the pCAS9-TPC vector that was digested and miniprepped for positive coloni 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 h at 37°C before spending the night at 4°C (Fig 2).

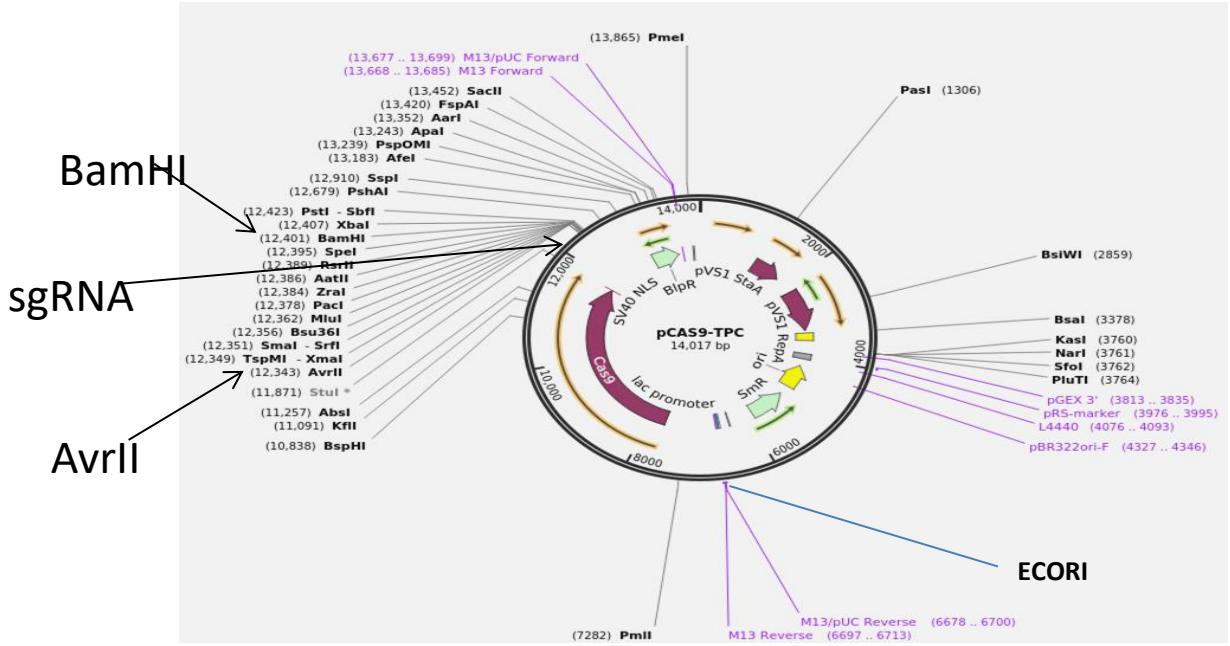

Fig 2. A pCAS9-TPC binary expression vector that was used for *Agrobacterium*- mediated transformation.

Purified digested pCAS9-TPC construct was prepared directly and was eluted in a 30µl of ddH_2O. The pCAS9-TPC vector was dephosphorylated by adding a 3.5µl of phosphatase buffer and a 1.5µl of alkaline phosphatase. Heat was used to inactivate the enzyme in the mixture for 20 minutes at 37°C and prepared ligation 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. Incubation lasted for at least three hours, but ideally overnight. A 10 lμ of the reaction were converted into *E. coli* (DH10 β) and then plated on LB with 100 mg/l of spectinomycin. The SS42 forward and SS43 reverse primers with an annealing temperature of 60°C were used to check for coloni using coloni-PCR. pCAS9-TPC vector which carried sgRNA sequence was transformed into *Agrobacterium* cells and transformed colonies using forward ss42 and reverse ss43 primers were detected.

Transformation plants (Desiree cultivar) using *Agrobacterium*-mediated transformation system:

Using the nodal cutting technique, the Desiree cultivar was in vitro micropropagated, as described by (Roca et al. 1978). Every 3 to 4 weeks, nodal cuttings were routinely subcultured on a new media (3% sucrose was added to the MS salts medium (Murashige and Skoog 1962) and the pH was raised to 5.6).

Transformation in plants by *Agrobacterium tumefaciens*:

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

Harvested in vitro leaves from plantlets were incubated for 5 mins with gentle shaking in a medium containing 30 ml of an overnight *A. tumefaciens* culture. The extra bacteria were then removed using sterile filter paper and 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 GA3, and a 1 ml/L of MS basal salt combination. The plantlets were subcultured once more after three weeks at a temperature of 20 ± 2°C. The antibiotics were added to the autoclaved medium under sterile circumstances.

Genomic DNA Isolation:

Samples were collected from 30-40 days old of editing potato Desiree cultivar plants and their control (non-editied plants) from *in vitro* cultures. Isolation of DNA samples was conducted using QIAGEN (cat. Nos. 69104 and 69106). Plasmids mini-prep for sgRNA vector and pCAS9-TPC plasmids 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 used for PCR purification in accordance with the manufacturer's instructions.

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 performed in a volume of 25 μl comprising a 1 μl of template DNA, 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). For molecular analysis and bioinformatic analysis, which employed the identical reaction conditions with forward sd1 and reverse sd2 primers, amplification was scheduled at 94 °C for

3 min, 94 °C for 30 sec, 58 °C for 40 sec, and 72 °C for 1 min for 35 cycles, and the final cycle was at 72 °C for 7 min (Table 2).

Polymerase chain reaction (PCR) was used to check for the presence or absence of the *bar* gene. The DNA amplification was carried out in a volume of 25 µl that comprised a 1 µl of DNA, 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 for both modified and unedited plants (promega, Madison, USA). For molecular analysis and bioinformatic analysis which employed the identical conditions of reaction with forward sd1 and reverse sd2 primers, for 35 cycles of amplification, the temperature was set to 94 °C for 3 min, 94 °C for 30 sec, 58 °C for 40 sec, and 72 °C for 1 min. The final cycle was 72 °C for 7 min (Table 2).

Table (2): The primers 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.

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

Nucleotide sequencing analysis:

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

Staining Potato micro tubers with iodine solution:

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

Results

Gene cloning:

By digesting the sgRNA vector (pChimera) with the *Bbs*I restriction enzyme and allowing the ligation of the vector and sgRNA with T4 DNA ligase, the results of cloning in the pChimera binary vector were examined in the current study. The ligation reaction was then inserted into DH 10β competent *E. coli* cells. Fn1 forward and M13 reverse primers were used in coloni PCR analysis to screen for positive (transformed) colonies, the negative control lane did not display any fragment markers at 270 bp size (Fig 3).

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

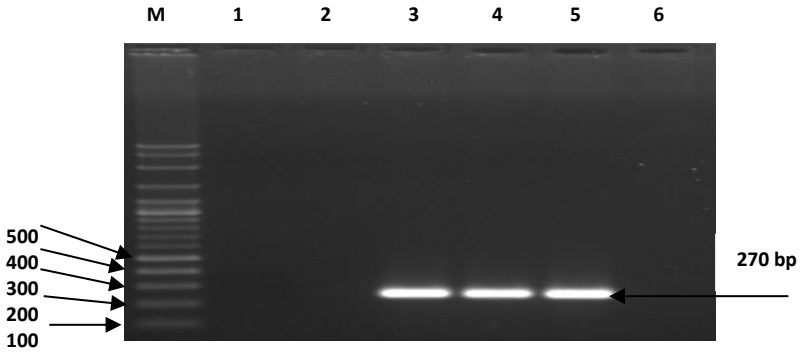

Fig 3. Screening of the converted colonies after pChimera transformation in *E.coli*.
Lane M: 100 bp ladder.
Lane 1: Negative control.
Lane 2 and 6: Negative coloni.
Lane 3, 4 and 5: Positive colonies.

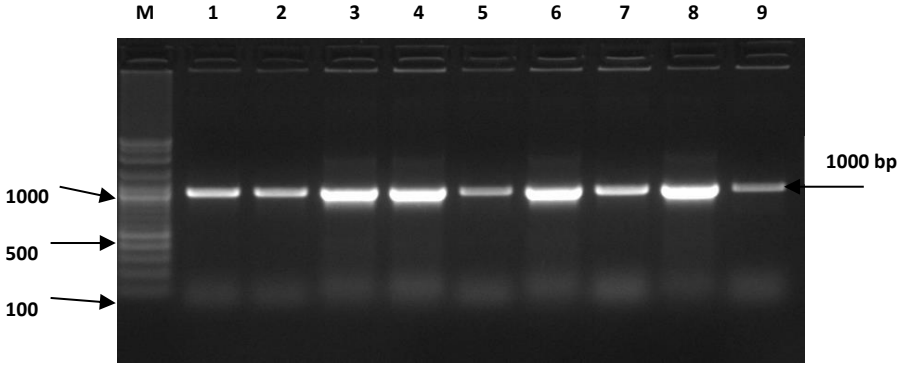

Fig 4. Verification of transformed colonies in *E. coli* following pCAS9-TPC transformation.
Lane M: 100 bp ladder.
Lane 1: Positive control.
Lane 2 to 9: Positive colonies.

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

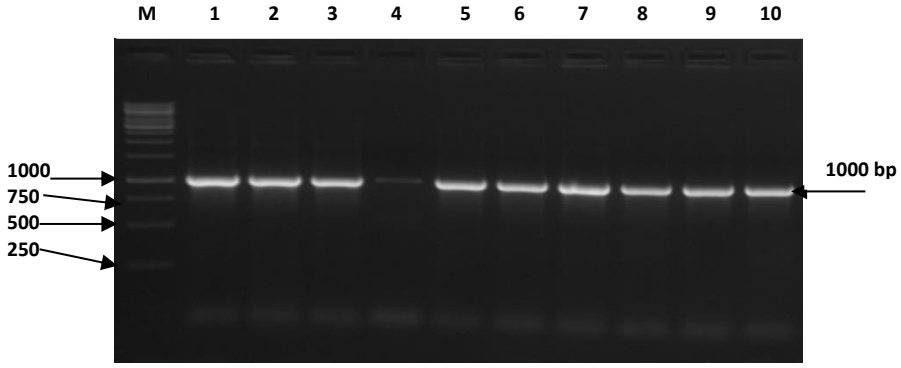

Fig 5. Verification of transformed colonies in *Agrobacterium* following pCAS9-TPC transformation.
Lane M: 1 kp ladder.
Lane 1: Positive control.
Lane 2 to 10: Positive colonies.

Transformation into Potato plants:

The Desiree cultivar of potatoes 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 media (MS basal salt with a 5 mg of 2,4-Dichlorophenoxyacetic acid /L concentration) in the dark for 6 days at 20 ± 2°C. This was done by the LBA4404 strain of *Agrobacterium tumefaciens*, which included the pCAS9-TPC vector to induce callus. On regeneration media, 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 in (Fig 7).

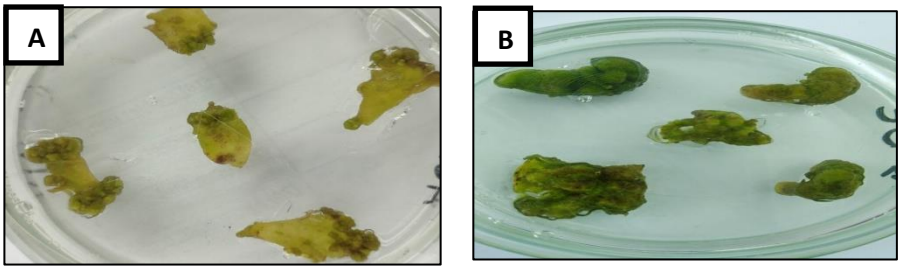

Fig 6. Formation of callus-on callus induction media from leaf explants of Desiree cultivar.

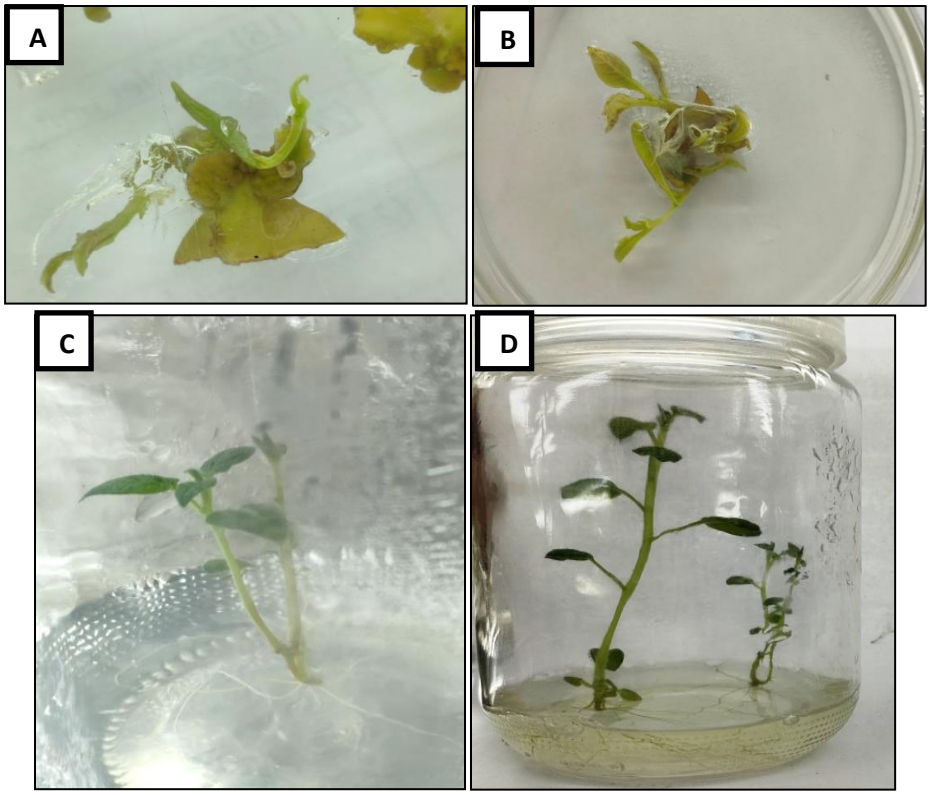

Fig 7. Stages of shoot formation on regeneration media from callus of Desiree cultivar.

PCR reaction for edited plants:

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

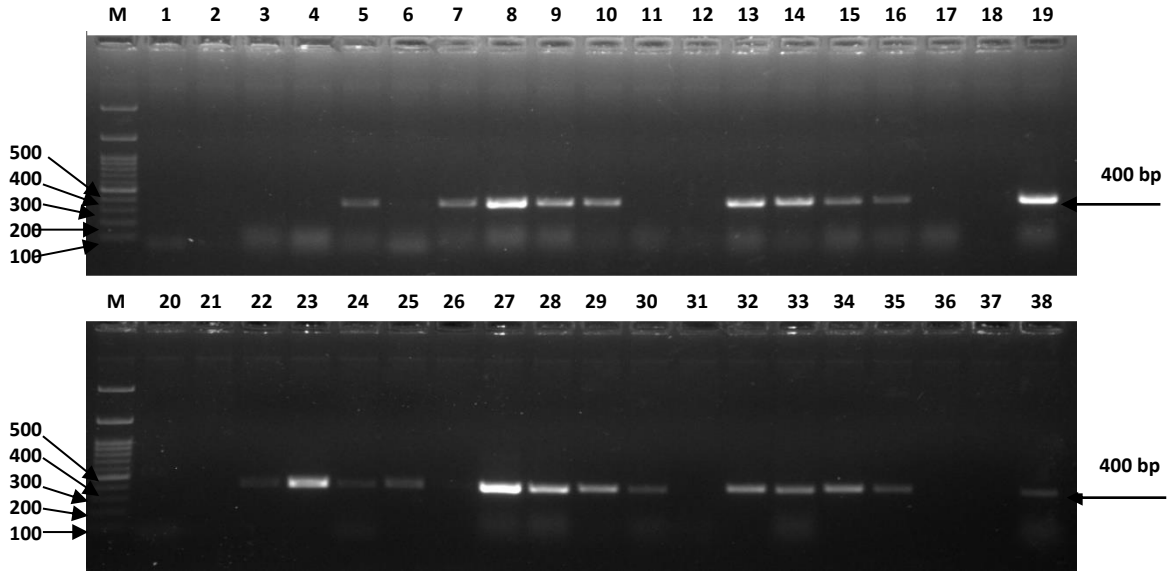

Fig 8. Screening of *bar* gene in edited potato Desiree cultivar.
Lane M: 100 bp ladder.
Lane 1and 20: Negative control.
Lane 2 and 21: Positive control
Lane 5,7,8,9,10,13,14,15,16,19,22,23,24,25,27,28,29,30,32,33,34,35 and 38: Contained the *bar* gene.

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 forwarded for nucleotide sequencing analysis (Butler et al. 2015). 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 a 21% of regenerated shoots. Since only one 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. It was possible that it would only partially cut the genomic regions that were complementary to the gRNA sequence. Due to the various target DNA locations' nucleotide compositions and genomic contexts, these off-target activities vary amongst them (Zhang et al. 2016).

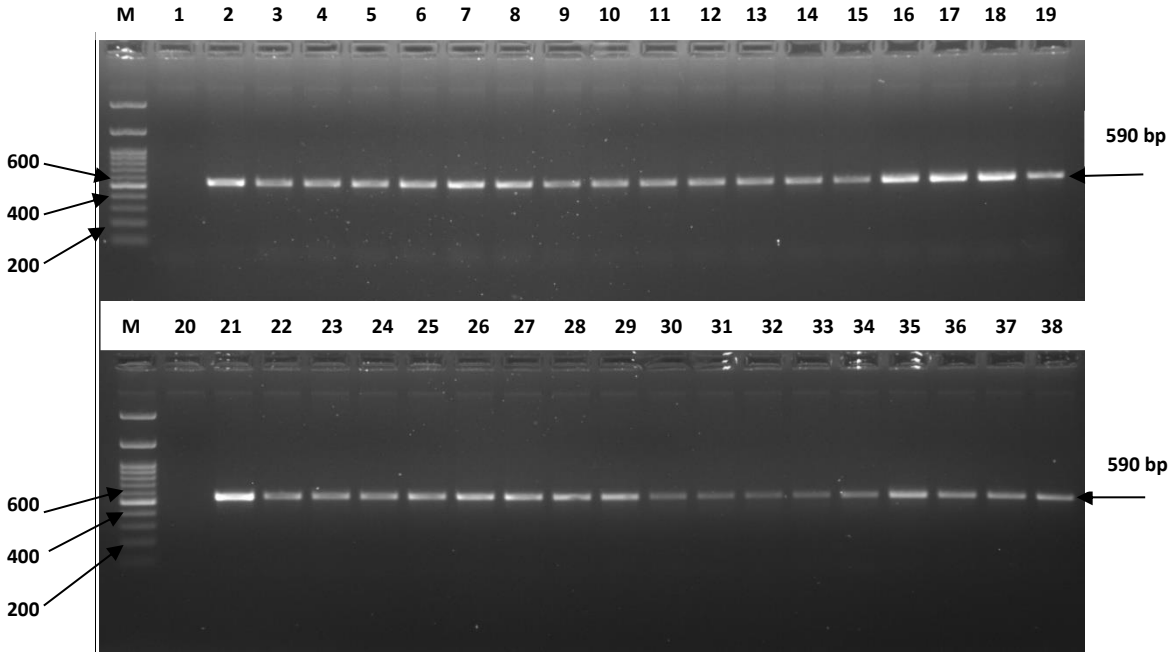

Fig 9. Confirmation of edited plants by using nd1 forward and nd2 reverse primers.
Lane M: 100 bp ladder.
Lane 1 and 20: Negative control.
Lane 2 and 21: Positive control.
Lane 3 to 19: Edited plants.
Lane 22 to 38: Edited plants.

In order to identify the mutant alleles and their locus, reads from a sequence were compared with a wild-type sequence that they belong to (Fig 10). 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 GBSS 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.

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

Starch phenotyping:

The starch phenotypes of mutant potato lines 19, 28, 34, and 35 were examined. Light microscopy research on starch was conducted using in vitro micro tubers. Decreased GBSS activity of the enzyme results to a starch with decreased amylose production and an increase in the amylopectin/amylose proportion. Iodine staining produces reddish-brown starch granules that are indicative of an amylopectin starch as opposed to an amylose starch, which coloured blue (Toinga-Villafuerte et al. 2022). The red-brown staining with iodine in this study's four mutant alleles verified that the starch was of amylopectin quality (Fig. 11), showing that the GBSS gene and enzyme activity had been knocked out.

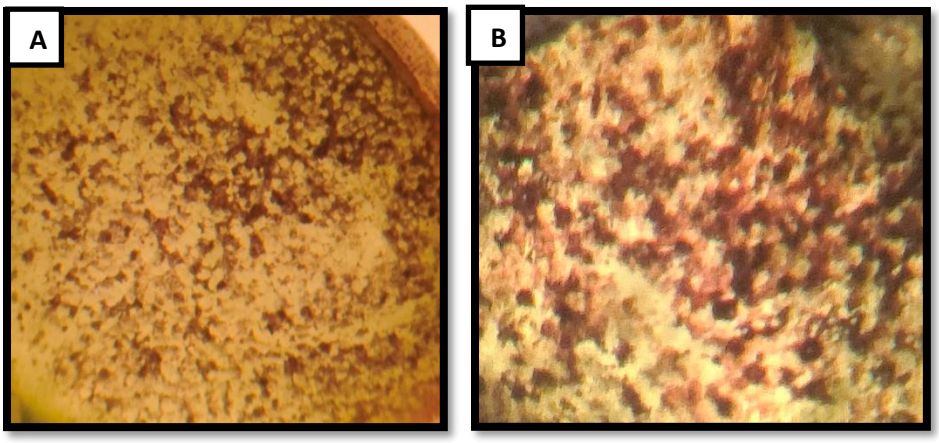

Fig 11. Sections of histochemical iodine staining for *invitro* microtubers that visualized under light microscopy and showing two different phenotypes. (A) wild-type (Desiree cv.) microtuber section was stained to blue from normal amylase:amylopectin ratio. (B) mutated line microtuber section was stained to red-brown that lacksed amylase and was rich in amylopectin.

Discussion

In this study, the GBSS gene was damaged in a potato mutant via the CRISPR/Cas9 system as well as gRNAs which matched the site in the GBSS gene's ninth exon.. A recently developed method called genome editing allows for the change of a particular gene using an artificial nuclease that recognises certain sequences (Osakabe and Osakabe 2015).

In tetraploid potato Desiree cultivars, we demonstrated that the introduction of CRISPR-Cas9 modules by transient expression of plasmids or stably integrated transgene(s) can successfully result in directed alterations through into GBSS gene. Using *Agrobacterium*-mediated transformation, we were successful in creating tetra-allelic mutations with reduced amylose synthesis. The persistence of the transgene may not be a concern for fundamental research when (Tang et al. 2018) demonstrated that the ongoing utilization CRISPR-Cas9 technologies in rice doesn't result in off-target mutations provided sgRNAs are rigorously generated. 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 a effective system for producing a mutation in the specific location with a rise editing frequency. Here, we indicate that a

binary sgRNA methodology on competing DNA strands is not always result in a large portion of the gene being efficiently deleted. This is likely because of breaking efficiency at the two sgRNA-targeted locations are not equivalent. We used a Sanger sequencing of positive plants to quickly and cheaply screen mutations.

When CRISPR-Cas9 ingredients are transmitted by plasmids, it's possible for damaged DNA fragments to be integrated into the target site in addition to random locations during the transfection process (Salomon and Puchta 1998). As a result, we reported that the insertions in the region of interest were present 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 are consistent with past studies on transient expression in potato, which revealed a significant increase of DNA insertions (Andersson et al. 2018; Clasen et al. 2016). Additionally, the unexpected insertion may make it harder to recognise the foreign DNA, which insertion into plant genomes, 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 tomato (Nekrasov et al. 2017) and rice (Tang et al. 2018).

Numerous mutations were found in the GBSS gene's nucleotide sequences. The GBSS gene had indel mutations in these mutants (Fig. 10). Four of these, mutant lines #19, #28, #34, and #35, had no wild-type sequences and four mutant sequences (Fig. 10). These findings support the hypothesis that they were four-allele mutants. There has not been a GBSS mutant potato yet. We created a four-allele mutant of the GBSS gene (GBSS mutant), which developed normally and generated an adequate number of potatoes with morphological identical to a wild type (Fig. 11). It is believed that a mutant potato like this one will aid in understanding how the genes responsible for starch

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 the backbone. Since one or a few features can be introduced to commercially appealing potato types, targeted mutations created using in vivo techniques are crucial for future potato breeding. This can then be achieved by avoiding change 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 the studies yielded rise of 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 technique to produce the 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 a 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).

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Author contribution

N. M.: conceived the study, carried out all the genetic transformation and vector confirmation tests, collected data, confirmed genome editing, and authored the first draught of the publication. M. A.: supervised the overall research, critically reviewed the text, and presented the results in their current form. A. H.: reviewing the paper, H. M.: plasmids that have been created and offer technical assistance. N. E.: data evaluation. E. A.: created the study, revised the manuscript, and evaluated it.

Ethics declarations

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

All authors declare no conflict of interest