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Manipulating genome of diploid potato inbred line *Solanum chacoense* M6 using selectable marker gene

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Abstract: The development of transgenic potatoes is imperative to investigate various gene functions as well as to develop robust potato varieties resistant to different biotic and abiotic stresses. Directing a potato breeding program from cultivated tetraploids to self-compatible diploid lines will be highly helpful for potato breeders. However, diploid potatoes are considered recalcitrant to regeneration. We aimed to develop a protocol for the transformation of diploid *Solanum chacoense* M6 potatoes using leaf, internodes, and microtubers as the explants. *Agrobacterium tumefaciens* strain GV2260 harboring pBIN19 expression vector containing *gusA* gene (interrupted by an intron to induce expression from eukaryotic cells) was used for this purpose. Different inoculation times (10, 20, 30 min) were applied in aforementioned explants for gene transfer. After cocultivation, explants were transferred to the media with various hormone concentrations [6–benzylaminopurine (BAP), 1–naphthaleneacetic acid (NAA), trans-zeatin, kinetin, and 2, 4–Dichlorophenoxyacetic acid (2,4–D)]. The calli generated were then transferred to shoot generating medium supplemented with thidiazuron (TDZ) and gibberellic acid (GA_3). According to histochemical GUS analysis, we found a 20 min inoculation time to be optimal for gene transfer and the medium containing 2 mg L^{-1} BAP and 2 mg L^{-1} NAA was the most suitable medium for callus induction from 20 min inoculated explants (41% callus formation for internodes and 65% for leaf explants). Abundant transcripts levels of *gusA* gene was found in primary transformants when subjected to RT-qPCR. GUS fluorometric assay further confirmed the primary transformants at protein level. The present study can serve as a gateway to transfer gene(s) of interest in diploid potatoes.

Keywords: Diploid potatoes, genetic improvement, Agrobacterium-mediated transformation, Solanum chacoense M6

1. Introduction

Potato is a non-cereal tuber-bearing food crop crucial in terms of food security worldwide. Potato is primitively domesticated and consumed in North America, Europe, former USSR nations, and now a large number of cultivations are carried out in South Africa, Asia, and South America. This makes potato the world's fourth most imperative crop in terms of production (Hong et al., 2017; Craze et al., 2018). It has been reported that worldwide potato production was 388,190,674 tonnes with the area cultivated on 19,302,642 hectares (FAOSTAT, 2017). In Turkey, potato was sowed on an area of 1,359,373 hectares with a production of 4,550,000 tons in 2018 (TÜİK, 2018). Domesticated potatoes are tetraploids, and the problem with the cultivated tetraploid potato (2n = 4x = 48) (Nadolska–Orczyk et al., 2007) is that it is highly heterozygous in nature and the impediments that challenge its breeding program is contributed by several factors such as inbreeding depression and its ploidy levels with reduced fertility (Reviewed in Leisner et al., 2018).

The heterozygous and tetraploid potato further perplex the modern bioinformatics tools that are used to differentiate real mutations generated by gene editing technologies and those that arise from the errors generated from sequencing (Dangol et al., 2019). A diploid potato (2n = 2x = 24) such as *Solanum chacoense* M6 (Reg. No. GP-1, BS 228; hereafter will be written as M6) could be a candidate for researchers as it is a self-compatible, homozygous inbred line. Its homozygosity was achieved by self-pollinating the diploid wild potato relative *S. chacoense* for seven generations (Jansky et al., 2014) The self-compatibility in M6 is due to the *Sli* gene (S-locus inhibitor) which precludes the gametophytic self-incompatibility (Hosaka and Hanneman, 1998; Jansky et al., 2014; Enciso–Rodriguez et al., 2019). Further, M6 could be a good choice in generating gene edited plants as there could be difficulty in obtaining homozygous mutants in cultivated tetraploid with all four alleles of the targeted gene knocked out simultaneously at a given time (Dangol et al., 2019).

Diploid M6 potato further has beneficial qualities such as increased dry matter content, resistance against the

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diseases and the quite suitable quality of chips processing (Jansky et al., 2014; Leisner et al., 2018). Potato breeding can be reinvented in that we can direct its breeding in diploid inbred lines as a hybrid F1 variety and with introgression of desired traits in germplasms of diploid potato (Enciso–Rodriguez et al., 2019). However, M6 lines still possess residual heterozygosity (Jansky et al., 2014; Leisner et al., 2018). The high glycoalkaloid content of the M6 tubers can be toxic imparting bitter taste quality (McKenzie and Corrigan, 2016; Uluwaduge, 2018; Enciso–Rodriguez et al., 2019).

Nonetheless, it will be interesting to further interrogate the genes of interest and other molecular questions with the use of M6 diploid potato as a candidate potato with available molecular biology techniques and gene editing technologies. With gene editing technology, we can fix targeted mutations in self-compatible diploid lines such as M6 with more opportunities in functional genomics (Butler et al., 2015). The undesired characters can further be eliminated by crossing it with other diploid potatoes and/or eliminating them via gene editing technologies and transgenic manipulations. So far, three whole genome sequences for potato crop are available, namely doubled monoploid *S. tuberosum* Phujera DM1–3 in 2011, diploid *Solanum commersonii* in 2015 which is a wild potato species and recently diploid M6 in 2018 (The Potato Genome Sequencing Consortium, 2011; Aversano et al., 2015; Leisner et al., 2018). The availability of M6 genome database is an added advantage in the field of functional genomics and gene editing technologies. Although several studies have been conducted in diploid potatoes (Ducreux et al., 2005; Nadolska–Orczyk et al., 2007; Cardi et al., 1992), diploid inbred line M6 provides greater opportunity in functional genomics studies of potato and in fixing targeted mutations (Butler et al., 2015). In this study, we have attempted to optimize the efficient *Agrobacterium* mediated transformation for diploid M6 potato to open gateway for any further genetic improvement in diploid potatoes.

2. Materials and methods

2.1. Plant material

Diploid inbred potato clone (M6) was kindly provided by Dr. Abdellah Barakate and Dr. Csaba Hornyik (Cell and Molecular Sciences, James Hutton Institute, Dundee, UK). M6 plantlets were subcultured every 3–4 weeks on MS (Murashige and Skoog, 1962) medium for micropropagation and maintenance. The M6 plants were grown at 24 ± 2 °C under fluorescent light with a 16/8 h (light/dark) photoperiod in the growth chamber. For transformation of M6 line, internodes, leaf and microtuber discs were used as explants. Microtubers were produced in semisolid MS medium (liquid MS dipped in sterile cotton) with different TDZ concentrations (0 mg L^{-1}, 0.1 mg L^{-1}, 0.5 mg L^{-1} and 1 mg L^{-1}) under the dark condition (covered with aluminum foil) for 6 weeks as described earlier according to Türkmen et al. (2017).

2.2. *Agrobacterium*-mediated transformation and GUS histochemical analyses

Agrobacterium strain GV2260 was electroporated (using electroporation device Bio-RAD GenePulser Xcell) with pBIN19 vector that harbored *gusA* reporter gene and *nptII* that encoded resistance against kanamycin for plant selection (Figure 1). The positive clones were identified using the *gusA* gene-specific primers (FP**:** 5' CCCTTACGCTGAAGAGATGC 3' and RP: 5' GAGCGTCGCAGAACATTACA 3') and chosen for *Agrobacterium*-mediated transformation experiments.

From our previous study comparing transformation efficiencies in tetraploid potatoes using five different *Agrobacterium* strains, we found that GV2260 gave the best histochemical GUS assay as well as callus induction rates (Dönmez et al., 2019). Hence, we considered GV2260 for our further experiments. The transformed GV2260 *Agrobacterium* strain harboring recombinant plasmid was grown overnight in LB broth containing 50 mg L^{-1} kanamycin. After the bacterial culture reached to an OD_{600} value of 0.8, they were centrifuged at 5000 rpm at 4 °C for 10 min and resuspended in liquid MS medium. The leaf disc, microtuber and internode explants were wounded and pre-treated with 15 mL of liquid MS medium and inoculated with 2 mL of the bacterial suspension and shaken intermittently. We optimized the inoculation time at various intervals (10, 20, 30 min) for leaf discs and internode explants, whereas 20 min inoculation time was used for microtuber explants. We did not optimize the inoculation time for microtuber explants due to its limited number. According to the histochemical GUS analyses conducted for leaf and internode explants in three replicates, a 20 min inoculation time was applied for each of the three explants in further experiments (three replicates each for leaf and internode explants, each of the three replicates containing 30 explants). After inoculation, explants were dried on sterile filter paper and transferred to cocultivation medium (MS medium with 100 mg L^{-1} acetosyringone for two days). Randomly selected explants were used for histochemical GUS assay (three replicates) by immersing the explants into GUS staining solution (100 mg L^{-1} X-gluc, 10 mM EDTA, 100 mM NaH_2PO_4, 0.1% Triton X100, 50% methanol (pH adjusted to 8)) overnight at 37 °C. The next day, explants were washed with 70% ethanol and observed under the microscope. After one month, explants were subcultured into the fresh callus inducing media (CIM). Three different CIM each containing 25 mg L^{-1} kanamycin and 150 mg L^{-1} Sulcid (CIM–1: 0.1 mg L^{-1} kinetin and 1 mg L^{-1} 2,4–D; CIM–2: 2 mg L^{-1} BAP, 0.2 mg L^{-1} NAA, 1 mg L^{-1} trans-zeatin and 1 mg L^{-1} kinetin; CIM–3: 2 mg L^{-1} BAP and 2 mg L^{-1} NAA) were

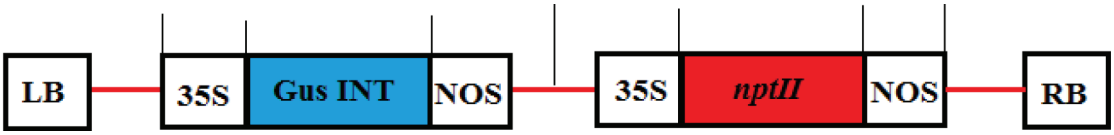

Figure 1. Schematic representation of pBIN19 containing *gusA* and *nptII* driven by CaMV35S promoter and nos terminator within T–DNA (The construct has *nptII* gene that encodes resistance to kanamycin used at100 mg L^{-1} concentration).

used for calli selection and formation. For regeneration, shoot induction medium (SIM: 2 mg L^{-1} TDZ, 0.2 mg L^{-1} GA_3, 25 mg L^{-1} kanamycin and 75 mg L^{-1} sulcid antiobiotic) was used, followed by subculture in SIM every two weeks.

2.3. Molecular Evaluation of Primary Transformants

The genomic DNA was extracted using CTAB method from randomly selected calli and the regenerated plantlets for molecular analyses. Gene-specific primers were used for confirming *nptII* gene (FP:5'TTGCTCCTGCCGAGAAAG 3'; RP: 5' GAAGGCGATAGAAGGCGA 3') and *gusA* gene (FP**:** 5' CCCTTACGCTGAAGAGATGC 3' and RP: 5' GAGCGTCGCAGAACATTACA 3').

Total RNA was extracted from the control non-transgenic M6 potato and transgenic plants (Y001 and Y008) using Aurum™ Total RNA Mini Kit (Bio-RAD, Cat no: #732-6820), quantified using spectrophotometer and confirmed via agarose gel electrophoresis. *DNase* I (Thermo Scientific) treatment was performed to get rid of genomic DNA from the RNA preparations. First strand cDNA was synthesized using oligo $(dT)_{18}$ primer according to the instructions provided by Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (#K1621). The first strand cDNA preparations from control and transgenic samples were diluted (1:10) and used as template in RT–qPCR assay to quantify transcripts levels of *gusA* gene. The specific *gusA* primers used for this assay were FP: 5' CACACCGATACCATCAGCGA 3' and RP: 5' GCTAACGTATCCACGCCGTA 3'. The reaction mixture was incubated at 95 °C for 15 min, followed by 40 cycles at 95 °C for 10 s, 55 °C for 15 s, 72 °C for 20 s by using Rotor Gene Q. After the amplification was completed, a melting curve analysis was performed to determine whether PCR produced only one product. The melting curve analysis was carried out by incubation at 99 °C to 70 °C with a transition rate of 1 °C min^{-1}.

For normalization, elongation factor 1α (*ef1α*) was selected as reference gene for quantifying the expression of genes (Nicot et al., 2005). The threshold values of samples in target gene expression analysis were analyzed by software supplied with the Rotor Gene instrument (QIAGEN). According to the standard deviations of the RT–qPCR, resulting data was calculated and the expression level of the genes was determined according to the $2^{-\Delta\Delta C}{}_T$ proportional calculation method (Livak and Schmittgen, 2001).

2.4. Fluorogenic assay protocol

Gene reporter systems have become an invaluable tool for the study of gene expression regulation in plant research. According to GoldBio (USA) protocol, 100 mg of *non-transgenic* and transgenic plant samples were ground to powder using liquid nitrogen. The powder from each sample was homogenized in protein extraction buffer (50 mM phosphate buffer, pH 7; 10 mM dithiothreitol; 1 mM Na_2EDTA; 0.1% sodium lauryl sarcosine; 0.1% triton X100). The protein extract was obtained by centrifugation (15000 rpm for 5 min at 4 °C). Pre-warmed (37 °C) 500 µL assay buffer (1 mM 4–methyl umbelliferyl beta–D–glucuronide) was added to the 50 µL extract, mixed by pipetting and vortexed. The samples were kept at 37 °C and GUS activity was observed under UV light during 1 h, 3 h and overnight time intervals (GoldBio, 2018).

3. Results

Histochemical GUS assay was performed from the explants (internodes and leaves) inoculated at different times. Leaf and internode explants inoculated for 20 min produced the best result compared to other inoculation times (Figure 2). It was found that 30 min of inoculation time was better than 10 min in internode explants and 10 min was better than 30 min in leaf explants. We compared the histochemical GUS staining of different explants with 20 min inoculation time and we observed that leaf explants have the best staining and it was followed by internode and microtuber explants respectively (Figure 3).

The callus formation in leaf and internodal explants was recorded only in CIM–2 and CIM–3, whereas no callus induction was noticed in CIM–1 supplemented with 2,4–D and kinetin. For internodal explants, CIM–3 had the highest calli inducing rates inoculated for 10 min (75%) and it was followed by 20 min (67%). CIM–2 showed equal response at a 10 min and 30 min (60%) inoculation time, whereas the least response was obtained from a 20 min inoculation time (45%). A 30 min inoculation time cultured in CIM–3 induced the fewest calli (33%) (Figure 4).

No callus formation was recorded in CIM–1 for leaf explants. For leaf explants, CIM–3 was found to be the best CIM when inoculated at 30 min (83%) and 10 min (80%). Callus induction of leaf explants in CIM–2 and CIM–3 was found to be similar for a 20 min inoculation time (66%). Leaf explants inoculated for 30 min (40%) showed better

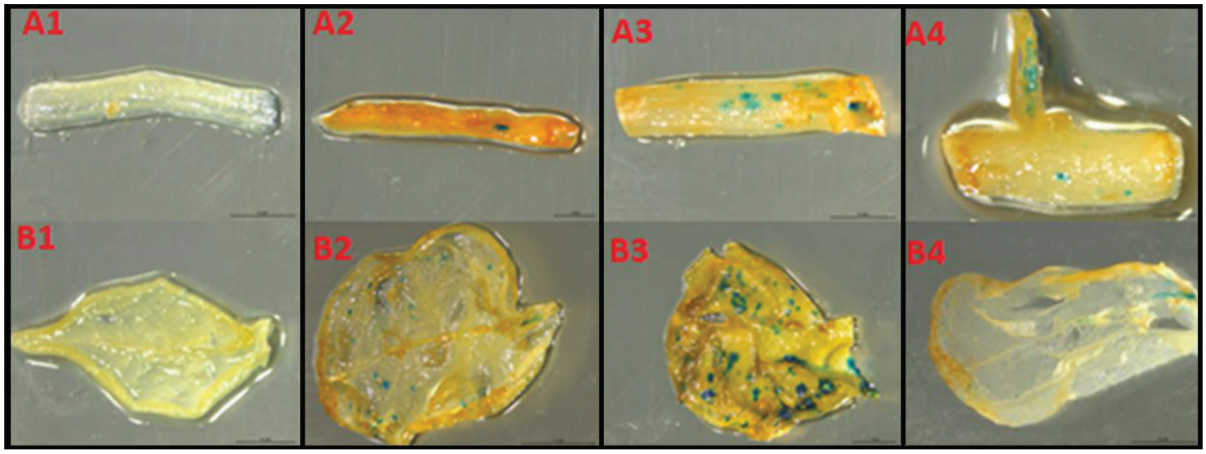

Figure 2. GUS histochemical assay of M6 explants under the microscope infected with GV2260 strain harboring recombinant plasmid (A line: internode explants, B line: leaf explants; 1: control explants, 2: 10 min inoculated explants, 3: 20 min inoculated explants, and 4: 30 min inoculated explants).

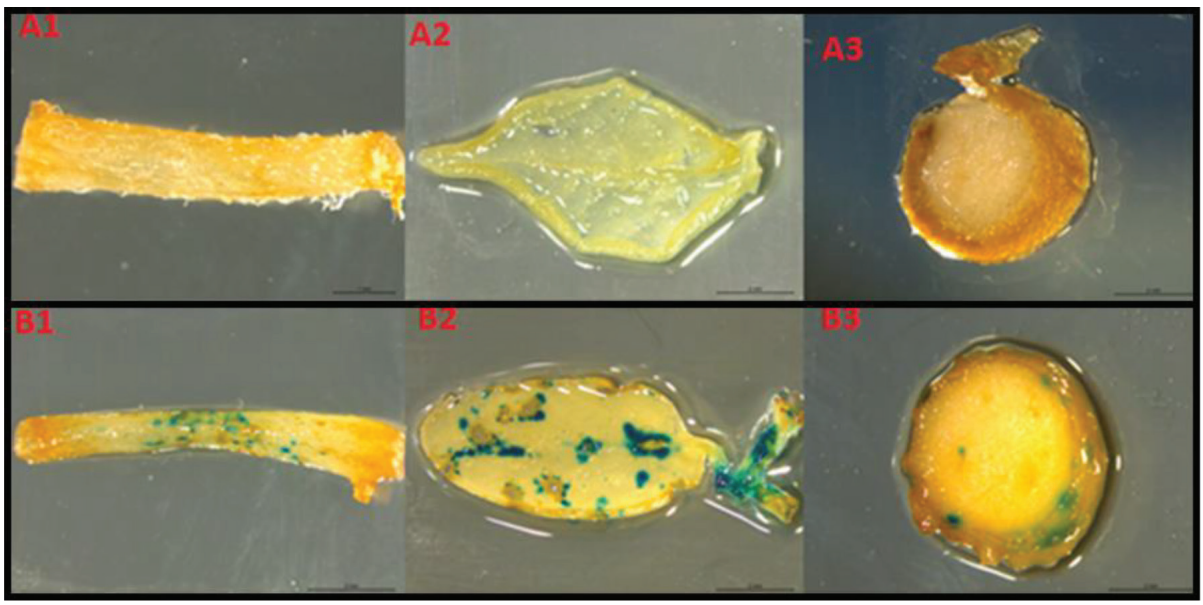

Figure 3. GUS histochemical assay of M6 explants under the microscope (A line: control explants of internode, leaf and microtuber, B line: 20 min inoculated explants; 1: internode explants, 2: leaf explants and 3: micro tuber disc).

callus induction than 10 min (33%). CIM–2 inoculated for 10 min showed the least callus induction rate (Figure 5).

CIM-1 did not induce calli formation in all explants used. CIM–2 gave the best result at 20 min of inoculation time for microtuber explants (73%), whereas internode had the least response in CIM–2 (36%). Leaf explants exhibited more callus induction in CIM–3 (65%) compared to CIM–2 (57%). For microtuber, CIM–3 (60%) showed the least callus induction compared to CIM–2, suggesting microtuber explant to be better in CIM–2 for callus induction. Nonetheless, our experiments showed that internode explants induced the fewest calli compared to the other explants.

Callus efficiency for microtuber was found to be the highest (73%) in CIM–2 compared to the other explants used, whereas leaf explants induced the best callus induction formation in CIM–3 (Figure 6).

Randomly selected calli were subjected to PCR assay using *nptII* gene-specific primers and positive results were observed at expected band sizes of 450 bp (Figure 7).

There was no shoot regeneration from the calli induced from internode and microtuber explants. However, shoot

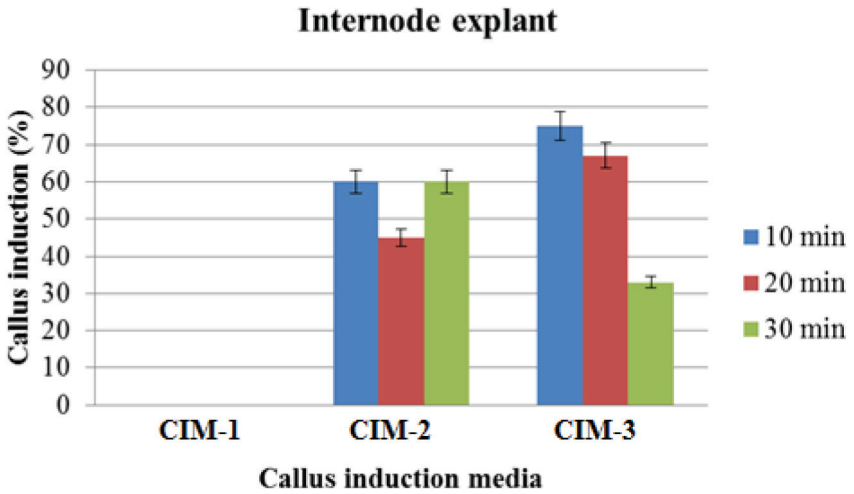

Figure 4. Callus induction efficiency of internode explants in different CIM inoculated in different times.

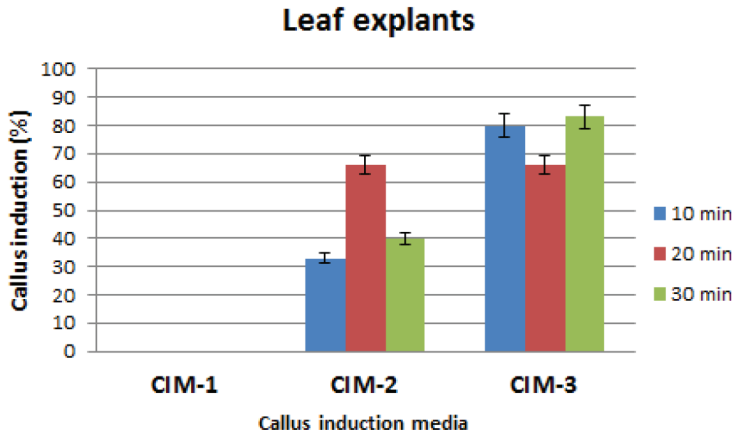

Figure 5. Callus induction efficiency of leaf explants in different CIM inoculated in different times.

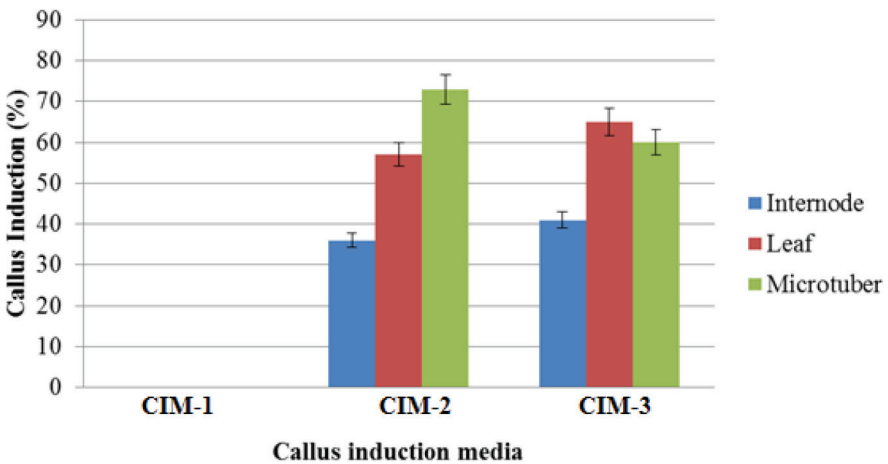

Figure 6. Callus induction efficiency from internode, leaf and micro tuber explants inoculated for 20 min in different CIM.

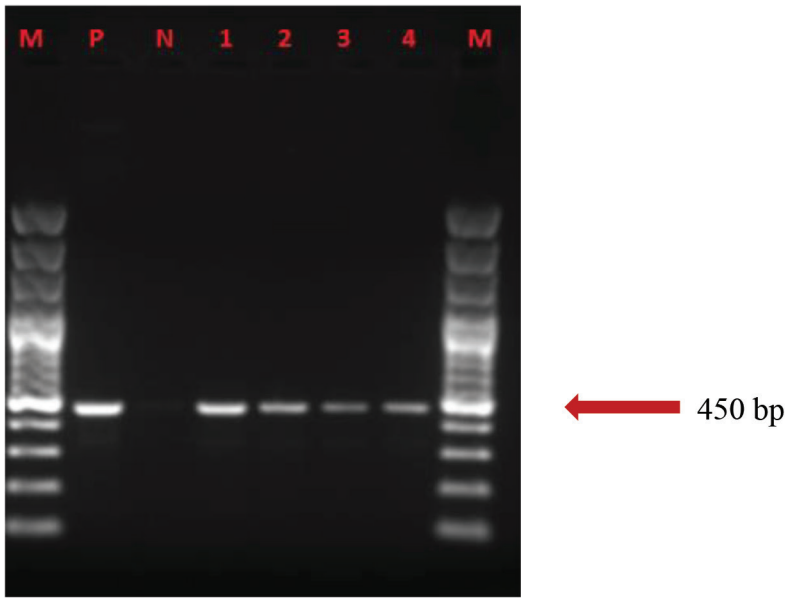

Figure 7. Agarose gel electrophoresis result of PCR using *nptII* gene-specific primers from calli (N: Negative control, P: Positive control, M: 100 bp Marker (Thermoscientific); 1: leaf calli in CIM–2 inoculated for 30 min, 2: leaf calli in CIM–3 inoculated for 30 min, 3: internode calli in CIM–2 inoculated for 20 min and 4: internode calli in CIM–3 inoculated for 20 min).

regeneration was induced only from leaf explant in CIM–2. One of the regenerated shoots, regenerated from the leaf calli, showed positive GUS staining and was observed under the microscope (Figure 8). Many of the regenerated shoots could not survive and few of them grew into a plant. The selected two transformants (Y001 and Y008) survived well enough and were used for gene expression and fluorometric analyses.

RT–qPCR showed that *gusA* expression levels in transgenic plants were significantly higher as compared to control (Figure 9). We observed 690–fold gusA expression in Y001 and 708–fold in Y008 transgenic plants compared to the control. This clearly indicates the manifold gusA gene expression in the primary transformants. The fluorogenic assays showed higher GUS activity in transformed plants as revealed by relative fluorescence establishing functionality of gusA protein (Figure 10).

4. Discussion

Due to the highly heterozygous nature of cultivated tetraploids, further analyses in molecular biology and functional genomics analyses become confounding and tedious. Such an intricate genomic structure of the alleles can be perplexing for the molecular biologists. Further, the choice of diploid potato plants will be best for conducting gene-edited technology and to redirect the potato breeding program into the diploid inbred lines (Dangol et al., 2019; Enciso–Rodriguez et al., 2019).

M6 is a diploid potato plant and its whole genomic sequence analysis has been published (Leisner et al., 2018). Due to its homozygosity and self-compatibility properties, M6 could be a suitable candidate plant for all molecular works that can be extrapolated to the domesticated cultivars. At the same time, M6 possesses desired qualities in chip processing and resistance against diseases (Jansky et al., 2014; Enciso–Rodriguez et al., 2019). However, the problem of high glycoalkaloid content (Leisner et al., 2018) in M6 tubers can be addressed via breeding programs and/or transgenic/gene editing analyses. With so many opportunities residing in diploid M6, there are no reports so far on diploid M6 plant transformation.

There are many reports on *Agrobacterium* -mediated plant transformation of diploid potatoes such as *S. phureja* (Ducreux et al., 2005), DG 88–596 (3C)/DG 82-330 (10J) (Nadolska–Orczyk et al., 2007) and *S. commersonii* (Cardi et al., 1992). However, M6 has been considered recalcitrant to plant transformation and regeneration (personal communication) and CRISPR work has not been performed on diploid M6, rather used in crosses with gene edited diploid event MSX914–10 (generated by crossing *S. tuberosum* Group Phureja with 84SD22). Furthermore, M6 as being a diploid self-compatible line has an extraordinary advantage in fixing targeted mutations and performing potato functional genomics (Butler et al., 2015).

Previously, we had attempted to find out the best *Agrobacterium* strain for the diploid M6 potato using

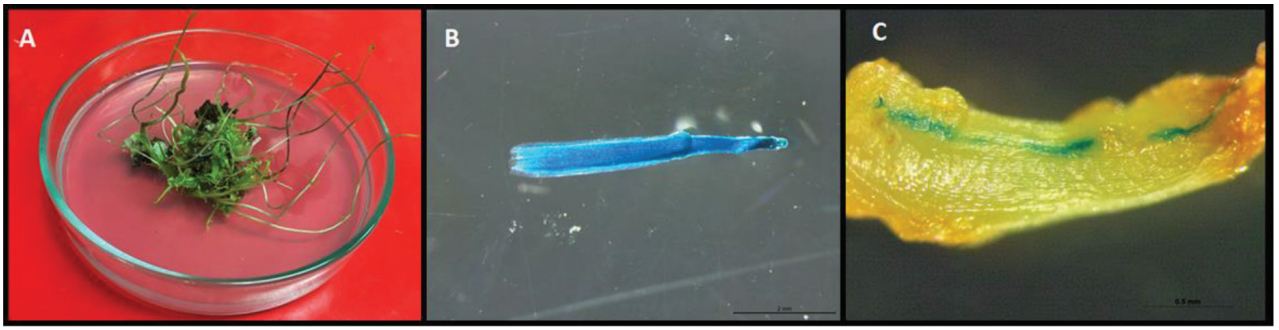

Figure 8. A) Regeneration of shoots from the leaf callus induced in CIM–2; B–C) Histochemical GUS assay conducted on regenerated transgenic plant shoots derived from leaf calli.

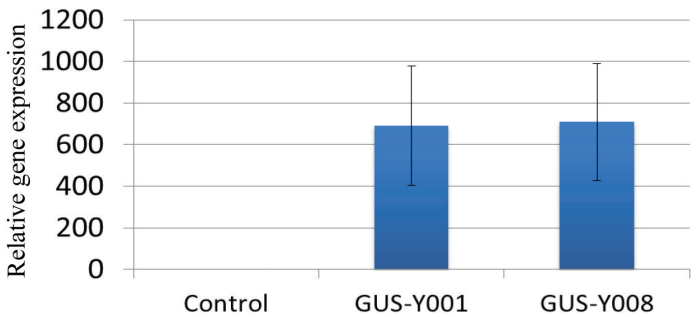

Figure 9. Graph showing variable gene expression of *gusA* gene in plants Y001 and Y008 compared with non-transgenic control via real time PCR method (EF1α gene was used as a housekeeping gene).

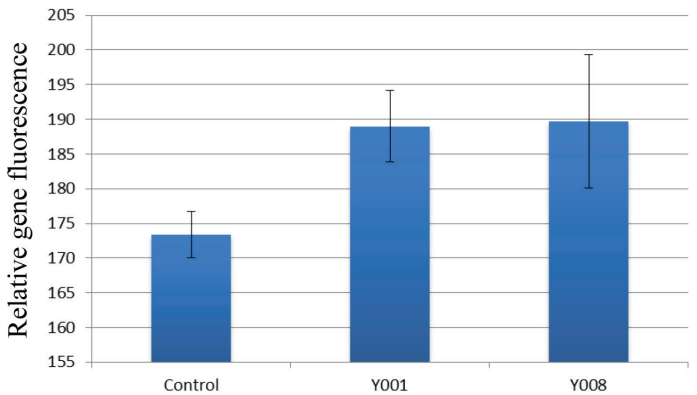

Figure 10. Graph depicting GUS activity in primary transformants using fluorogenic analysis compared with the non-transgenic control.

"Desiree" as control by scrutinizing transformation efficiencies of five different *Agrobacterium* strains (AGL1, GV2260, GV3101, LBA4404 and EHA105) (Dönmez et al., 2019). From this study, we further determined well-responsive explant, based on their histochemical GUS assays and calli inducing rates for optimization of M6 transformation. Also, we have optimized the appropriate inoculation time with the most suitable *Agrobacterium* strain, that contained amalgamation of various hormones, and finally the best medium composition for shoot regeneration with GV2260 *Agrobacterium* strain.

Firstly, we sought to find out the most suitable inoculation time for the GV2260 infection in the various explants of diploid M6 using the data generated from GUS histochemical staining from leaf and internode explants (Figures 2 and 3). We found more GUS staining at 20 min of inoculation time in both leaf and internode explants; however, the 30 min inoculation time was better than 10

min in internode explants and 10 min was better than 30 min in leaf explants. Hence, 20 min was selected as the most suitable inoculation time for our explants.

Secondly, we compared various explants (leaf, internodes and micro tubers) with a 20 min inoculation time infected with GV2260 (Figure 3). The best staining was visualized in the leaf explants under the microsope and it was followed by internode and microtubers. Hence, we can recommend leaf explants to be more suitable in terms of gene transfer. Nonetheless, we proceeded with all three explants for our further experiments to interrogate the calli inducing rate as well as the regeneration ability.

For callus induction ability with different combinations of hormones, an experiment with three different media CIM-1, CIM–2 and CIM–3 was performed. No callus induction was recorded in the treatment of hormones 2,4–D and kinetin, and eventually the explants (both leaf and internodes) dessicated in just two weeks of time. It was believed that 2,4–D would trigger the calli induction of explants as it has been previously reported as a rapid elicitor of callus induction (Elaleem et al., 2009; Khalafalla et al., 2010; Byarugaba et al., 2018). However, all these studies were conducted in tetraploid potatoes while in the present study, diploid potatoes did not show any response to callus induction using different explants in medium supplemented with 2,4–D. There is a genotype-specific requirement in terms of hormonal composition and their concentrations. This is why prediction of the suitable transformation and regeneration protocol in advance for a particular genotype is fundamentally impossible (Visser, 1991). Only CIM–2 and CIM–3 were considered for the next experiments. For leaf explants, CIM–3 was found to be the best CIM when inoculated at 30 min (83%) and 10 min (80%). CIM–2 inoculated for 10 min showed the least callus induction rate (Figure 5). For internodes, CIM–3 exhibited the highest calli inducing rates at 10 min inoculation time (75%) and it was followed by 20 min (67%), thereby indicating CIM–3 to be the best CIM for both leaf and internodes with some variation in inoculation timings. For instance, a 30 min inoculation time was the best for CIM–3 in leaf explants, however the least for internode explants**.** Next, we attempted to compare various explants with GV2260 inoculation for 20 min based on our results**.** CIM–2 (73%) and CIM–3 (65%) gave the best result in 20 min of inoculation time for microtuber and leaf explants respectively, whereas internodes showed the least response in CIM–2 (36%) and CIM–3 (41%). Internode explants exhibited the least callus induction rate overall (Figure 6).

We obtained callus induction from leaf explants in CIM–2 (composed of BAP, NAA, kinetin and trans-zeatin hormones) and regeneration in SIM which was composed of MS medium supplemented with GA_3 and TDZ hormones. The upshot of our results indicated that CIM–2 induced regeneration in internodes and leaf explants. This result indicated that CIM–2 can be a suitable medium for leaf and internode explants both in terms of callus induction and subsequent regeneration. The overall transformation efficiency was obtained as 0.80%.

RT–qPCR has emerged as a standard molecular technique to analyze relative expression of targeted genes (Anayol et al., 2016). The positive transformants were assayed by RT–qPCR and results revealed that transgenic plants had increased expression of *gusA* compared to control (Figure 9). The results showed abundant transcript levels (690–708 fold) of *gusA* gene in transgenic plants. Likewise, GUS fluorogenic assays established the functionality of *gusA* gene in transformed plants.

5. Conclusion

We have reported suitable strain, infection time and callus induction medium using different explants for genetic modification in diploid M6 line that have been reported as recalcitrant to *in vitro* culture conditions based on scientific literature. The regenerated plants were confirmed for integration and expression of *gusA* gene in M6. We believe that the present study leads to new avenues for genetic improvement of diploid potatoes.

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Contribution of authors

The data presented in the manuscript is a part of PhD thesis work of Mr. Sarbesh Das Dangol who conducted transformation experiments, recorded data, and wrote the manuscript. İlknur Yel helped in establishing transformation experiments and data analysis and completed her BS project using some part of data. Mehmet Emin Çalışkan and Allah Bakhsh supervised the study, critically read the manuscript, and presented it in its current form.

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