Research Article

Transformation Efficiency of Five *Agrobacterium* Strains in Diploid and Tetraploid Potatoes

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Abstract | 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, specially in diploid potato and to redirect the breeding program away from tetraploids to the diploids. We aimed to develop the protocol for transformation of *Solanum chacoense* diploid M6 potato using tetraploid *S. tuberorsum* cv. Desiree as a control using five different *Agrobacterium* strains harboring pBIN19 binary vector that further contains *gusA* gene, interrupted by an intronic sequence, under the control of 35S CaMV promoter. After transformation, we analyzed the transformation efficiencies of each of the *Agrobacterium* strains using the histochemical GUS analysis. The highest *gusA* gene transfer efficiency rate both for leaf (60%) and internode (100%) explants was observed in cv. Desiree using *Agrobacterium* strain GV2260, while the highest gene transfer efficiency rate for leaf and internode explants in *S. chacoense* M6 were obtained with AGL1. The highest callus formation for both cv. Desiree and *S. chacoense* M6 was obtained on cv. Desiree leaf explants, transformed via the *Agrobacterium* strain GV2260. The lowest callus formation, for both *S. chacoense* M6 and cv. Desiree, were obtained from internode explants. About 33% calli induction was achieved from transformation mediated by AGL1 strain in cv. Desiree, and about 23 % from LBA4404 strain in *S. chacoense* M6. This study will further aid in the development of stable transgenic potatoes, especially in diploid cultivars.

Received | September 02, 2019; **Accepted** | November 15, 2019; **Published** | November 26, 2019
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Citation | Dönmez, B.A., S.D. Dangol and A. Bakhsh. 2019. Transformation efficiency of five *Agrobacterium* strains in diploid and tetraploid potatoes. *Sarhad Journal of Agriculture,* 35(4): 1344-1350.
DOI | http://dx.doi.org/10.17582/journal.sja/2019/35.4.1344.1350
Keywords | Potato, Diploid, Tetraploid, *Agrobacterium*-mediated transformation

Introduction

Potato is a food crop bearing tubers and a very important crop when it comes to food security, historically cultivated in Europe, North America, former soviet countries, later adopted in other parts of the world. Potato is now the fourth-most crucial crop globally (Hong et al., 2017; Craze et al., 2018). According to FAOSTAT (2017), 388.19 megatonnes of potatoes are being produced globally, grown in an area of 193026.42 square kilometers. As per Turkish Statistical Institute (TUIK, 2017), 4.55 megatonnes of potatoes are grown in Turkey in an area of 13,593.73 square kilometers annually.

The cultivated potatoes are tetraploids (2n=4x=48) and heterozygous, as well as its breeding is challenged by inbreeding depression and its ploidy level has been implicated to decreased fertility (Nadolska-Orczyk et al., 2006; Leisner et al., 2018). In addition, cultivated potatoes are highly tetrasomic, and its tetraploidy makes it a challenging crop in molecular biology research. This is specially true in gene editing studies, where targeting all alleles at a given time to generate homozygous mutation is quite challenging compared to diploids. Heterozygosity of tetraploid potato can

also be challenging during gene editing experiments and subsequent next generation sequencing (NGS). Sufficient information is available on the failure of bioinformatic tools to detect CRISPR-mediated real mutation, confusing them with errors (Dangol et al., 2019).

Solanum chacoense M6 potato can be a good choice owing to its diploidy, homozygosity (developed by selfing *S. chacoense* for seven generations), self-compatibility (*Sli* gene that inhibits gametophytic self-incompatibility), elevated dry matter content, disease resistances and chip-processing qualities. The diploid potatoes could be beneficial if we can generate inbred lines for cultivated breeding with desired traits (Jansky et al., 2014; Enciso-Rodriguez et al., 2019; Hosaka and Hanneman, 1998). Very recently, whole genome sequencing of diploid *S. chacoense* M6 potato has been performed (Leisner et al., 2018). The availability of whole genome sequence makes *S. chacoense* M6 potato more important in molecular biology research. Therefore, focusing potato research on diploid *S. chacoense* M6 potatoes seems a way forward in achieving food security.

Agrobacterium-mediated transformation of *S. tuberosum* cv. Desiree has been pioneered long ago (Ooms et al., 1985; Tavazza et al., 1988; Visser, 1991) and several efficient transformation protocols have been described since then (Haesaert et al., 2015; Craze et al., 2018). Therefore, it has become a choice of cultivar as a control. So far, no reports have been made on diploid *S. chacoense* M6 potato with respect to its genetic transformation using *Agrobacterium*-mediated transformation. A similar study has been conducted in *Nicotiana tabacum* plant using the five different *Agrobacterium* strains containing *nptII* and *gusA* genes (Bakhsh et al., 2014) and in tomato using four different *Agrobacterium* strains harboring *nptII* and *uidA* gene in the binary vector (Chetty et al., 2013).

In this paper, we compared efficiencies of five different *Agrobacterium* strains in *S. chacoense* M6 diploids and cultivated tetraploid potato cv. Desiree, scrutinized its calli inducing rates and percentages of histochemical GUS stainings for each of the five *Agrobaterium* strain.

Materials and Methods

Plant material

Tetraploid cv. Desiree and diploid inbred line *S. chacoense* M6 potatoes were cultured in MS medium in growth chamber at 24±2°C under fluorescent light at 100 µmol $m^{-2}s^{-1}$ with 16/8 hour (light/dark) photoperiod. Internodes and leaves were used as explants for the transformation experiments.

Agrobacterium-mediated transformation

Five different *Agrobacterium* strains were used for the experiment, namely GV2260, GV3101, LBA4404, AGL1 and EHA105. Electroporation of the plasmid pBIN19 vector (Figure 1) containing reporter gene p35S Gus-INT and *nptII* gene as a selection marker, was conducted in each of the *Agrobacterium* competent strains using Bio-Rad electroporation device 'Gene Pulser XCell'. The transformed cells were plated on LB medium containing kanamycin (50 mg L^{-1}) at 28 °C for 2 days. The transformation of cells was confirmed using *gusA* gene specific primers (FP**:** 5' CCCTTACGCTGAAGAGATGC 3' and RP: 5' GAGCGTCGCAGAACATTACA 3'). One single isolated colony from each of the positive transformed strains were inoculated to 10 mL LB broth (kanamycin 50 mg L^{-1}), grown overnight and used for transformation of leaf explants at the OD_{600} value of 0.8. Each of the cultures was centrifuged at 5000 rpm for 10 mins at 4° C and the pellet was resuspended in 10 mL of MS broth. Following infection for 30 min, co-cultivation was performed for two days on MS medium containing 100 mg L^{-1} acetosyringone and 0.8 % agar.

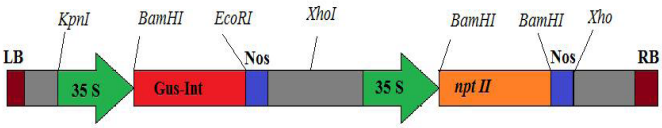

Figure 1: *pBIN19 vector consists of the gusA gene with intron driven by Cauliflower Mosaic Virus (35S) promoter and nptii under the control of 35S Promoter. Each of these genes has nopaline sytnhase (NOS) terminator. The left and right borders are shown as LB and RB, respectively, in the figure.*

Callus induction media (CIM)

The transformation was performed in three replicates for each of the five strains for leaf and internode explants, each replicate contained both explants.

After two days, explants were washed in distilled water containing antibiotic (Sulcid) for 15 minutes to kill *Agrobacterium* from the surface of the explants. After drying the explants on filter papers, two different callus inducing media (CIM) were used for each variety CIM-1 (2 mg L^{-1} BAP, 0.2 mg L^{-1}

NAA, 1 mgL^{-1} transzeatin, 1 mg L^{-1} kinetin, 25 mg L^{-1} kanamycin and300 mgL^{-1} sulcid) composition was used for cv. Desiree explants while CIM-2 1 (2 mg L^{-1} BAP, 2 mg L^{-1} NAA, 25 mg L^{-1} kanamycin and 300 mgL^{-1} sulcid) was used for *S. chacoense* M6 explants. Data for callus induction was recorded and presented as %age.

Histochemical gus analyses

For detection of *gusA* gene, GUS solution was prepared (1 Mm X-gluc, 10mM EDTA, 100mM NaH_2PO_4, 0.1% Triton X-100, 50% methanol and 100 µg/ml Chloramphenicol; pH 8). We checked the histochemical GUS staining of leaf and internode explants co-cultivated for 2 days (one experiment), and on CIM cultivated for 6 weeks in three replicates. The explants grown on CIM were used for the calculation of GUS positive transformation rate. Explants grown on co-cultivation media as well as three replicates of each of the five different *Agrobacterium* transformed explants were treated with GUS solution in an eppendorf tube at 37° C overnight under dark condition. Next day, the explants were washed twice with ethanol and observed under the microscope.

Results and Discussion

Colony PCR was performed using *gusA* gene specific primers to confirm positive clones prior to the transformation of the explants (Data, not shown).

Callus induction rate

Three to four weeks later, callus formation from various explants in each of the three replicates was observed and relevant data (histochemical gus analysis percentages and calli inducing rates) was recorded from each of the plates. Percentage of callus induction from leaf and internode explants for *S. chacoense* M6 and cv. Desiree potatoes were calculated (Figures 2 and 3). In cv. Desiree, AGL1 *Agrobacterium* strain showed maximum callus induction efficiency from internodes (33.33%), followed by GV2260 (16.7%), EHA105 (15.56%), LBA4404 (13.46%), and GV3101 (12.07%). The percentage of maximum callus induction efficiency for leaf explants was 64.58% for GV2260 strain followed by LBA4404 (51.79%), EHA105 (47.73%), GV3101 (18%) and AGL1 (16.33%) (Figure 2).

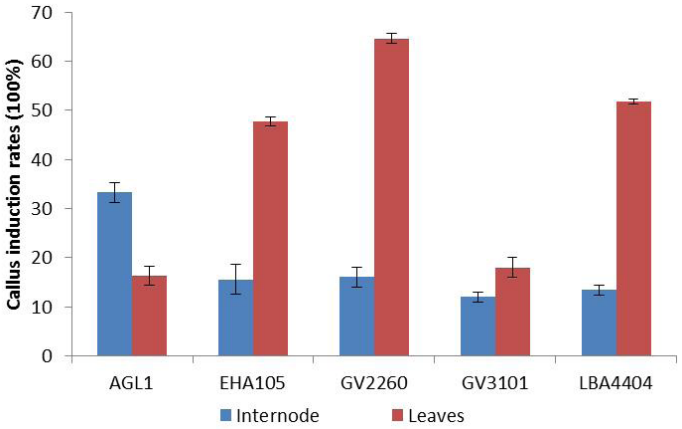

Figure 2: *The graph shows the percentages of callus induction for Desiree explants transformed with different Agrobacterium strains.*

In *S. chacoense* M6, maximum callus induction efficiency was induced by LBA4404 *Agrobacterium* strain from internodes (23.81%) followed by GV3101 (21.82%), GV2260 (21.43%), EHA105 (11.9%), and AGL1 (8.51%). The percentage of maximum callus induction efficiency for leaf explants in *S. chacoense* M6 was achieved at 88.1% in GV2260 strain followed by LBA4404 (77.78%), GV3101 (60.78%), AGL1 (54.84%) and EHA105 (53.33%) (Figure 3).

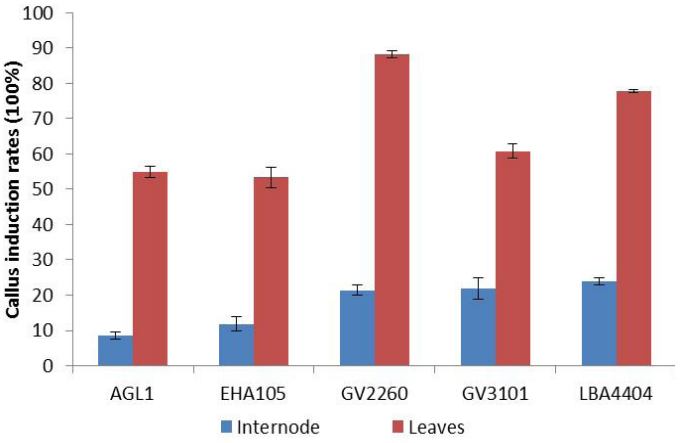

Figure 3: *The percentage of callus induction for M6 explants transformed by the different Agrobacterium strains.*

Histochemical GUS analysis

Transformed explants were tested for GUS expression using histochemical analysis according to the procedure described by Jefferson et al. (1987). For cv. Desiree internodal explants, *Agrobacterium* strain GV2260 gave the best results with 100% of GUS positive transformation efficiency, followed by AGL1 (87%), LBA4404 (60%), EHA105 (47%) and GV3101 (7%). No GUS staining was found on leaf explants transformed with AGL1 and GV3101. However, GV2260 gave the best GUS result in leaf explants with 60% GUS transformation, followed by

EHA105 (13.33%) and LBA4404 (6.67%) (Figures 4 and 5).

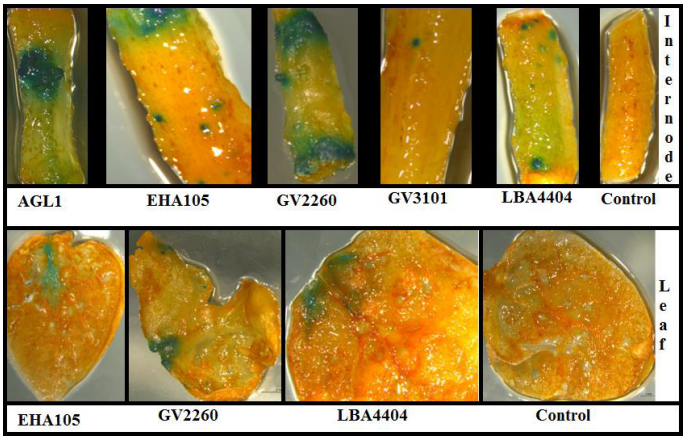

Figure 4: *Histochemical GUS analyses of Desiree leaf and internode explants using five different Agrobacterium strains.*

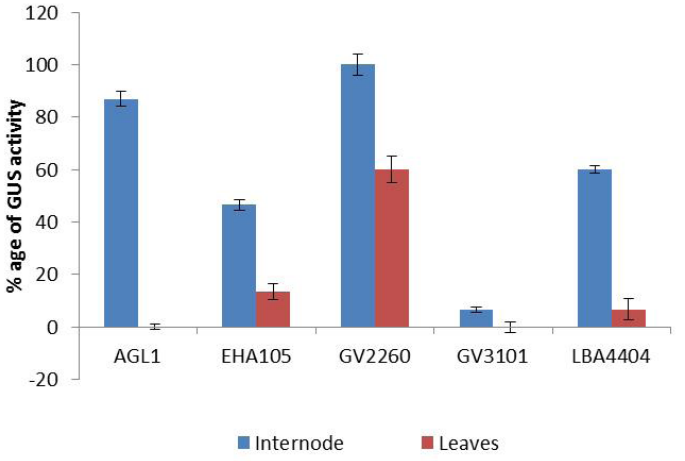

Figure 5: *Desiree explants depicting the percentage of GUS positive transformed explants with different Agrobacterium strains in the study.*

GUS transformation rates in leaf and internode explants with different *Agrobacterium* strains were calculated for both *S. chacoense* M6 and cv. Desiree to elucidate the best *Agrobacterium* strain (among five) suitable for higher transformation rate in cv. Desiree and *S. chacoense* M6. For *S. chacoense* M6 internodal explants, *Agrobacterium* strain AGL1 showed the best GUS analysis result (100%), followed by GV2260 (93.33%), LBA4404 (93.33%), EHA105 (66.67%) and GV3101 (6.67%). For leaf explants, again AGL-1 and GV2260 *Agrobacterium* strains showed the highest percentage of GUS positive transformed plants (100%), EHA105 (93.33%), LBA4404 (80%), and GV3101 (20%) (Figures 6 and 7).

The high heterozygous nature of domesticated tetraploid potatoes renders challenges for functional genomics studies due to its allelic diversity. In such crop species, diploid potato such as *S. chacoense* M6 could be a viable option both in molecular biology as well as breeding program (Dangol et al., 2019; Enciso-Rodriguez et al., 2019). Further, when the whole genomic sequence of diploid *S. chacoense* M6 is already available in the potato genomic database (Leisner et al., 2018), it can be a model crop plant for the study of potatoes. As *S. chacoense* M6 transformation protocol has not been established yet, and as cv. Desiree transformation is more efficient and well-established (Ooms et al., 1985; Tavazza et al., 1988; Visser, 1991; Haesaert et al., 2015; Craze et al., 2018), we aimed to compare the *Agrobacterium* strains for *S. chacoense* M6 potato using cv. Desiree as a control.

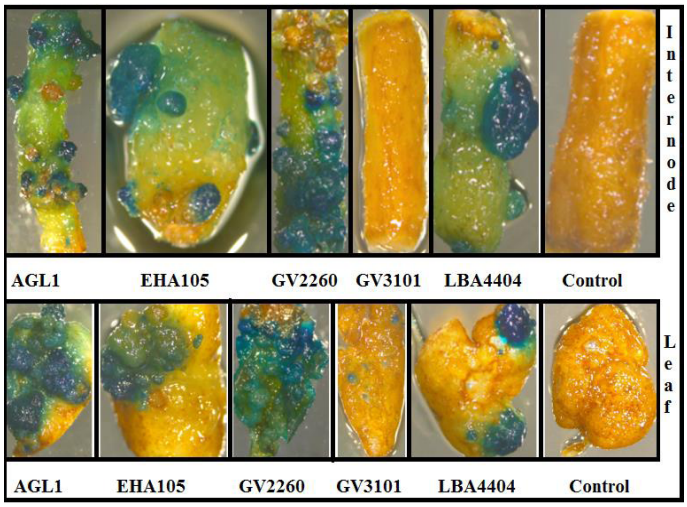

Figure 6: *Histochemical GUS analyses of M6 leaf and internode explants using five different Agrobacterium strains.*

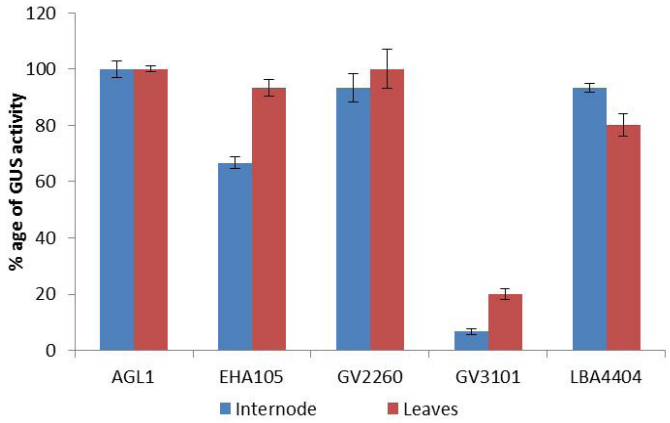

Figure 7: *M6 leaf and internode explants depicting the percentage of GUS positive transformed explants with different Agrobacterium strains in the study.*

In this study, various *Agrobacterium* strains (harboring beta-glucuronidase *gusA* gene interrupted by an intronic sequence under the control of 35S CaMV

promoter in the plasmid pBIN19) were used to transform *S. chacoense* M6 leaf and internode explants using cv. Desiree cultivar as control. For this, we used various strains available in our laboratory (AGL1, GV2260, GV3101, LBA4404 and EHA105) and in-house protocol was followed (Bakhsh et al., 2014). The CaMV 35S promoter was for ubiquitous expression of the *gusA* gene. In this experiment during the transformation process, kanamycin antibiotic for antimicrobial resistance was used as a selective marker as it has been found to have higher efficiency in transformation experiments (McCormick et al., 1986; Bakhsh et al., 2014). GUS solution was used for the screening of the transformed explants and the stained explants were observed under the microscope.

The highest *gusA* gene transfer efficiency rate in cv. Desiree was found in leaf (60%) and internode (100%) explants mediated by the *Agrobacterium* strain GV2260. In *S. chacoense* M6, the highest gene transfer efficiency rate both for both leaf and internode explants was obtained from *Agrobacterium* strain AGL1 (100%). Combining both the gene transfer efficiency data for cv. Desiree and *S. chacoense* M6, the highest *gusA* gene efficiency was obtained with *Agrobacterium* strain GV2260, and the lowest *gusA* gene efficiency was obtained with *Agrobacterium* strain GV3101. The highest callus formation for explants was obtained on *S. chacoense* M6 leaf explants (88%) using *Agrobacterium* strain GV2260 and with cv. Desiree leaf explants (64%) using GV2260 strain. In cv. Desiree, the highest callus formation data was obtained from internode explants using AGL1 strain (with 33%). In *S. chacoense* M6, the highest callus formation data was obtained from internode explants (23%) using LBA4404 strain. The reason for obtaining high callus formation data from leaf explants could be due to the inoculation process, for its appropriate surface area for transformation as compared to the internode explants which were quite thinner, lacked usual thickness and tender.

In previous studies on tomato using various *Agrobacterium* strains (Chetty et al., 2013), highest transformation rate (65%) was observed with the *Agrobacterium* strain GV3101. In their study, *Agrobacterium* strain EHA105 was defined to be more efficient compared with GV3101 strain. In their experiment, inoculation time was limited to 20 minutes. The differences arising between these two studies could be mainly related to the type of strains used, plasmid type, and the inoculation time used for transformation. Hence, the discrepancy might have arisen in these two studies. In the study conducted by Chetty et al. (2013), the highest transformation efficiency rate was obtained in cotyledon explants with GV3101 strain. In our experiment, we didn't use the cotyledon explants; the lowest transformation efficiency rate was found in internode explants with GV3101 strain in cv. Desiree, whereas in *S. chacoense* M6 the lowest efficiency rate was obtained in the internode explants with GV3101 strain.

From our experiment overall, we can see that GV2260 is the most suitable strain for callus induction in cv. Desiree and *S. chacoense* M6 explants, although LBA4404 was found to be better in *S. chacoense* M6 internode explants and AGL1 in cv. Desiree internode explants. However, the efficiency of LBA4404 is not consistenly high for all the explants in cv. Desiree and *S. chacoense* M6. The highest callus formation data obtained from internode explants inoculated with LBA4404 in *S. chacoense* M6 internode explant was 23%.

GUS histochemical analysis revelaed that AGL1 was the best for transformation in near similarity with GV2260 strain in *S. chacoense* M6. In cv. Desiree, the best histochemical GUS transformation was found to be in GV2260. The highest gene transfer efficiency rate for all the explants used in diploid *S. chacoense* M6 was obtained from AGL1 (100%). The highest callus induction rate for explants was obtained in *S. chacoense* M6 leaf explants with the inoculation from GV2260 (88%). Furthermore, there is a genotype-specific requirement in terms of hormonal composition and their concentrations, which renders the suitable transformation and regeneration protocol to be predicted in advance for a particular genotype fundamentally impossible (Visser, 1991). This could be the reason for different responses of *Agrobacterium* strains in various explants in terms of their transformation efficiency.

We recommend GV2260 and AGL1 to be better strain for further experiments in optimizing the transformation protocol for diploid *S. chacoense* M6 potato. However, AGL1 has been reported to be a hypervirulent strain (Anand et al., 2019).

Conclusions and Recommendations

We have compared the transformation efficiency of diploid *S. chacoense* M6 potato using cv. Desiree as

a control using five different *Agrobacterium* strains. We found that GV2260 could be the best strain to transform diploid *S. chacoense* M6 potato based on their overall callus inducing performance as well as histochemical data records. This study can further aid in developing the optimization protocol for the stable plant transformation in diploid *S. chacoense* M6 potato using the strain we have recommended.

Acknowledgments

Mr. Sarbesh Das Dangol is a TUBITAK (Scientific and Technological Council of Turkey) fellow availing Graduate Scholarship Programme for International Students-2215 (PhD). The support of TUBITAK is highly acknowledged. We are very grateful to Dr. Abdellah Barakate for his generous willingness to provide us with the diploid inbred line *S. chacoense* M6 seeds from the James Hutton Institute, Dundee, UK, for which we also sought permission from United States Department of Agriculture (USDA) for the germplasm transfer.

Novelty Statement

The present study focuses on the development of the transgenic Solanum chacoense diploid M6 potato, which could be helpful in developing potato varieties resistant to biotic/abiotic stresses; the development of transgenic potato especially M6 is imperative in that the whole genome has been sequenced and this plant is homozygous as well as self-compatible. Developing a transgenic line for such a crop is quite important in po-tato breeding and hasn't been reported before.

Author's Contribution

Betul Ayça Dönmez completed her BS thesis out of the data obtained from this research work. The establishment of genetic transformation system in diploid inbred line M6 is one of objective of PhD thesis work of Sarbesh Das Dangol who wrote the article. The research work was conceptualized and supervised by Allah Bakhsh.

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