

Salt Stress-Induced Genetic Variation in Calli and Shoot Cultures of Economically Important *Stevia Rebaudiana*

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Research Article

Keywords: *Stevia rebaudiana*, callus, in vitro shoots, salt stress, genetic variation, UDP glycosyltransferase

Posted Date: February 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-161357/v1>

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Abstract

Stevia rebaudiana is one of the potent members of family *Asteraceae* and is famous for the synthesis of Steviol glycosides. Recently it is one of the economically important plants with high market demand. Seed infertility and stem cuttings are the major issues in obtaining homogeneous population, therefore, the best alternative is plant cell, tissue and organ culture. Currently, salt stress is one of the major issues worldwide. The overall objective of the current study was to investigate the effect of salt stress (100 mM NaCl) on genetic variation in calli and shoot cultures of *S. rebaudiana*. Sterilized leaf explants were inoculated on MS media augmented with 2,4-D (2.0 mg/L) and BAP (2.0 mg/L) for callogenesis. Shoots were obtained by using BAP (2.0 mg/L) and GA₃ (0.5 mg/L) after 30 days. Callus and shoots were exposed to salt stress for 30 days. These fresh calli and shoot cultures were analyzed for genetic variation including three genes. 1: Leaf sample UDP glycosyltransferase gene restricted with *ScaI* 2: Callus sample gene restricted with *ScaI* 3: Leaf sample UDP glycosyltransferase mRNA restricted with *NcoI* 4: Callus sample mRNA restricted with *NcoI* 5: Leaf sample UDP glycosyltransferase mRNA restricted with *DraIII* 6: Callus sample mRNA restricted with *DraIII*. Only the callus sample digested with *DraIII* showed a variation from the mother plant. Others genes did not show any variation. It is concluded that genetic variation occurs in callus which is undifferentiated mass of cells and the variation caused may be due to salt stress. This study further need sequencing to identify the genes responsible for genetic and Somaclonal variation.

Introduction

Stevia rebaudiana is one of the potential sweet herbs in the family *Asteraceae* and commonly known as sweet leaf and commercially cultivated in many countries for multiple purposes (Amin et al. 2017). Obesity and overweight are one of the major risk factors, which causes several diseases such as hypertension, hyperlipidemia, diabetes, surgical risks, pulmonary and renal problems, pregnancy complications and certain type of cancer. Therefore, steviosides is one of the best strategies to control obesity and overweight (Gupta et al. 2013).

Plant tissue culture plays a key role in propagation, secondary metabolites production, genetic manipulation and conservation of plants. However, the in-vitro cultures of plant cells showed a genetic variation and it is also a characteristic of regenerated cells upon exposure to multiple culture conditions (Braun 1959). PGRs such as 2-4-D, NAA, BAP and salt stress are the major elicitors for genetic variability in plants which cause chromosomal instability, high level of polyploidy and shows visible differences in morphology (Sun et al. 2013; Pasqual et al. 2014; Silva and Carvalho, 2014; Khan et al. 2014). The genetic variation in tissue cultured-derived tissues received a great importance and was proposed by Larkin and Scowcroft (1981). The culture conditions might be mutagenic which induce genetic variation in regenerated plants obtained from somatic embryos, calli and tissues of plants that show phenotypic and as well as genotypic variation (Orbović et al. 2008). Moreover, dehydration stress and salt stress indicate a high level of similarity with respect to genetical, molecular biochemical and physiological effects. Many of salt responsive genes are involved in the regulation and encodes proteins of other salt

responsive gene. Mostly transcription factors¹⁴² and protein kinase¹⁴³, these are the salt response regulatory genes and exposure of *Stevia* to salt stress and mannitol cause genetic variation in cultures *in vitro* (Pandey and Chikara, 2015). Therefore, the overall objective of the current study was to investigate the effect of salt stress on genetic variation in calli and shoot cultures of *Stevia rebaudiana*. The current study will help in understanding the genes responsible for salt stress and the calli cultures can be manipulated for such genes that can minimize the salt induced-stress in *Stevia* as well as in other medicinal plants.

Callus induction and shoot formation was obtained using the hormonal combination of previous research (Ahmad et al. 2016). The *Stevia* plant were taken from PCSIR Laboratories Complex, Peshawar. Callus formation was obtained from leaf explant inoculated on MS medium containing 2,4-D (2.0 mg/L) in combination with BAP (2.0 mg/L). The shoots were obtained by exposing the callus to fresh media augmented with BAP (2.0 mg/L) and GA₃ (0.5 mg/L). All cultured conditions were provided according to the protocol of Ahmad et al. (2016).

The calli and shoot cultures of *Stevia* were exposed to salt stress (100 mM NaCl). The flasks were placed in growth room for 30 days under complete cultural conditions. After 30 days, the cultures exposed to salt stress were removed from the flasks and washed with sterile distilled water and kept in liquid nitrogen for further analysis. Gene responsible for salinity stress was identified from previously available literature. The sequences of the gene and mRNA were retrieved from NCBI using the GenBank accession numbers. The total sequences were uploaded in the SnapGene (Molecular Biology software) for finding of potential restriction enzymes site to be digested with. The targeted genes UGT76G1, UGT85C2, UGT74G1 were taken from NCBI with GenBankID (LC312448.1, AY345978.1, AY345982.1). All the three sequences were BLAST with a shotgun transcriptome assembly, the blast result of these three genes match with *Helianthus annuus* UDP-glycosyltransferase (GenBank ID; XM-022127757.1). The sequence of UGT76G1, UGT85C2, UGT74G1 and *Helianthus annuus* UDP-glycosyltransferase showed 100 % similarity (Supplementary figure 1a, 1b and 1c).

Specific Primers were designed manually for the salinity stress responsible gene for the amplification of the total gDNA (genomic DNA) pool. The primers sequence was analyzed by OligoAnalyzer 3.1 (<https://eu.idtdna.com/calc/analyzer>) of "Integrate DNA Technology". Primers were synthesized commercially from Macrogen, Advance Bioscience International (Supplementary table 1).

Further, the DNA was also isolated from control plant leaves, callus and *in vitro* shoots by CTAB method. The quantity of gDNA was analyzed by gel electrophoresis. The DNA was diluted up to 50 ng/μl for amplification of UDP-glycosyltransferases. Genomic DNA was used for PCR based amplification of the three genes using gene specific primers. For the amplification of the PCR, Dream taq, PCR Master Mix, was used. The PCR was operated according to the profile given in Supplementary table 2. Each set of samples was restricted with same enzyme for finding any difference in the size of fragment and restriction site. Three different enzymes including *ScaI*, *NcoI* and *DraIII* were used in the current study for finding any differences among the genes. For restriction of other enzymes same protocol was followed

(Supplementary table 3). A 25 μL reaction mixture comprised of 12.5 μL Dream Taq buffer with 0.5 mM MgCl_2 and 10 μL of distilled water, 0.5 μL each of forward and reverse primers was added with 2 μL of template DNA was added. The reaction was placed in 37 °C incubator for four hours for complete digestion with restriction enzymes.

Statistical analysis

Selected software's, Statistix 8.1 (USA) and OriginLab 8.5 (USA) was used for measuring mean values and standard deviation.

It was observed that the 100 mM salt and PGRs induced 73.3 % callogenesis as compared to control media (93 %). The same media produced mean shoot length of 9 cm and shoot number (14) as compared to control cultures (11 cm and 20) after 30 days of inoculation. Hence from these results it is observed that saline stress has an effect on gene manipulation during *in vitro* induction of callus and shoot regeneration of stevia.

The genes responsible for salinity stress was identified from the previously available literature and was found that UDP-glycosyltransferase gene was responsible for salinity stress in *S. rebaudiana*. The complete gene of length 1377 bp and its mRNA sequences 1586 and 1554 was retrieved from NCBI using their GenBank ID as (for gene cds LC312448.1, for mRNA as AY345978.1 and AY345982.1; supplementary figure 2a, 2b and 2c).

Total gDNA was successfully isolated from the cultured callus as well as from the callus induced shootings/leaves of *S. rebaudiana*. The quantity of total gDNA was confirmed by Gel Electrophoresis and NanoDrop. RNA was removed from gDNA by treating with RNase mix. Again, the quality was checked by Gel electrophoresis and good quality DNA was diluted to 60 ng/ μL . The PCR was operated according the PCR profile. The fragments lengths of actual size were amplified by PCR as shown in figure 3.

The PCR amplified fragments containing the primers dimers seen at the bottom of the gel image was subjected to cleanup by using Axygen PCR clean up kit. PCR cleanup was successfully done by following the manufacturer protocol. The products were again analyzed by Gel Electrophoresis to test the quality of the products as shown in the Figure 4.

In order to determine the variation caused by salt stress. These samples were subjected to restriction with same enzyme as shown in Supplementary table 3 to analyze the fragments length. The UDP glycosyltransferase of both shoots from leaf and callus was successfully restricted with *ScaI* and the desired restricted fragments of 944 bp and 573 was obtained the same fragments length was also obtained when restricted the said sequence with SnapGene software. UDP glycosyltransferase mRNA was restricted with *NcoI* and again the same fragments length of 909 bp and 677 bp from both leaf and callus samples were obtained like previously. The same fragments were obtained when the sequence was analyzed in silico with SnapGene. mRNA was also restricted with *DraIII* successfully and the results was strange. In the mother plants the restricted fragments were of 1063 bp and 320 bp, and the same length

that was expected from the restriction with SnapGene, while in the callus which was inoculated in salt containing media the product does not restricted. And we found only single band in the gel. The same experiment was repeated thrice but the results remain the same. Hence, from these results we assume that there is some type mutation arise due to which the restriction site for *DraIII* enzymes have been damaged and the fragment cannot be restricted with this enzyme anymore as the figure 5 elaborates all the restriction results.

In this study, the callus and shoot cultures were grown under *in vitro* condition these calli and shoot cultures were exposed to 100 mM salt stress and then analyzed for the identification of three genes including gene cds LC312448.1, for mRNA as AY345978.1 and AY345982. The exposure of these cultures to salts stress significantly affect the expression of these genes. The current results are in agreement with the data reported by Pandey and Chikara (2015). They also observed that the addition of different salt concentrations effected plant growth parameters and gene expression level involved in plant development and biosynthesis of secondary cell products. In Steviol glycoside biosynthesis pathway the transcript expression profiling of genes showed an increase in expression genes; UGT85C2, UGT76G1 and UGT74G1 in the 50, 75 and 100 mM NaCl concentration compared with control. As compared to UGT74G1 (Sativoside) the expression ratio of UGT76G1 (Rebaudioside A) was significantly increased by 67 %. Due to this it was concluded that salinity stress increases the sweetness of the plant than normal plant. while in drought stress condition, all the three gene were down regulated (Pandey and Chikara, 2015).

In this study, salt stress inhibited the plant growth parameters which are an agreement with the reports of [Taffouo et al. \(2009\)](#) and [Kapoor and Srivastava \(2010\)](#). The salt negatively affected photosynthetic pigments, growth hormones and enzymatic activities which showed inhibitory effect on plant growth and development. Furthermore, the decrease in stomatal conductance and evapotranspiration were also affected in Stevia exposed to salt stress ([Acosta-Motos et al. 2015](#)). In the previous study, Stevia was considered as a moderate salinity tolerant plant ([Cantabella et al. 2017](#)). Further it is concluded that lower concentration of NaCl caused a decreased in Steviol glycoside contents. [Ceunen and Geuns \(2013\)](#) found that under salt stress condition these metabolites play an important role in osmoprotactant molecule. [Cantabella et al. \(2017\)](#) found a great contribution in Stevia plant under salt stress to osmotic adjustment. The previous results showed that expression of KAH were increased at each concentration (50, 75, 100 mM) of NaCl. The expression ratio of the three genes may also be repressed by the varying concentration of NaCl. Some significant increase in their respective glucosides were observed with the over expression of some glycosyltransferase (Pandey and Chikara, 2015). In conclusion, three genes (UGT76GI, UGT85GI, UGT76GI) responsible for UDP glycoside transferase were isolated. Only the callus sample digested with *DraIII* showed a variation from the mother plant. Others genes did not show any variation.

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Figures

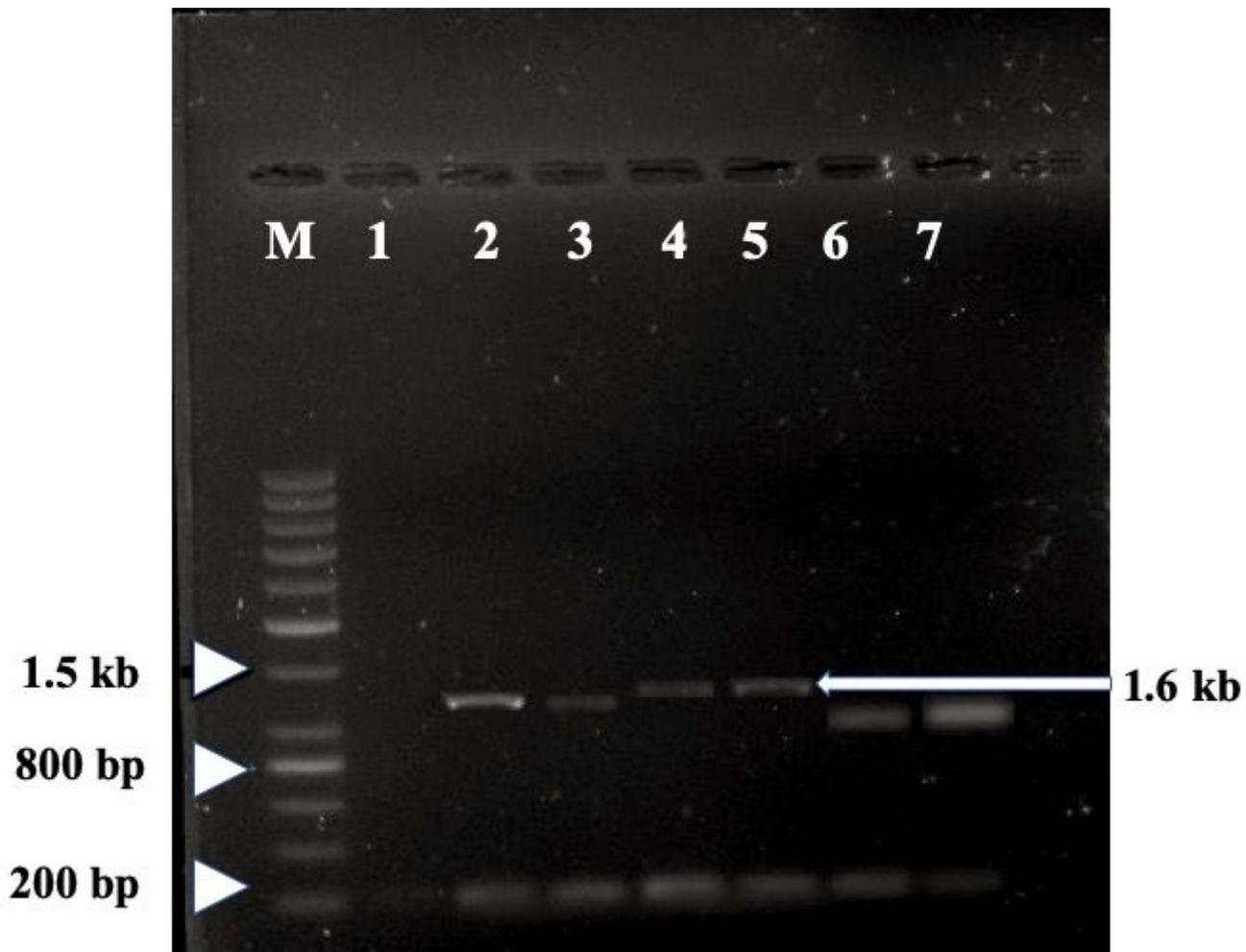


Figure 1

The Gel image of genomic DNA (M: 200 bp) ladder; (1) Non template control (2) leaf sample from callus induced shooting UDP glycosyltransferase gene (3) Callus sample gene (4) Leaf sample UDP glycosyltransferase mRNA (5) Callus Sample mRNA (6) Leaf sample UDP glycosyltransferase mRNA (7) Callus sample mRNA.

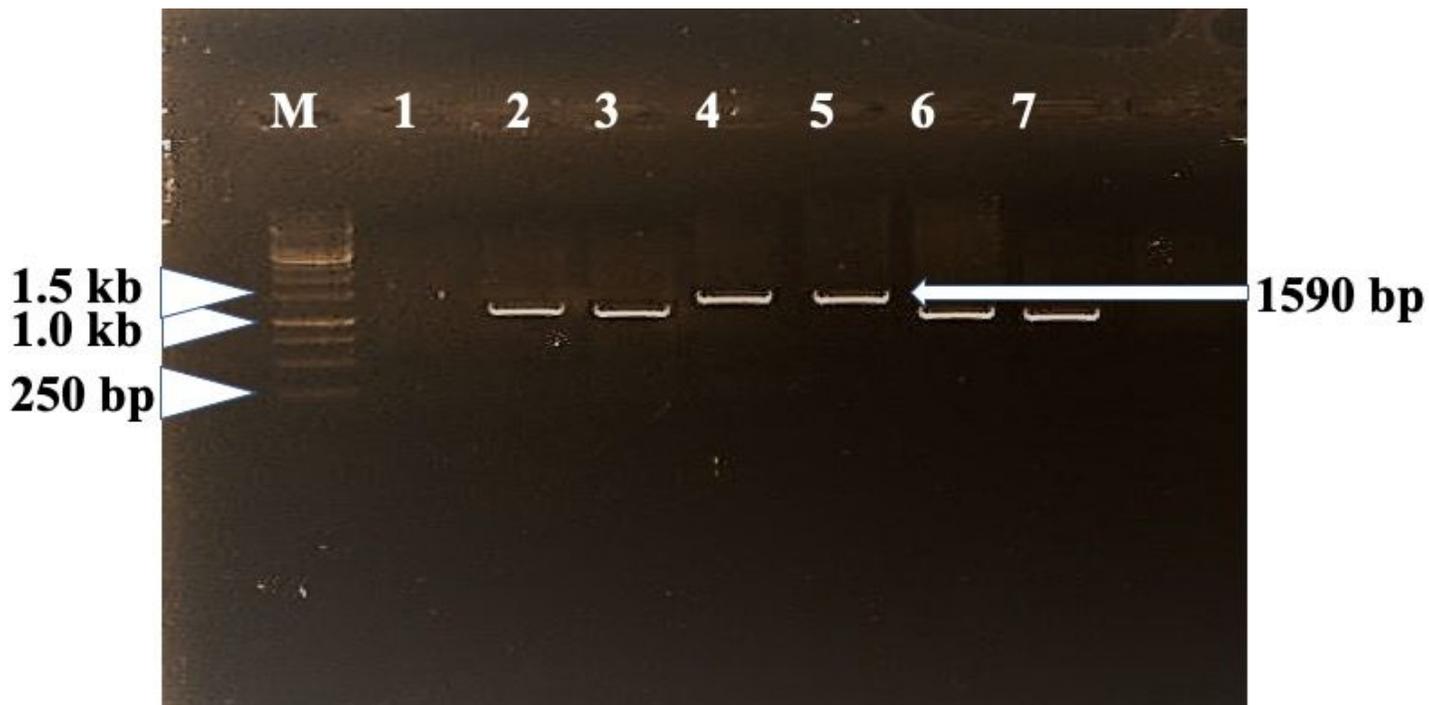


Figure 2

Cleaned PCR fragment after elution from Axygen PCR cleanup kit column. M: 1 kb DNA ladder (1) Non template control (2) leaf sample from callus induced shooting UDP glycosyltransferase gene (3) Callus sample gene (4) Leaf sample UDP glycosyltransferase mRNA (5) Callus Sample mRNA (6) Leaf sample UDP glycosyltransferase mRNA (7) Callus sample mRNA.

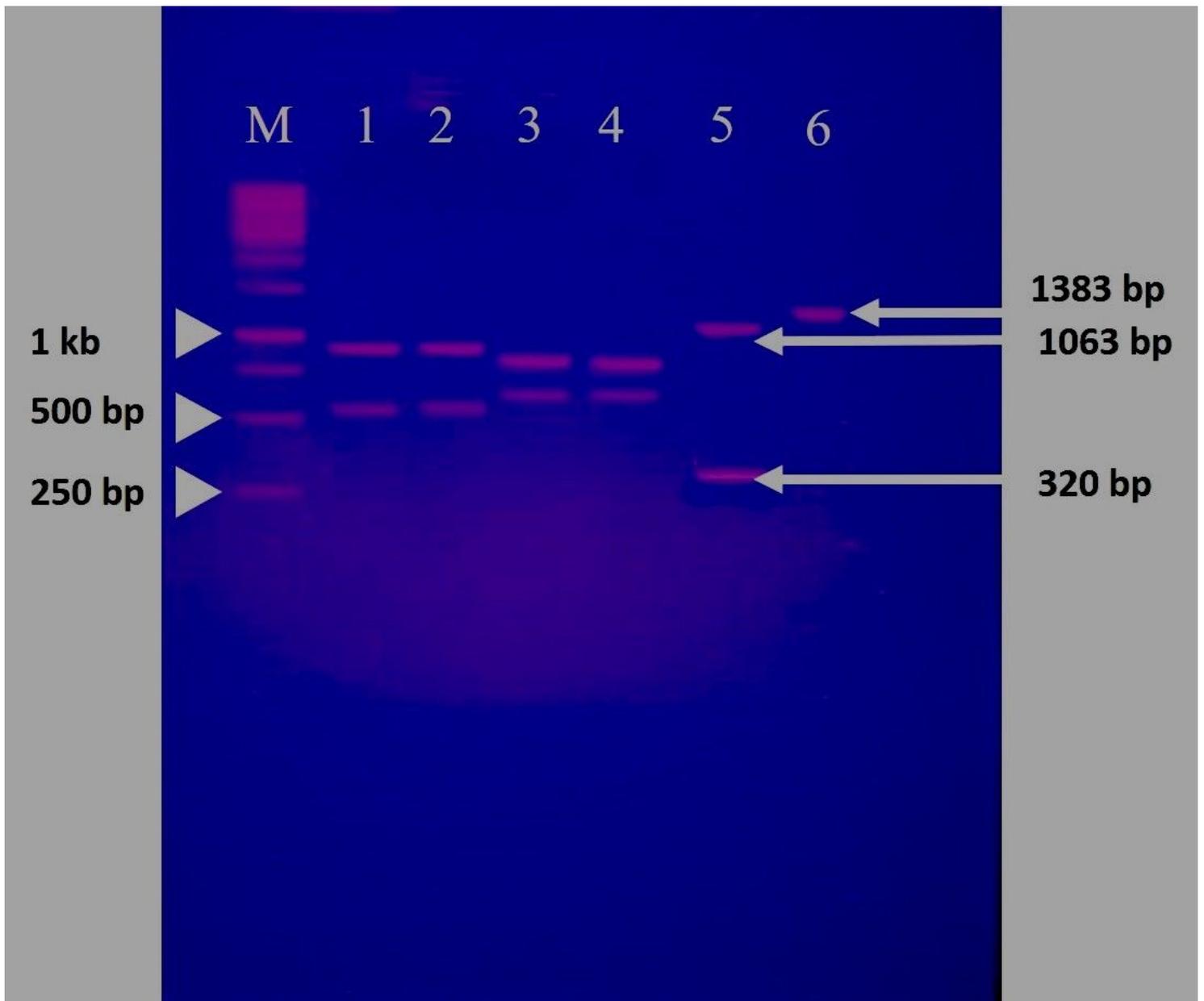


Figure 3

Restriction analysis of the genes. M: 1 kb DNA ladder (1) Leaf sample UDP glycosyltransferase gene restricted with Scal (2) Callus sample gene restricted with Scal (3) Leaf sample UDP glycosyltransferase mRNA restricted with NcoI (4) Callus sample mRNA restricted with NcoI (5) Leaf sample UDP glycosyltransferase mRNA restricted with DraIII (6) Callus sample mRNA restricted with DraIII.

Supplementary Files

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