Reduced gene expression of potato apoplastic invertase inhibitor gene on CRISPR/Cas9 targeting and analyzing its transformation efficiency parameters

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Research Article

Keywords: potato, Solanum chacoense M6, Solanum tuberosum cv. Desiree, CRISPR/Cas9, apoplastic invertase inhibitor

Posted Date: January 8th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-3832361/v1

Additional Declarations: No competing interests reported.

Abstract

Background

Pathogen infections that affect potato yield cause severe economic losses every year. Several studies point the role of apoplastic (cell wall) invertase (CWIN) enzyme in plant defense mechanisms, and that apoplastic invertase inhibitor (INVINH1) post-translationally regulates CWIN. Nevertheless, the role of *INVINH1* needs to be elucidated for several effects in plant transformation parameters and its gene expression which we sought to explore using CRISPR/Cas9 technology.

Methods and Results

In this study, we sequenced the first exon of *INVINH1* gene in cv. Desiree and *Solanum chacoense* M6. We identified in the first exon two alleles for *StINVINH1* gene in cv. Desiree and one allele for *ScINVINH1* gene in *S. chacoense* M6. We designed two single-guided RNAs (sgRNAs) to target *INVINH1* gene from diploid *S. chacoense* M6 and tetraploid *S. tuberosum* cv. Desiree using CRISPR/Cas9 based technology. In our earlier study, we have already optimized transformation protocol for M6 and cv. Desiree using *Agrobacterium* strains, based on which *Agrobacterium* strain AGL1 was chosen for CRISPR/Cas9 experiment. Our experimentation showed that heat stress at 37°C could increase the mutagenesis capability, and CRISPR/Cas9 targeting affected plant transformation parameters. It was found from the knockout experiment that the indels were present in the calli, and the candidate regenerated plants showed reduced gene expression level conducted via RT-qPCR.

Conclusion

Our study demonstrated that *INVINH1* targeting affected the calli induction and regeneration rates, was effective under heat stress, and reduced its gene expression level. More studies are required to comprehend the function of INVINH1 enzyme in potato stress response and defense mechanism.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the fourth most important food crop globally after maize, rice and wheat [1–3] and its breeding suffers from its ploidy level, inbreeding depression, poor wild species adaptation and low rate of recombination and sexual fertility [4]. Cultivated potatoes are tetraploid, highly heterozygous and have tetrasomic inheritance which makes potato conventional breeding and research challenging, therefore requiring the use of gene editing accelerated breeding approach [5]. Gene editing technology such as CRISPR (Clustered regularly interspaced short palindromic repeat)-Cas9 (CRISPR-associated nuclease 9) system on the other hand is an inexpensive, convenient, suitable for multiplexing and DNA methylation insensitive method [6, 7]. Using such a useful technology in potato breeding, we

sought to explore if targeting cell wall (apoplastic) invertase inhibitor (*INVINH1*) gene using CRISPR/Cas9 technology would affect plant transformation parameters and its gene expression.

Post-translational targeting of cell wall (apoplastic) invertase (CWIN) is performed by INVINH1 enzyme [8, 9]. CWIN has crucial plant functions such as plant physiology, stress-related response, fruit set and seed filling [10]. CWIN is also modulated on interaction between plants and pathogens, mostly upregulation of CWIN mRNA when infected by virus, fungus, bacteria, oomycete and nematodes [11]. Overexpression of CWIN in rice showed increased resistance to fungal and bacterial pathogens [12]; while in pepper, *Xanthomonas campestris* pv. *Vesicatoria* was found to inhibit CWIN [13]. Silencing of *INVINH1* has been shown to increase hexose level and seed weight in fruit [8], increased tolerance to chilling [14] and delay in leaf senescence [8] in tomato, and accelerated seed germination in *Arabidopsis* [15] as well as improved soybean seed weight [9, 10]. Elevation of CWIN post-translationally and its components by the associated inhibitors in *Arabidopsis* led to lowered fungal/bacterial susceptibility as well as disease index [10]. A drop in *INVINH1* mRNA was observed upon *Pseudomonas syringae* pv. tomato DC3000 infection in *Arabidopsis*, which declined activity of invertase inhibitor due to plant defense response; acarbose inhibition of CWIN also showed increased pathogen susceptibility [16]. In diploid and tetraploid potatoes, 8 different alleles of highly AT-rich *INVINH1* gene (comprising of two exons separated by an intron) have been reported. Substitution polymorphisms were mainly seen in exons, whereas high polymorphism was observed in the single intron [17]. Both INVINH1 and vacuolar invertase inhibitor (INVINH2) could be required in regulation of invertase activity in cold-stored tuber [18].

In this study, we targeted *INVINH1* gene using CRISPR/Cas9 system in *S. chacoense* M6 and potato cv. Desiree and explored the plant transformation parameters and the gene expression levels in the resulting plants. Diploid potato *S. chacoense* M6 (Reg. No. GP-1, BS 228) is homozygous, generated by seven generation of selfing [19, 20], has its whole genome sequenced [4], and optimized transformation protocol [21]. Tetraploid potato cv. Desiree [22] (Twell and Ooms, 1988) has a well-known transformation protocol [23–25]. We sequenced a part of the first exon of *INVINH1*, on the basis of which we designed two sgRNAs to target the first exon of *StINVINH1*gene in cv. Desiree and *ScINVINH1*gene in *S. chacoense* M6.

Materials and Methods

Plant material

MS medium [26] (Duchefa Biochemie, Cat. No. M0222.0050) was used to propagate cv. Desiree and *S. chacoense* M6 at 24 ± 2 ºC in the growth chamber (100 µmol m^{-2} s^{-1} fluorescent light, photoperiod of 16/8 h light/dark). Cell and Molecular Sciences, James Hutton Institute, Dundee, UK provided *S. chacoense* M6, while cv. Desiree was obtained from Nigde Omer Halisdemir University, Nigde, Turkiye, for the experimentation. Every three to four weeks, subculture was performed, and explants used were leaves, internodes and microtubers [27]. Microtuber production was done from cv. Desiree and *S. chacoense* M6 as described in [28], with variation in thidiazuron (Duchefa Biochemie, CAS No. 51707-55-2) concentrations: 0, 0.1, 0.5 and 1 mg L^{-1} performed in triplicates.

Sequencing of the first exon of INVINH1 gene and allelic polymorphism determination

To unravel the alleles of *StINVINH1* and *ScINVINH1*, DNA extraction was performed using CTAB (cetyl trimethylammonium bromide) method, amplified using the primers NFE-F 5'-CCACATTTAGTTCTTAATTTCCCAA-3' and NFE-R 5'-GAAAAGGCACAATTCTTCAAAGG-3' [17] via proofreading polymerase, followed by gel-excision, purification, A-tailing and cloning to pGEM®-T Easy or pTZ57R/T. For *INVINH1* sequencing, *E. coli* strains Top10 and JM109 were used. Several clones were sent for sequencing at Nitta Laboratuvar Ürünleri İthalat İhracat Ltd., Ankara, Turkiye for Sanger sequencing. CLC Main Workbench 8 (Qiagen) was used to align the clone sequences and allelic diversity was analyzed.

Scoring of gRNA spacer sequence, design and cloning to CRISPR vector

Various online tools were used to score gRNA spacer sequences: https://crispr.med.harvard.edu/sgRNAScorer, http://broadinstitute.org/rnai/public/analysis-tools/sgrna-design and http://cistrome.org/SSC. The two best scored gRNA1 and gRNA2 were picked and separately cloned to pGNK-LeCas9-AtU6PgRNA CRISPR vector. The spacer sequence targeted the first exon and one of the gRNAs also targeted the functional domain analyzed by ExPASy-PROSITE (https://prosite.expasy.org/) of the INVINH1 protein. For cloning of gRNA1 and gRNA2, XL10-Gold Ultracompetent Cells (Agilent) were used.

Control experiment

Triplicate experiments were performed for internode explants on callus inducing media CIM-2 as described in [21, 27] (with and without kanamycin) as follows: 1) with and without *Agrobacterium* AGL1 (empty) infection, 2) AGL1 infection that harbored an empty pGNK-LeCas9-AtU6p-sgRNA (without gRNA). Callus induction and regeneration frequency were calculated [21, 27, 29].

$$\text{Callus induction rate } (\%) = \frac{\text{Total number of calli}}{\text{Total number of explants}} \times 100\% \qquad (1)$$

$$\text{Regeneration frequency } (\%) = \frac{\text{Total number of regenerated shoots}}{\text{Total number of calli incubated}} \times 100\% \qquad (2)$$

Gene editing experiment

Agrobacterium strain AGL1 separately harboring gRNA1 and gRNA2 were used for gene editing experimentation. AGL1 that harbored gRNA1 in the CRISPR vector was called A1 construct and AGL1 harboring gRNA2 in the CRISPR vector was called A2 construct. Two different CIM were prepared: CIM-2 for cv. Desiree and CIM-3 for *S. chacoense* M6 as described in [21, 27]. The explants were placed on MS liquid, wounded, infected using A1 construct and A2 construct separately, co-cultivated for 2 d. After 2 d, the explants were either incubated on CIM-2 or CIM-3 in plant growth chamber, or intermittently heat-

stressed at 37°C for 1–2 d under dark. Shoot regeneration media (SIM) was prepared as described in [21]. For rooting, root generating media (RGM) was prepared, RGM1 [1 mg L^{-1} IBA (indole-3-butyric acid), 0.2 mg L^{-1} GA_3 (Gibberellic acid), 25 mg L^{-1} kanamycin sulfate and 40 mg L^{-1} sulcid] and RGM-2 [1 mg L^{-1} IAA (indole-3-acetic acid), 0.2 mg L^{-1} GA_3, kanamycin sulfate (25 mg L^{-1}] and 40 mg L^{-1} sulcid).

Molecular analysis of calli and T_0 plants

DNA was extracted from calli and regenerated plants using GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific, Cat. No. K0792). Selection of transgenic calli and plants was performed using *nptII* primers (*nptII*-F: 5'-TTGCTCCTGCCGAGAAAG-3' and *nptII*-R: 5'-GAAGGCGATAGAAGGCGA-3'). For sequencing purpose, PCR was done with proofreading polymerase (*EasyPfu* DNA polymerase, TRANS, Cat. No. AP211) via NFE-F and NFE-R or InInt-R (5'-TAAGATAAACATAACTCCTTATTCA-3') primers. Gel extraction was done using *EasyPure*® Quick Gel Extraction Kit (TRANS, Cat. No. EG101-01), A-tailed and cloned using QIAGEN PCR Cloning Kit (Cat. No. 231122). Some samples were directly sequenced from the PCR product. Direct sequencing and cloned vector sequencing was performed using Sanger sequencing at Sentebiolab, Ankara, Turkiye. Sequence analysis of calli as well as regenerated shoots was done using the online tool http://multalin.toulouse.inra.fr/multalin/ and software CLC Main Workbench 8.

Gene expression analysis of regenerated plants

After growing the rooted regenerated plants in the soil, RNA was isolated from each independent putative plants and wild-type control and treated with DNase I (Thermo Scientific, Cat. No. EN0521). Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (#K1612) was used for first strand cDNA synthesis with 1 µL Oligo $(dT)_{18}$ primer and a total RNA of 0.5 µg from control and putative samples. Dilution of first strand equal concentrations of cDNA in 1:10 ratio was performed for template in RT-qPCR with the use of iTaq^{TM}Universal SYBR GreenSupermix (BIO-RAD, Cat. No. 172–5121). InvInhRT-F (5'-GTGTGTGAAAACTTTGTC-3') and InvInhRT-R (5'-GAAAAGGCACAATTCTTC-3') primers were used with 2.5 µL of diluted cDNA. The program reaction was as follows: 95°C for 15 min, followed by 40 cycles at 95°C for 10 s, 51°C for 15 s, 72°C for 20 s by using Qiagen Rotor-Gene Q. Melting curve analysis (incubation at 99°C to 70°C with a transition rate of 1°C min^{-1}) was performed to determine if PCR generated only single product. For the purpose of normalization, *18S rRNA* was used as a reference gene to quantify gene expression [30]. Threshold values were generated using Rotor-Gene QRT-PCR instrument (QIAGEN) software in analysis of target gene expression. Data calculation was done using RT-qPCR standard deviations, and gene expression level was calculated using $2^{-\Delta\Delta C}{}_T$ proportional calculation method [31, 21].

RESULTS

Allelic determination of INVINH1 gene and spacer sequence design

Sequencing of 17 different clones from cv. Desiree showed two alleles of *StINVINH1* gene, while only one allele in 10 different clones from *S. chacoense* M6 for *ScINVINH1* gene. Allelic variation was analyzed

using Qiagen CLC Main Workbench 8 analysis. From the scoring analysis, “GACAAAAGAAGTGAAACAGC” with PAM sequence AGG was chosen as gRNA1 and antisense spacer “GGATCTTTCCAAGCTTGAGG” with PAM AGG was chosen as gRNA2—both of which start with “G” nucleotide at their 5’ ends. gRNA1 that targeted 1st exon of *INVINH1* also targeted phosphorylation sites of INVINH1 enzyme.

Microtuberization for explant generation in plant transformation

Adding thidiazuron was found to generate more microtubers, specially at 0.5 mg L^{-1} for cv. Desiree (26 microtubers) and *S. chacoense* M6 (31) against the control (9 for cv. Desiree and 0 for *S. chacoense* M6). This was followed by 1 mg L^{-1} thidiazuron that yielded 26 microtubers in cv. Desiree and 27 in *S. chacoense* M6, and finally 0.1 mg L^{-1} thidiazuron (23 microtubers in cv. Desiree and 24 in *S. chacoense* M6) (Fig. 1).

Control experiment results

Control experiment was performed to measure the extent of damage to the plant explants, calli induction and regeneration brought by selection pressure, *Agrobacterium* and CRISPR vector. In absence of both kanamycin and *Agrobacterium*, it was found that *S. chacoense* M6 showed 88.46% calli induction and cv. Desiree showed 100% calli induction (Fig. 3a). For those grown in CIM with kanamycin, there was no callus induction. Four different conditions were also used: 1st infection (empty AGL1 cells without kanamycin), 2nd infection (empty AGL1 cells with kanamycin), 3rd infection (AGL1 cells harboring empty CRISPR vector without kanamycin), 4th infection (AGL1 cells harboring the control vector with kanamycin).

In the case of cv. Desiree, 1st infection condition gave the most calli induction (86.67%), followed by 3rd (63.33%), 4th (61.9%) and 2nd (12.5%). For *S. chacoense* M6, 1st infection condition gave the highest calli induction (72.5%), followed by 3rd (65.6%), 4th (38.09%) and 2nd (4.17%). Nevertheless, it was found that *S. chacoense* M6 had lower calli induction than cv. Desiree in all conditions except for 3rd infection condition (Fig. 3b).

Without bacteria and without kanamycin, regeneration was the highest for cv. Desiree (54.54%), and the least in *S. chacoense* M6 (26.08%). No regeneration was observed for cv. Desiree with empty AGL1 cells and without kanamycin, while for *S. chacoense* M6 it was 31.03%. Regeneration frequency was 31.57% for cv. Desiree infected with CRISPR vector harbored AGL1 cells without kanamycin selection, while with kanamycin selection it was 30.77%. For *S. chacoense* M6 infected with CRISPR vector harbored AGL1 cells and without kanamycin selection, regeneration was 14.28%, while with kanamycin selection it was 0%.

Gene editing using A1 and A2 constructs

The gene editing experiment with A1 construct (AGL1 harboring CRISPR vector with gRNA1) and A2 construct (AGL1 harboring CRISPR vector with gRNA2) was conducted in cv. Desiree and named DA1 and

DA2, respectively. For *S. chacoense* M6 infected using A1 and A2 constructs were named M6A1 and M6A2, respectively. For M6A1, internode explants showed the highest calli induction (88.63%), followed by leaf (72.09%) and microtuber (23.33%) explants. For M6A2, again internodes showed highest calli induction (50.88%), followed by leaf (49.22%) and microtuber (25%) explants. DA2 also showed a similar trend: the highest calli in internode (77.74%), leaf (77.69%) and followed by microtuber (33.33%); however, not in DA1: the highest in leaf (48.08%), followed by internode (37.85%) and microtuber (16.67%) explants. Overall, internode explants could be considered the best among all explants, constructs and varieties used, except for DA1 (Fig. 4a).

It was found that the regenerants for M6A1 leaf, internode and microtuber calli were 0, 10 and 18, respectively. This gave the regeneration frequencies of 0%, 2.14% and 128.57% for leaf, internode and microtuber explants, respectively. For M6A2, regenerant number was 0, 5 and 4 for leaf, internode and microtuber calli, respectively (regeneration frequencies of 0%, 26.67% and 26.67%, respectively), while for DA1 it was 9, 6 and 1 for leaf, internode and microtuber calli, respectively (3.6%, 2.99% and 10%, respectively), and for DA2 it was 0, 3 and 1 for leaf, internode and microtuber calli, respectively (0% ,0.72% and 5%, respectively). Overall, it can be seen that microtubers gave the highest regeneration frequency among all the explants, constructs and varieties used, while leaf gave no regeneration at all in M6A1, M6A2 and DA2, and the least frequency in DA1 with 3.6% (Fig. 4b).

PCR screening and sequencing of calli and regenerants

Calli that showed positive PCR results for *nptII* screening were sent for sequencing. Only the heat-stressed calli, co-cultivated at 28°C for 2 d and intermittently heat-stressed at 37°C, showed mutation. The amplicons cloned to pDrive cloning vector were sent for Sanger sequencing which showed indel mutation in three of the clones, deletion of “C” in M6A2 calli, insertion of “A” in DA1 calli and insertion of “A” and “C” in DA2 (Fig. 6).

PCR screening done to detect transgenic regenerants resulted in positive samples from M6A1 regenerants: A11, A13, A19, A20, A21 and A22. However, even though A14 was found to be negative on PCR screening, one of the *ScINVINH1* alleles showed an extra peak, a base substitution. For M6A2, samples B8 and B9 were positive, For DA1, C1 was negative, however survived kanamycin selection, hence C1 was sent for molecular analysis. In DA1 regenerants, C2, C3, C4, C6 and C7 were positive, and for DA2, D1, D2 and D3 were positive. On sequencing the positive regenerants, several peak overlaps were noticed, as compared to the wild-type.

Real time PCR data

These putative plants found from sequencing were then potted in soil (Fig. 7a) and analyzed for *INVINH1* gene expression using real-time PCR. Regenerated plants C8, C9, C10 and C12 had reduced gene expression of *StINVINH1* as compared to wild-type cv. Desiree, except D3 regenerant. Regenerant A22 had reduced gene expression of *ScINVINH1* as compared to the wild-type *S. chacoense* M6 (Fig. 7b).

DISCUSSION

Potato is an imperative food crop possessing high nutritional value [1, 32], and an ever-increasing population makes potato more crucial to fight hunger and poverty [32–34]. However, potato is challenged by biotic and abiotic stress tolerances [35, 36], and the genetic makeup of potato makes its conventional breeding challenging, calling for the need of genome editing [5]. It has been known that CWIN enzymes play a role in immune and defense responses during plant-pathogen interactions [10] and are targeted post-translationally by INVINH1 enzyme [8, 9, 17], suggesting that INVINH1 might possibly regulate plant defense mechanism. Silencing *INVINH1* has also been associated with improvement in seed weight, seed germination, fruit hexose level, chilling tolerance, and delay in leaf senescence [8, 9, 10, 14, 15]. Moreover, potato has several isoforms of *CWIN* genes [18], which makes CRISPR/Cas9 targeting *INVINH1* a viable option.

Our study indicated that there are two alleles in *StINVINH1* gene for cv. Desiree and homozygous *ScINVINH1* gene for *S. chacoense* M6. Similar allelic sequences were also reported by [17]. The control experiments also clearly indicated that the wild-type potato explants are sensitive to kanamycin. On infection with empty AGL1 cells, there was a slight decrease in calli induction, and a further decrease when infected with AGL1 cells harboring empty pGNK-LeCas9-AtU6p-sgRNA vector. This indicated that AGL1 cells affected calli induction and furthermore when the CRISPR vector is harbored by AGL1. Moreover, as compared to the explants treated with empty AGL1 cells, frequent sulcid treatment to kill the growth of *Agrobacterium* was required when infected with AGL1 cells harboring either CRISPR vector or gRNA cloned CRISPR vector. This might indicate that CRISPR vector might have some role in *Agrobacteirum* overgrowth. This overgrowth of *Agrobacterium* was problematic, especially in cv. Desiree, as it affected calli development and regeneration, further exacerbated by the frequent sulcid treatment. Cell mutation due to selection pressure may also cause *Agrobacterium* overgrowth, and this could be addressed by using mutant strain of *Agrobacterium* with sucrose sensitivity, such as GV2260-*SacB/R* [37].

It was clear from the experiment that the highest calli was produced by internode explants for both construct types in *S. chacoense* M6 and cv. Desiree (except for DA1); microtuber was found to be the best explant for regeneration from the calli generated, even though the calli generation from microtuber explant was found to be the least. The highest regeneration frequency in cv. Desiree and *S. chacoense* M6 incubated without bacterial infection and kanamycin pressure denoted that explants from both are capable of regenerating. It was also observed that the regeneration frequency for internode explant infected with AGL1 harboring empty CRISPR vector was much above in contrast to the ones infected with AGL1 harboring gRNA clone CRISPR vectors, indicating gRNA effect on regeneration.

The heat-stressed calli showed mutation probably because Cas9 enzymes perform optimally at higher temperatures [38]. It was also shown by [38] that the heat stress temperature of 37°C was beneficial to improve mutation frequency generated by CRISPR in *Arabidopsis* than those grown at 22°C. Heat stress effect at 37°C was also demonstrated in citrus [38]. It has also been found that the mutagenesis

frequency improved from 50%-63% in transgenic rice lines on heat stress treatment [39]. As the positive transgenic regenerated plants sent for direct sequencing showed overlapping peaks, which was difficult to analyze if it were either substitution, heterozygous or chimeric mutations considering that there was overrepresentation of wild-type sequence. As there is also the cloning option, it would require a great investment of time, tedious cloning and large number of sequencing. Hence, in this study, the putative plants that happened to show peak overlaps were transferred to potted soil and analyzed using RT-qPCR. Our study showed a decrease in *INVINH1* gene expression in C8, C9, C10, C12 (cv. Desiree) and A22 (*S. chacoense* M6) indicating that the *INVINH1* gene expression level of these plants may have been affected as a result of the targeted mutagenesis. As the gRNA1 targeting site is in proximity to the start codon, and also targets phosphorylation sites, this might have resulted in the gene expression differences between D3 and the other regenerants.

Our study examined the sequencing data of putative lines from direct sequencing only without cloning the alleles. A study obtained genetically chimeric plants [40] and has reported reduced SBE1 protein in their study, which may explain the reduced gene expression in our study. There was a decline in poplar chimeric mutation with “second round of regeneration” which resulted in homozygous mutants [41]; similar work can be done in the future. Veillet et al. (2019) [42] has also generated lines which could be potentially chimerical event [42]. In potato genome editing via CRISPR-SaCas9 and cytosine base editors (SaCBE), similar type of sequencing trace data as obtained in our study can be observed [43].

CONCLUSION

In this study, we reported two alleles of *StINVINH1* gene in cv. Desiree and homozygosity in case of *ScINVINH1* gene in in *S. chacoense* M6. The study showed that the CRISPR vector and gRNA may affect calli induction and regeneration and the heat stress treatment can lead to chances of successful mutagenesis. Furthermore, Sanger sequencing of calli showed presence of mutagenesis, and the putative regenerants showed decreased *INVINH1* gene expression. Further studies are needed at the protein level, and to unravel the role of INVINH1 enzyme in plant defense and/or stress response.

Abbreviations

A1 construct: AGL-1 cells harboring gRNA1 in CRISPR/Cas9 construct; **A2 construct**: AGL-1 cells harboring gRNA2 in CRISPR/Cas9 construct; **CIM**: callus inducing media; **CRISPR**: clustered regularly interspaced short palindromic repeat/**Cas9**: CRISPR-associated nuclease 9; **CTAB**: cetyl trimethylammonium bromide; **cv.**: cultivar; **CWIN**: apoplastic (cell wall) invertase; **d**: days; **DA1**: cv. Desiree infected with A1 construct; **DA2:** cv. Desiree infected with A2 construct; **GA_3**: Gibberellic acid; **gRNA**: guide RNA; **IAA**: indole-3-acetic; **IBA**: indole-3-butyric acid; **INVINH1**: apoplastic invertase inhibitor; **INVINH2**: vacuolar invertase inhibitor; **M6A1**: *S. chacoense* M6 infected with A1 construct; **M6A2**: *S. chacoense* M6 infected with A2 construct; ***nptII***: neomycin phosphotransferase II; **PCR**: polymerase chain reaction; **RGM**: root generating media; **RT-qPCR**: Reverse transcriptase quantitative PCR; **sgRNA**: single-guided RNA; **SIM:** shoot regeneration media.

Declarations

All authors declare that they have no conflict of interests.

Funding

We would like to thank TUBITAK for awarding the first author Sarbesh Das Dangol with Graduate Scholarship Programme for International Students-2215 (PhD).

Availability of data and materials

The data presented in this paper is original.

Acknowledgment

We are thankful to Dr. Abdellah Barakate who created the CRISPR vector, designed and supervised the protocols for gene editing plant transformation.

Contribution of authors

The work presented in this paper is a part of PhD thesis work of Sarbesh Das Dangol who conducted experiments, recorded data, and wrote the manuscript. Mehmet Emin Çalışkan (PhD supervisor) and Allah Bakhsh (PhD Co-Supervisor) supervised the study, designed the experimentations and critically read the manuscript, and presented it in its current form. The PhD thesis monitory committee of Sarbesh Das Dangol reviewed and evaluated the study and supervised the experimentation and protocols.

Ethical approval

There is no human participants or animals performed by any of the authors.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This research complies with all ethical standards required to manipulate plasmid vectors and bacterial cells and manipulation of potato plants without involvement of human participants nor animal candidates.

References

1. Hong Z, Fen X, Yu W, Hong-hai H, Xiao-feng D (2017) Progress of potato staple food research and industry development in China. J Integr Agric 16:2924-2932. https://doi.org/10.1016/S2095-3119(17)61736-2
2. Craze M, Bates R, Bowden S, Wallington EJ (2018) Highly efficient *Agrobacterium*-mediated transformation of potato (*Solanumtuberosum*) and production of transgenic microtubers. Curr Protoc Plant Biol 3:33–41. https://doi.org/10.1002/cppb.20065

3. Hameed A, Zaidi SS, Shakir S, Mansoor S (2018) Applications of new breeding technologies for potato improvement. Front Plant Sci 9:925. https://doi.org/10.3389/fpls.2018.00925
4. Leisner CP, Hamilton JP, Crisovan E, Manrique-Carpintero NC, Marand AP, Newton L, Pham GM, Jiang J, Douches DS, Jansky SH, Buell CR (2018) Genome sequence of M6, a diploid inbred clone of the high-glycoalkaloid-producing tuber-bearing potato species *Solanum chacoense*, reveals residual heterozygosity. Plant J 94:562-570. https://doi.org/10.1111/tpj.13857
5. Andersson M, Turesson H, Olsson N, Fält AS, Olsson P, Gonzalez MN, Samuelsson M, Hofvander P (2018) Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. Physiol Plant 164:378–384. https://doi.org/10.1111/ppl.12731
6. Zhou H, Liu B, Weeks DP, Spalding MH, Yang B (2014) Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. Nucleic Acids Res 42:10903-10914. https://doi.org/10.1093/nar/gku806
7. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science 337:816-821. https://doi.org/10.1126/science.1225829
8. Jin Y, Ni D-A, Ruan Y-L (2009) Posttranslational elevation of cell wall invertase activity by silencing its inhibitor in tomato delays leaf senescence and increases seed weight and fruit hexose level. Plant Cell 21:2072-2089. https://doi.org/10.1105/tpc.108.063719
9. Tang X, Su T, Han M, Wei L, Wang W, Yu Z, Xue Y, Wei H, Du Y, Greiner S, Rausch T, Liu L (2017) Suppression of extracellular invertase inhibitor gene expression improves seed weight in soybean (*Glycine max*). J Exp Bot 68:469-482. https://doi.org/10.1093/jxb/erw425
10. Su T, Han M, Min J, Zhou H, Zhang Q, Zhao J, Fang Y (2020) Functional characterization of invertase inhibitors PtC/VIF1 and 2 revealed their involvements in the defense response to fungal pathogen in *Populus trichocarpa*. Front Plant Sci 10:1654. https://doi.org/10.3389/fpls.2019.01654
11. Tauzin AS, Giardina T (2014) Sucrose and invertases, a part of the plant defense response to the biotic stresses. Front Plant Sci 5:293. https://doi.org/10.3389/fpls.2014.00293
12. Sun L, Yang DL, Kong Y, Chen Y, Li XZ, Zeng LJ, Li Q, Wang E-T, He Z-H (2014) Sugar homeostasis mediated by cell wall invertase GRAIN INCOMPLETE FILLING 1 (GIF1) plays a role in pre-existing and induced defence in rice. Mol Plant Pathol 15:161–173. https://doi.org/10.1111/mpp.12078
13. Sonnewald S, Priller JPR, Schuster J, Glickmann E, Hajirezaei MR, Siebig S, Mudgett MB, Sonnewald U (2012) Regulation of cell wall-bound invertase in pepper leaves by *Xanthomonas campestris* pv. *vesicatoria* type three effectors. PLoS One 7:e51763. https://doi.org/10.1371/journal.pone.0051763
14. Xu X, Hu Q, Yang W, Jin Y (2017) The roles of cell wall invertase inhibitor in regulating chilling tolerance in tomato, BMC Plant Biol 17:195. https://doi.org/10.1186/s12870-017-1145-9
15. Su T, Wolf S, Han M, Zhao H, Wei H, Greiner S, Rausch T (2016) Reassessment of an *Arabidopsis* cell wall invertase inhibitor AtCIF1 reveals its role in seed germination and early seedling growth. Plant Mol Biol 90:137–155. https://doi.org/10.1007/s11103-015-0402-2

16. Bonfig KB, Gabler A, Simon UK, Luschin-Ebengreuth N, Hatz M, Berger S, Muhammad N, Zeier J, Sinha AK, Roitsch T (2010) Post-translational derepression of invertase activity in source leaves via down-regulation of invertase inhibitor expression is part of the plant defense response. Mol Plant 3:1037–1048. https://doi.org/10.1093/mp/ssq053
17. Datir SS, Latimer JM, Thomson SJ, Ridgway HJ, Conner AJ, Jacobs JME (2012) Allele diversity for the apoplastic invertase inhibitor gene from potato. Mol Genet Genomics 287:451-460. https://doi.org/10.1007/s00438-012-0690-z
18. Datir S, Ghosh P (2020) In silico analysis of the structural diversity and interactions between invertases and invertase inhibitors from potato (*Solanum tuberosum* L.). 3 Biotech 10:178. https://doi.org/10.1007/s13205-020-02171-y
19. Jansky SH, Chung YS, Kittipadukal P (2014) M6: A diploid potato inbred line for use in breeding and genetics research. J Plant Regist 8:195-199. https://doi.org/10.3198/jpr2013.05.0024crg
20. Enciso-Rodriguez F, Manrique-Carpintero NC, Nadakuduti SS, Buell CR, Zarka D, Douches D (2019) Overcoming self-incompatibility in diploid potato using CRISPR-Cas9. Front Plant Sci 10:376. https://doi.org/10.3389/fpls.2019.00376
21. Dangol SD, Yel I, Caliskan ME, Bakhsh A (2020) Manipulating genome of diploid potato inbred line *Solanum chacoense* M6 using selectable marker gene. Turk J Agric For 44:399-407. https://doi.org/10.3906/tar-1910-13
22. Twell, D, Ooms G (1988) Structural diversity of the patatin gene family in potato cv. Desiree. Mol Gen Genet 212:325-336. https://doi.org/10.1007/BF00334703
23. Haesaert G, Vossen JH, Custers R, Loose MD, Haverkort A, Heremans B, Hutten R, Kessel G, Landschoot S, Droogenbroeck BV, Visser RGF, Gheysen G (2015) Transformation of the potato variety Desiree with single or multiple resistance genes increases resistance to late blight under field conditions. Crop Prot 77:163-175. https://doi.org/10.1016/j.cropro.2015.07.018
24. Wang ES, Kieu NP, Lenman M, Andreasson E (2020) Tissue culture and refreshment techniques for improvement of transformation in local tetraploid and diploid potato with late blight resistance as an example. Plants 9:695. https://doi.org/10.3390/plants9060695
25. Butler NM, Atkins PA, Voytas DF, Douches DS (2015) Generation and inheritance of targeted mutations in potato (*Solanum tuberosum* L.) using the CRISPR/Cas system. PLoS ONE 10:e0144591. https://doi.org/10.1371/journal.pone.0144591
26. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15:473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x
27. Donmez BA, Dangol SD, Bakhsh A (2019) Transformation efficiency of five *Agrobacterium* strains in diploid and tetraploid potatoes. Sarhad J Agric 35:1344-1350. http://dx.doi.org/10.17582/journal.sja/2019/35.4.1344.1350
28. Turkmen AK, Yavuz C, Dangol SD, Tarım C, Demirel U, Çalışkan ME (2017) Evaluation of micro tuberization performances of different genotypes. Turk J Agric- Food Sci Tech 5:353-357. https://doi.org/10.24925/turjaf.v5i4.353-357.1203

29. Sahoo KK, Tripathi AK, Pareek A, Sopory SK, Singla-Pareek SL (2011) An improved protocol for efficient transformation and regeneration of diverse indica rice cultivars. Plant Methods 7:49. https://doi.org/10.1186/1746-4811-7-49
30. Nicot N, Hausman JF, Hoffman L, Evers D (2005) Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J Exp Bot 56:2907-2914. https://doi.org/10.1093/jxb/eri285
31. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. Methods 25:402-408. https://doi.org/10.1006/meth.2001.1262
32. Caliskan ME, Onaran H, Arioglu H (2010) Overview of the Turkish potato sector: challenges, achievements and expectations. Potato Res 53:255-266. https://doi.org/10.1007/s11540-010-9170-1
33. Raymundo R, Asseng S, Robertson R, Petsakos A, Hoogenboom G, Quiroz R, Hareau G, Wolf J (2018) Climate change impact on global potato production. Eur J Agron 100:87-98. https://doi.org/10.1016/j.eja.2017.11.008
34. Anonymous (2011) FAO in the 21st century: Ensuring food security in a changing world. http://www.fao.org/3/i2307e/i2307e.pdf
35. Chacon-Cerdas R, Barboza-Barquero L, Albertazzi FJ, Rivera-Méndez W (2020) Transcription factors controlling biotic stress response in potato plants. Physiol Mol Plant Pathol 112:101527. https://doi.org/10.1016/j.pmpp.2020.101527
36. Handayani T, Watanabe K (2020) The combination of drought and heat stress has a greater effect on potato plants than single stresses. Plant Soil Environ 66:175-182. https://doi.org/10.17221/126/2020-PSE
37. Liu Y, Miao J, Traore S, Kong D, Liu Y, Zhang X, Nimchuk ZL, Liu Z, Zhao B (2016) *SacB-SacR* gene cassette as the negative selection marker to suppress *Agrobacterium* overgrowth in *Agrobacterium*-mediated plant transformation. Front Mol Biosci 3:70. https://doi.org/10.3389/fmolb.2016.00070
38. LeBlanc C, Zhang F, Mendez J, Lozano Y, Chatpar K, Irish VF, Jacob Y (2018) Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. Plant J 93:377-386. https://doi.org/10.1111/tpj.13782
39. Nandy S, Pathak B, Zhao S, Srivastava V (2019) Heat-shock-inducible CRISPR/Cas9 system generates heritable mutations in rice. Plant Direct 3:e00145. https://doi.org/10.1002/pld3.145
40. Tuncel A, Corbin KR, Ahn-Jarvis J, Harris S, Hawkins E, Smedley MA, Harwood W, Warren FJ, Patron NJ, Smith AM (2019) Cas9-mediated mutagenesis of potato starch-branching enzymes generates a range of tuber starch phenotypes. Plant Biotechnol J 17:2259-2271. https://doi.org/10.1111/pbi.13137
41. Ding L, Chen Y, Ma Y, Wang H, Wei J (2020) Effective reduction in chimeric mutants of poplar trees produced by CRISPR/Cas9 through a second round of shoot regeneration. Plant Biotechnol Rep 14:549–558. https://doi.org/10.1007/s11816-020-00629-2

42. Veillet F, Perrot L, Chauvin L, Kermarrec MP, Guyon-Debast A, Chauvin JE, Nogué F, Mazier M (2019) Transgene-free genome editing in tomato and potato plants using *Agrobacterium*-mediated delivery of a CRISPR/Cas9 cytidine base editor. *Int J Mol Sci 20*:402. http://doi.org/10.3390/ijms20020402
43. Veillet F, Kermarrec MP, Chauvin L, Chauvin JE, Nogué F (2020) CRISPR-induced indels and base editing using the *Staphylococcus aureus* Cas9 in potato. Plos one 15:e0235942. https://doi.org/10.1371/journal.pone.0235942

Figures

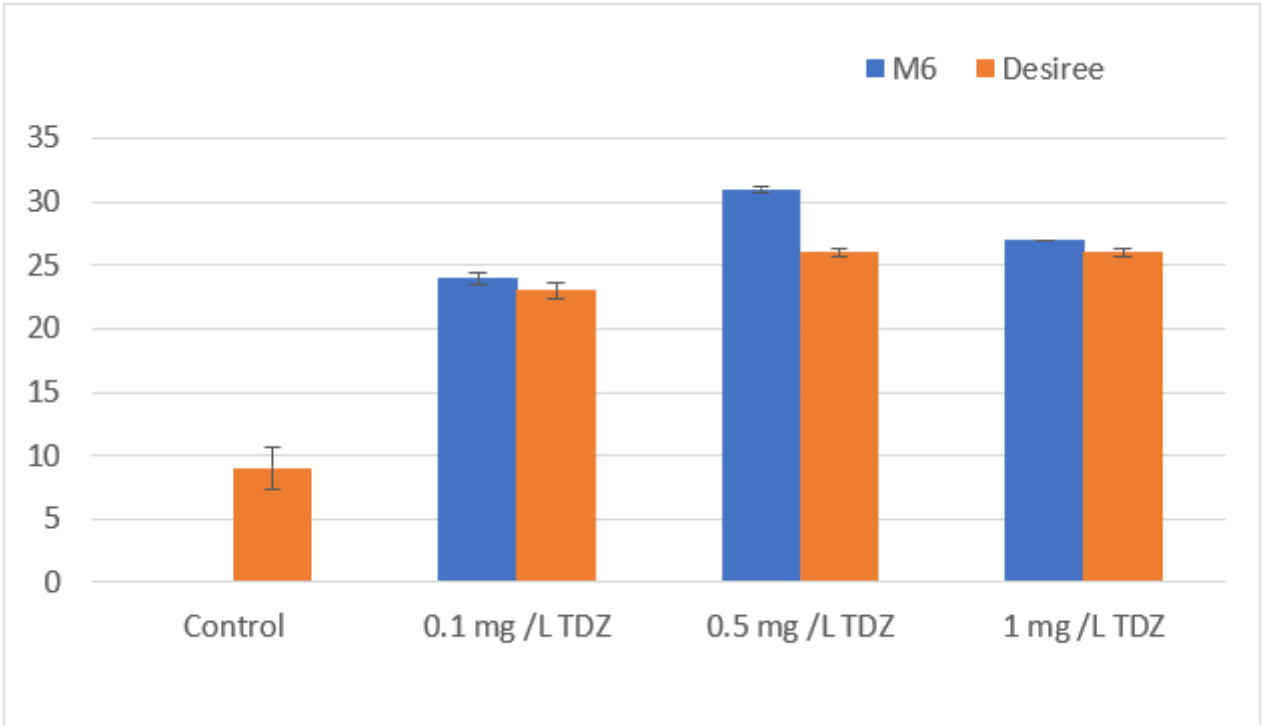


Figure 1

Number of microtubers in cv. Desiree and *S. chacoense* M6 under different concentrations of thidiazuron

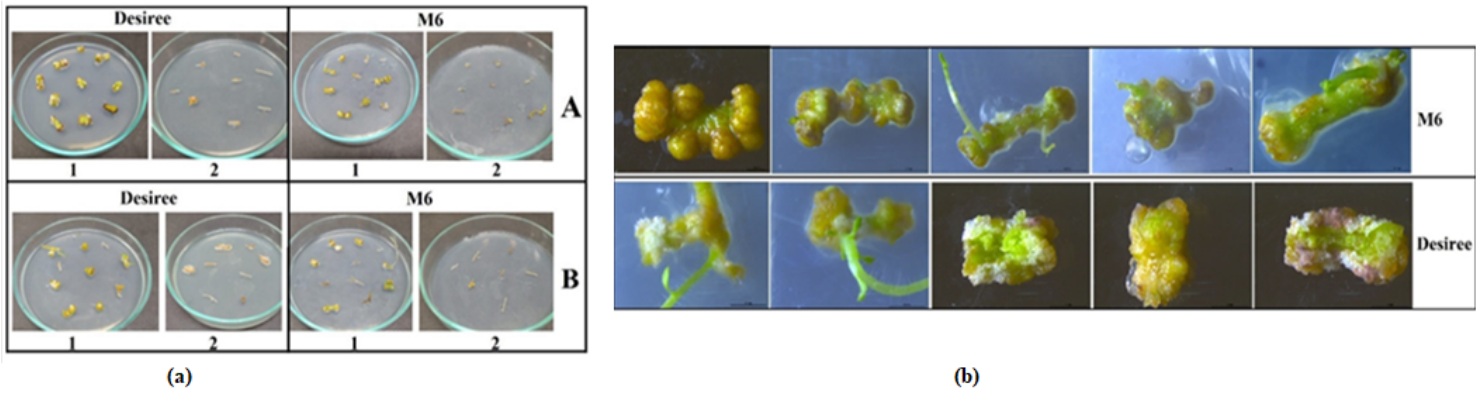


Figure 2

Control experiments in internode explants: calli induction on CIM- A line: AGL1-∅(empty) and B line: AGL1 with empty CRISPR vector (1: without kanamycin, 2: with kanamycin) (a), and calli developed from explants under microscope (b)

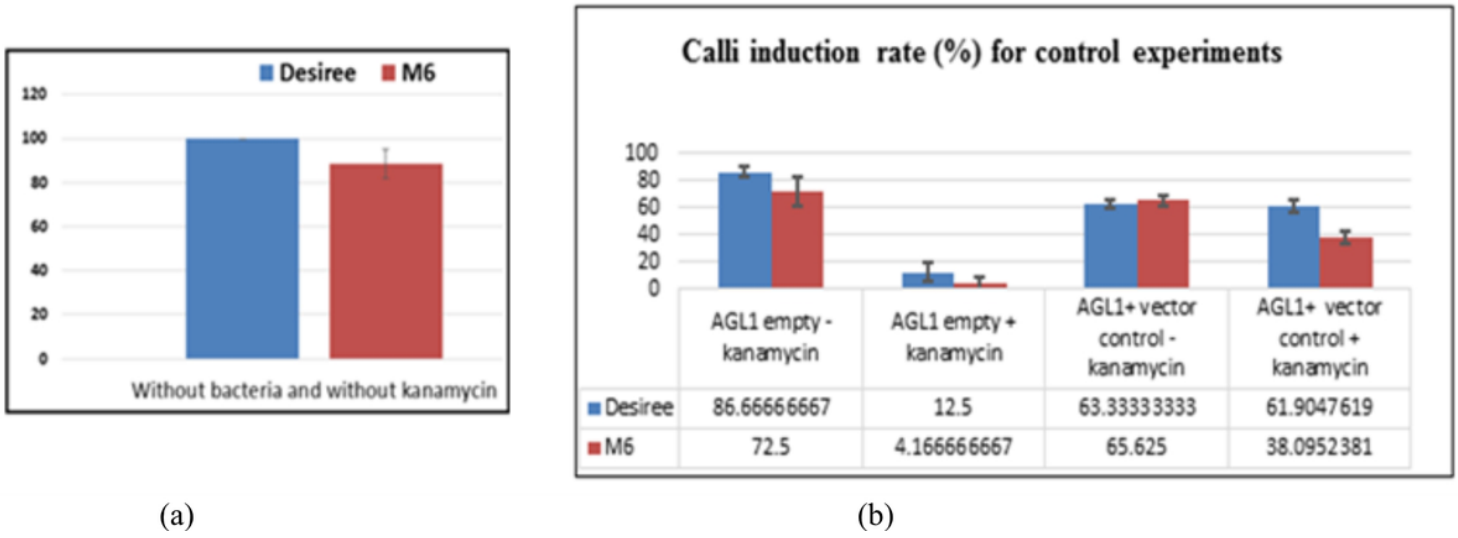


Figure 3

Control experiments in internode explants. Bar graph showing calli induction rate (%) without bacteria and without kanamycin (a), and bar graph showing calli induction rate (percentage) for control experiments (b)

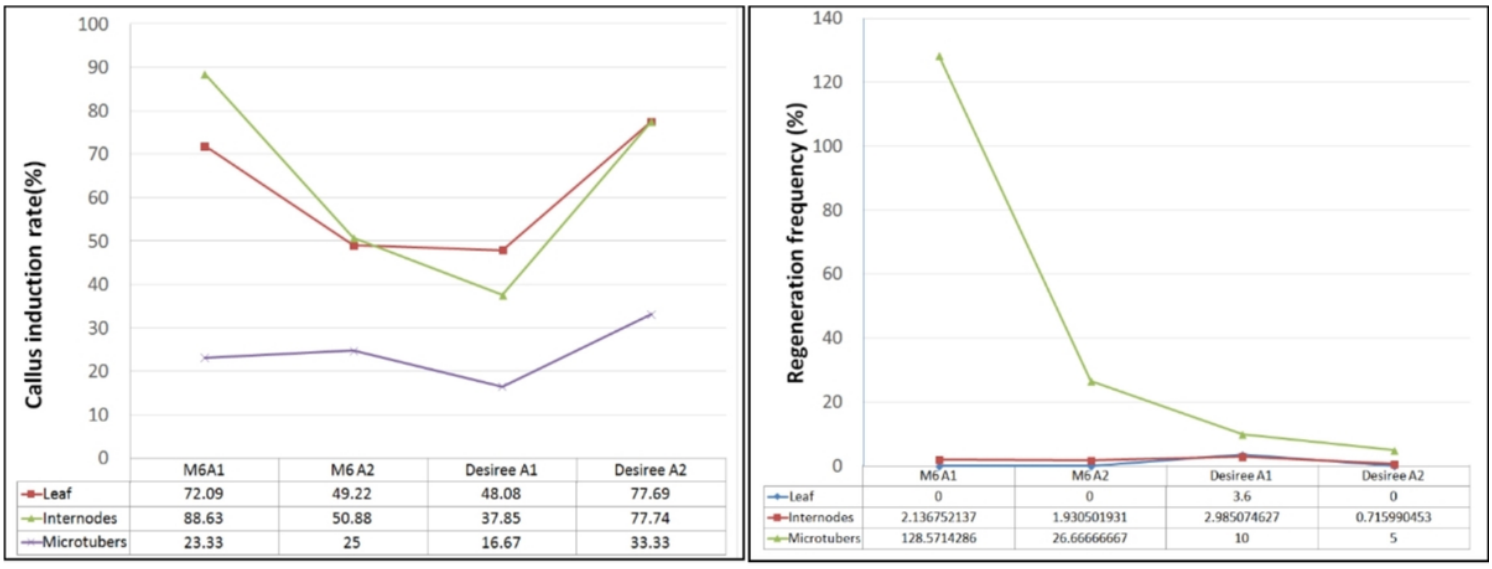


Figure 4

Line graph depicting callus induction (%) for various explants using A1 and A2 constructs in *S. chacoense* M6 and cv. Desiree (a), and line graph depicting regeneration frequency (%) in different explants infected with A1 and A2 constructs for *S. chacoense* M6 and cv. Desiree (b)

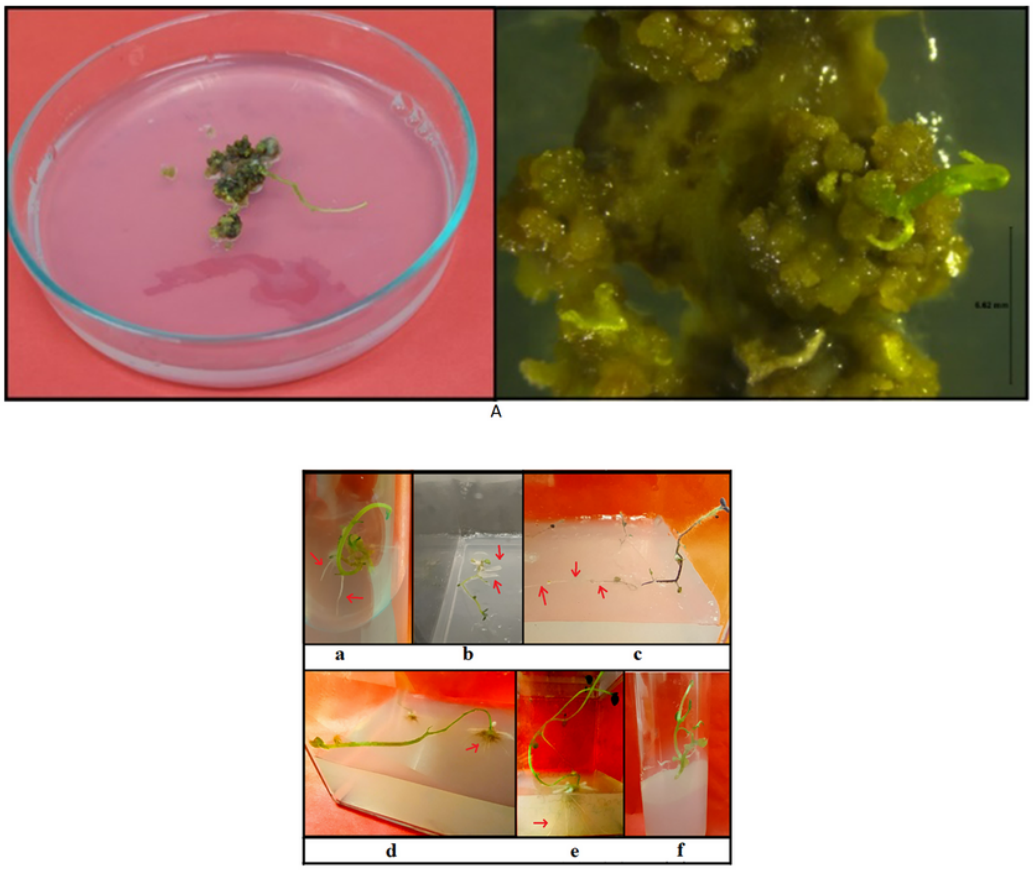


Figure 5

Regeneration from calli of leaf explant (a), and candidate regenerants cultured in RGM: rooting on media supplemented with IBA (roots pointed by red arrows), and no rooting on IAA supplementation ‘f’ (b)

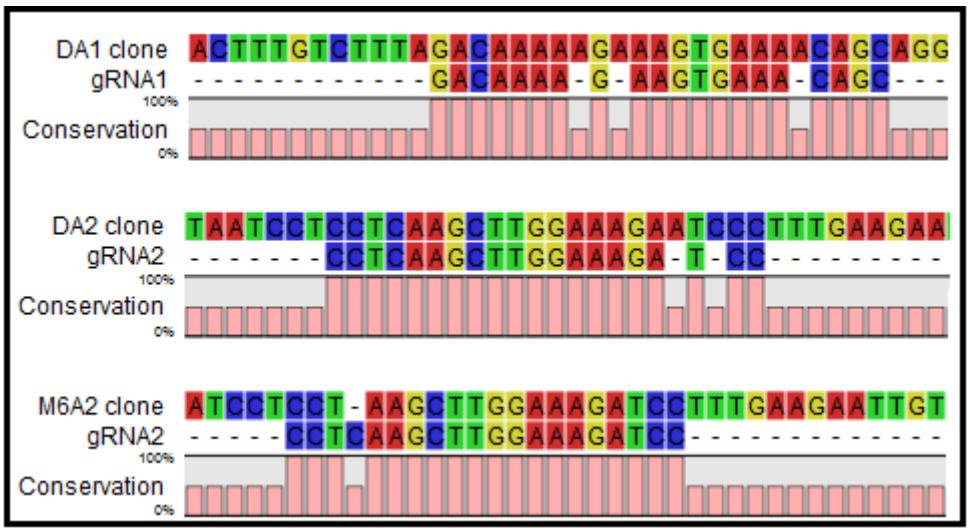
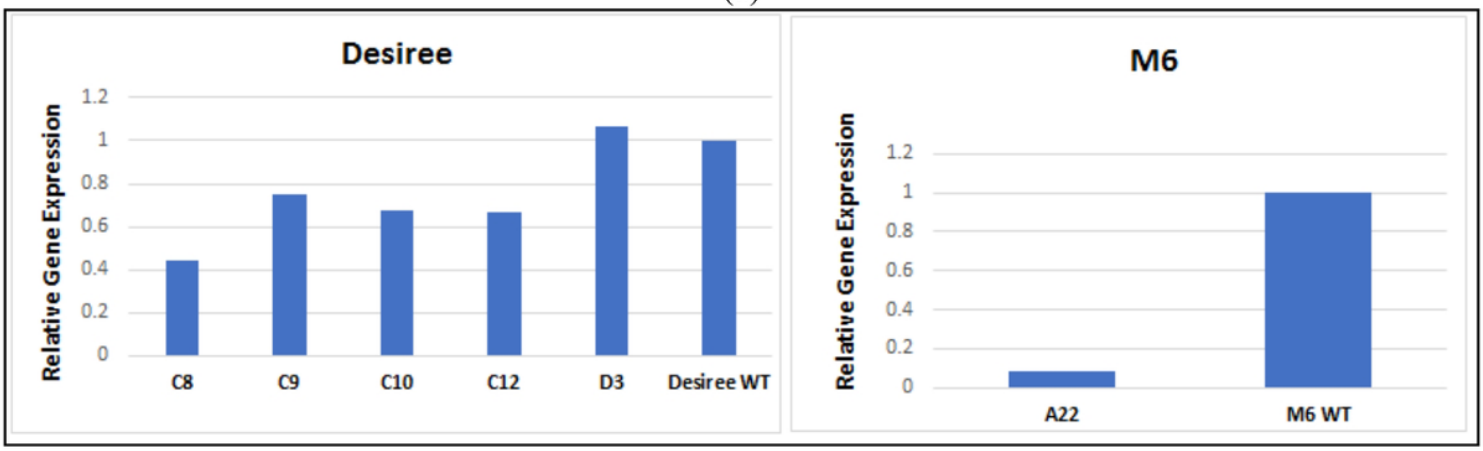


Figure 6

Cloned gene fragment of transformed calli were sent for sequencing and aligned to gRNA1 or gRNA2 (CLC Main Workbench 8, Qiagen). Sanger sequencing was performed by Sentebiolab, Ankara, Turkiye.

(a)



(b)

Figure 7

Transfer of the putative and wildtype potato plants to the pots (a), and relative gene expression analysis (*INVINH1* gene) of putative transgenics compared against the wildtypes using real-time PCR based method (b)