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TRANSFORMATION OF CHITINASE GENE TO RESIST EARLY BLIGHT DISEASE IN SOME POTATO VIRUS RESISTANT LINES

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Potato (*Solanum tuberosum* L.) is the fourth most important food crop in the world and is a critical crop in terms of food security. During the growing season and under storage conditions, potato usually suffers from several fungal and viral diseases that cause serious damage and losses in yield such as leaf diseases, wilts, diseases of young plants, tuber, virus and fungal diseases (Jalli *et al*., 2011).

Alternaria solani is a fungal pathogen that produces a disease in tomato and potato plants called early blight; it is also one of the most important foliar pathogens of potato. Yield losses attribute to foliar damage, which results in decrease tuber quality and yield reduction, reach 20 to 30% (Olanya *et al*., 2009).

The cell walls of some bacteria, fungi, mushrooms, the exoskeleton of crustaceans (crabs, shrimp, etc.) and insects have chitin compound (Araujo *et al*., 1993). Chitin is used by chitin-containing organisms for protection against the harsh conditions in their environment and host anti parasite/pathogen immune responses. Thus, the absence of chitin can lead to the death of the pathogen (Lee, 2009).

Chitinases are involved in the defense mechanism of plants and vertebrates. Baculoviruses, which are used for biological control of insect pests, produce chitinases for pathogenesis special a defense against fungal pathogens by hydrolyzing 1,4, ß-D glucosidic bonds which linked the basic component of chitin (repeated units of N-acetyl glucose amine) (Escott *et al*., 1996).

Genetic transformation is highly important to resist the diseases of plants. One of these diseases is early blight disease caused by *Alternaria Solani* and the major goal of this study is to produce resistant plants to early blight disease by transforming the chitinase gene into two different lines of potato resistant to virus Y (PVY5 and PVY15) and potato cultivar (Desiree).

MATERIALS AND METHODS

This study was performed in Micro Propagation Technology Lab., Agriculture Genetic Engineering Research Institute (AGERI), ARC, Giza-Egypt, during the period from 2012-2017.

1- Gene cloning

The PCR product of *CHI* gene (chitinase), which was obtained from barley and purified by using QIAquik®PCR Purification system (Cat. No. 28106) was cloned into pRI 201-AN binary vector (Fig. 1). After two steps of digestion by NdeI and SalI restriction enzymes, the product was ligated by Bio Labs T_4 DNA ligase kit.

2- Transformation into E. coli

A 10 µL of ligation reaction product (construct) was added to 150 µL of *E. coli* competent cell and incubated on ice for 30 min, then the vial was incubated in a water bath at 42°C for 45 sec, after that was directly transferred on ice for 2 min. Then, 850 µL of LB broth medium was added to the *vial* and incubated for 90-120 min at 37°C with shacking at 200 rpm.

Finally, 500 µL of transformed competent cells was spread on LB agar plates supplemented with the appropriate antibiotic (kanamycine) and incubated at 37°C overnight.

3- PCR analysis for detection of positive colonies

The PCR was performed in 25 µl of reaction volume containing 5 µl of 1X PCR buffer (10 mM of Tris-HCl, pH 8.3, 50 mM of KCl, 2 mM of $MgCl_2$, 0.01% (w/v) of gelatine), 0.5 µl of dNTPs, 0.25 µl of Taq DNA polymerase, 1 µl of DNA template (*CHI* gene), a 1.5 µl (10 pmol) of each forward and reverse *CHI* gene primers and pRI vector primer and complete the volume with 13.75 µl of H_2O. Sequance of *CHI*, pRI and NPT II primers are shown in Table (1). In this step, the PCR conditions were started at 94°C for 3 min, 30 cycles of denaturation stage at 94°C for 40 sec., annealing stage at 58°C for 50 sec and extension stage at 72°C for 90 sec, followed by 7 min extension at 72°C.

4- Transformation into Agrobacterium tumefaciens

A 10 µl of the mini prep was added to the vial of *Agrobacterium* competent cells and incubated in ice for 30 min. The vial(s) were transferred into water bath and incubated for exactly 45 sec at 37°C, then were directly transferred in ice for an additional 2 min. After that, an 850 µl of LB broth medium was added to each vial gently mixed, and incubated at 28°C with moderate shaking for 90-120 min. A 350 µl of transformed competent cells were added to each LB agar plate which supplemented with the kanamycine (25 mg/ml) and uniformly spread. The plates were incubated at 28°C for 2-3 days, after this period, transformation in the grown colonies was detected by PCR analysis.

5- Transformation of potato (Solanum tuberosum L.) by Agrobacterium tumefaciens containing pRI 201- AN vector

Desiree cultivar, PVY5 and PVY15 potato lines were *in vitro* micropropagated using nodal cutting technique as described by Roca *et al*. (1978). Nodal cuttings were routinely sub-cultured every 3 to 4 weeks on a fresh MS medium (Murashige and Skoog, 1962).

Leaves of Desiree cultivar, PVY5 and PVY15 were selected after four weeks from sub cultured and collected in sterile Petri dishes under aseptic conditions. The upper and lower parts of the leaflets were cut out with sharp blade, and then the leaves were transferred into Petri plate containing 50 ml of *Agrobacterium tumefaciens* culture and incubated for 10 min with shacking. After incubation, the excess of bacterium was blotted on a sterile filter paper and the leaves were spread out on the callus media.

6- Callus initiation

The transformed leaves were spread out on the callus medium contained MS salts, 5 ml/L of 2-4,D (1 mg/ml), 1 ml/L of kanamycin monosulfate (100 mg/L) and 1 ml/l of cefotaxime sodium salt (200 mg/l) and incubated at 25 ± 1°C in a dark for 5 days.

7- Regeneration from callus

Callus were transferred into regeneration medium containing 100 mg/L of kanamycin monosulfate, 200 mg/l of cefotaxime sodium salt, 1 mg/l of BA, 1mg/L of IAA and 10 mg/L of GA3 and incubated in the growth chamber at 25 ± 1°C which is illuminated with fluorescent tubes for 16 hours per day at 3000 Lux. After 72 days, shoots were transferred to MS medium supplemented with 3% of sucrose, 1 mg/L of GA3 and 2 g/L of phytagel and the pH was adjusted to 5.6-5.7. The number of regenerated shoots in each callus tissue was calculated and analyzed (Kumar *et al*., 2014).

8- DNA extraction from plant tissue

DNA from leaves of Desiree cultivar and callus of PVY5 and PVY15 lines were extracted using to DNAeasy Plant

Mini Kit (50) Cat. No. 69104 from QIAGEN.

9- Detection of transformed potato plants

PCR analysis was performed to detect the transgenic Desiree potato cultivar, PVY5 and PVY15 lines by using forward and reverse primer of *CHI* and *NPTII* genes.

RESULTS AND DISCUSSION

Cloning of CHI gene

The PCR product was purified from any traces to prepare the template (*CHI* gene) and vector (pRI 201-AN) to be digested by restriction enzymes (NdeI and SalI) after adding the sequences of restriction enzymes (NdeI and SalI) to the sequence of *CHI* gene.

After two steps of digestion, first by SalI enzyme, then by NdeI enzyme to both template and vector, the result of digestion to the template and the vector was measured on a nanodrop instrument after ligation of the vector and the gene together (1 to 4 ratio) by T4 DNA ligase but not as Ningaraju (2006) who used the ligation reaction with an optimal molar ratio of 1:3 (vector: insert).

1- Transformation in E. coli

The ligation reaction was inserted into DH10β competent cells of *E. coli*. Screening for positive (transformed) colonies was carried out by PCR analysis in which the negative control lane did not show any fragment marker at 798 bp size (Fig. 2). Comparable results were obtained by Ningaraju (2006) who transformed chitinase genes (*chi A*, *chi B* and *chi C*) from *Serratia marcescens* into DH5α competent cell of *E. coli*.

2- Confirmation of potential positive colonies

The combination primers of *CHI* gene and pRI vector were used to confirm the two positive colonies 1 and 4. The results showed a clear fragment with molecular size of 1761 bp using the pRI F & *CHI* R primers, while a fragment of 1187 bp appeared using the two primers of *CHI* F & pRI R. In addition, the fragments with 2115 bp and 798bp which were obtained when the primers pRI F & R and *CHI* F & R were used (Fig. 3).

3- Transformation of CHI gene into Agrobacterium

Plasmid pRI 201-AN vector which contained *CHI* gene was transformed into LBA4404 *Agrobacterium tumefaciens* competent strain cells according to Ningaraju (2006). While, this screening of transformed colonies was done by using *CHI* forward and vector reverse primer, the transformation carried out a clear fragment size at 1187 bp (Fig. 4) which indicated that the selected colony no.1 was a transformed colony and carried the *CHI* gene.

4- Transformation of potato (*Solanum tuberosum* L.)

Leaves of Desiree cultivars, PVY5 and PVY15 were *in vitro* micropropagated

using nodal cutting technique as described by Roca *et al*. (1978). After 3-4 weeks, leaves were selected and incubated on MS media for 1-3 days in dark at 28°C after transformation steps by LB4404 *Agrobacterium* which contained *CHI* gene cloned in pRI 201-AN vector. The same result was also reached by Singh *et al*. (2015) who used class I rice endo chitinase gene introduced into eggplant (*Solanum melongena* L.) under the control of a constitutive CaMV 35S promoter by *Agrobacterium*-mediated transformation.

5- Callus induction and regeneration

After 1-3 days, the transformed leaves of Desiree cultivars, PVY5 and PVY15lines were spread out on callus medium (MS with 5 mg/L of 2-4, D) and incubated at 25 ± 1°C in the dark for 5 days to induct the callus. The leaf explants derived calli were placed on a regeneration media containing 1 mg/L of BA, 1 mg/Lof IAA, 10 mg/L of GA3 with 25 mg/mL of kanamycin and 200 mg/L of cefotaxcime. After 10 weeks, leaves became shoots through callus induction, regeneration and elongation steps are shown in Fig. (5).

The callus induction results of Desiree cultivar, PVY5 and PVY15 lines after 3 weeks were a 100%, 53.3% and 77.1%, respectively. After 9-10 weeks regeneration results were 96.6%, 71.4% and 73.5%, respectively (Table 2). Comparable results were reached by Rahayu *et al*. (2016) who used 4 mg/l of 2-4, D which gave the highest fresh weight and callus growth on *Centella asiatica* L. plant and Shirin *et al*. (2007) who used leaf and inter-nodal explants of four potato cultivars which cultured on MS media containing 2-4, D growth hormone alone.

6- PCR detection for transformed plants

Genomic DNA was extracted from the transformed potato plants of cultivar Desiree, PVY5 and PVY15 and the transformed plants were detected by using CHI and NPT II primers.

The use of *CHI* gene specific primers revealed a clear fragment at molecular size of 798 bp in all transgenic plants compared with the non transgenic Desiree cultivar (Fig. 6). The same was reached by Chang *et al*. (2002) who used *Agrobacterium*-mediated co-transformation of a pea β-1,3-glucanase and chitinase genes in potato (*Solanum tuberosum* L.).

For more detection, the positive transgenic plants were screened by using *NPTII* forward and reverse primer and the results showed a clear fragment at molecular size of 650 bp as shown in Fig. (7), as expected for the presence of *CHI* gene into the transgenic plants.

The forward and reverse primer of *CHI* gene was also used to confirm the presence of *CHI* gene into the transgenic plants of PVY5 and PVY15 lines, which showed a clear fragment at molecular size of 798 bp (Fig. 8). The same was reached by Esfahani *et al*. (2010) who used five lines out of eight putative transgenic potato *Savalan* cultivar which contained the

end part of the *CHI*42 transgene and Nos terminator. The corresponding fragment, 700 bp of the *chit42* gene, was amplified using specific primers (F4/RENOS).

SUMMARY

Potato (*Solanum tuberosum* L.) an agro-economically important food crop in the world. It is sensitive to many fungal pathogens including *Alternaria solani*, the causal agent of early blight disease. In the present study, pRI 201-AN binary vector, used in potato transformation, containing the *NPT-II* selectable marker gene in plant, containing the *chitinase* gene. Desiree cultivar, PVY5 and PVY15 lines (resistant to potato virus Y) were transformed with the pRI construct *via* the *Agrobacterium* delivery system. *Chitinase* gene was transformed into leaves of potato gene types. Transformed leaves were incubated on MS medium with a 5 mg/L of 2-4,D. After that, leaves transferred to regeneration media which contained MS medium with a 1 mg/L of IAA, a 1 mg/L of BA, a 10 mg/L of GA3, a 1 mg/L of cefatoxine (200 mg/ml) and a 1 mg/L of kanamycine (25 mg/ml). After 10 weeks of transformation, the regeneration results of Desiree cultivar, PVY5 and PVY15 lines were 96.6%, 71.4% and 73.5%, respectively. Their expression at the transcriptional level was confirmed by polymerase chain reaction (PCR).

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Table (1): Oligonucleotide sequence of *CHI* gene, pRI vector and NPTII specific primers.

Primers	Nucleotide sequence	bp	Gene (*CHI*) expected size
CHI forward	(F): 5`- CCCGGGACATATGATGAGATC GCTCGCGGTGGTG -3`	34	798 bp
CHI reverse	(R): 5`- CCGGGTCGACTTAGGCGAAGG GTCTCTGGCTGTA -3`	34	
pRI Forward	(F) 5`- GCGCGCGCGGCCGCGCCTGCA GGTGCCCAG- 3`	30	2115 bp
pRI Reverse	(R) 5`- GCGCGCCCCGGGTTCCTTATC TTTAATCATATTCCA - 3`	36	
NPTII Forward	(F) 5`- CGCAGAAGGCAATGTCATAC -3`	20	650 bp
NPTII Reverse	(R) 5`- ACCGCTGCGTAAAAGATACG- 3`	20	

Table (2): The percentages of callus induction and regeneration of Desiree cultivars, PVY5 and PVY15.

Callus induction %			Regeneration %		
Desiree	PVY 5	PVY 15	Desiree	PVY 5	PVY 15
100%	53.3%	77.1%	96.6%	71.4%	73.5%

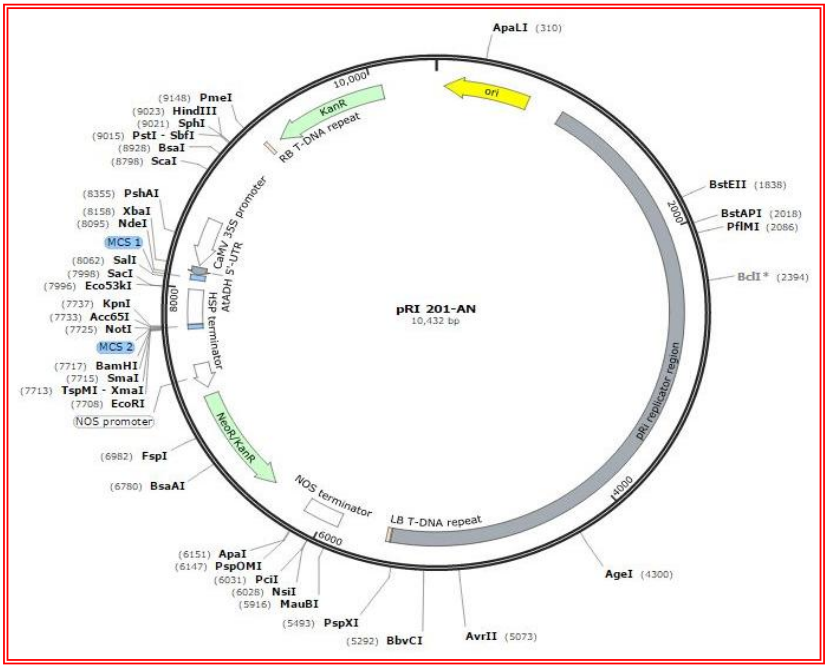

Fig. (1): pRI 201-AN binary vector, containing the NPT-II selectable marker of plant.

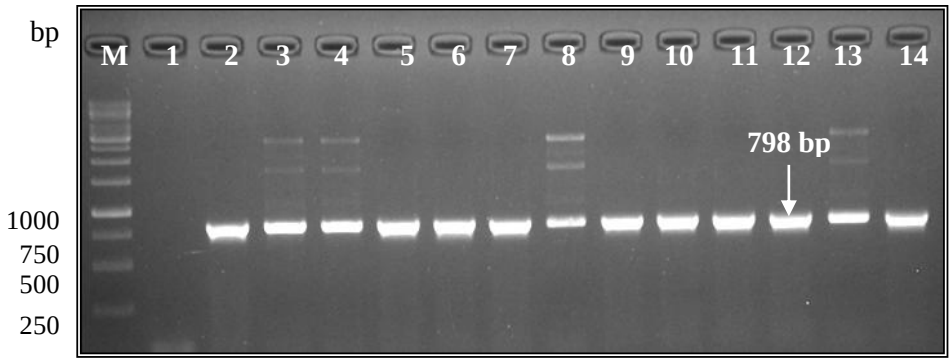

Fig. (2): Screening of transformed colonies after transformation by *E. coli*. 1: negative control, 2 to 13: positive colonies, 14: positive control and M: 1 kb Ladder.

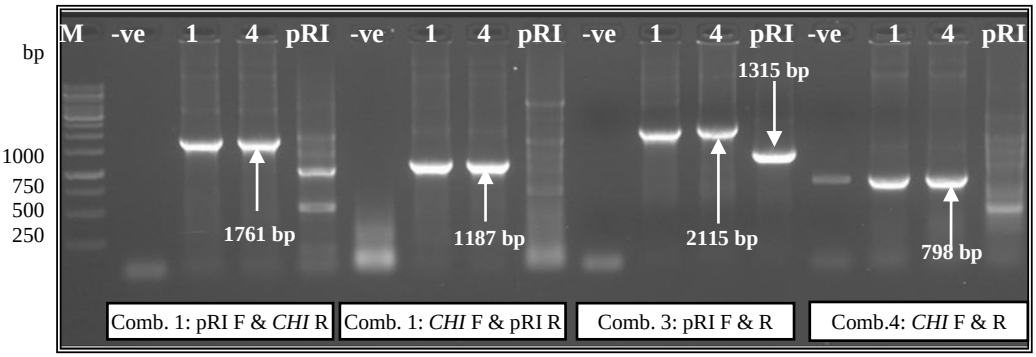

Fig. (3): Confirmation of potential positive colonies by different combination (comb.) of primers. -ve: Negative control, 1: coloni no. 1, 4: coloni no.4 and pRI: pRI vector. Comb.1: forward primer of pRI and reverse primer of *CHI* were used and expected size was 1761 bp. Comb.2: forward primer of *CHI* and reverse primer of pRI were used and expected size was 1187 bp. Comb.3: forward primer and reverse primer of pRI were used and expected size was 2115 bp. Comb.4: forward primer and reverse primer of *CHI* were used and expected size was 798 bp.

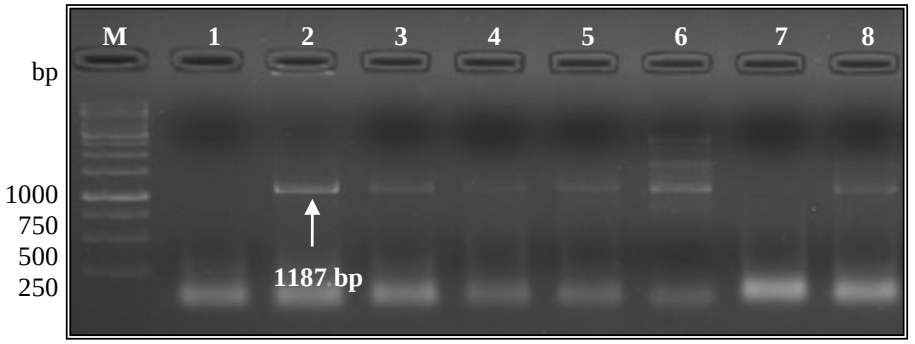

Fig. (4): Screening of transformed colonies after transformation by *Agrobacterium*, 1: negative control, 2 to 6: positive colonies, 7: negative coloni, 8: positive control and M: 1 kb Ladder.

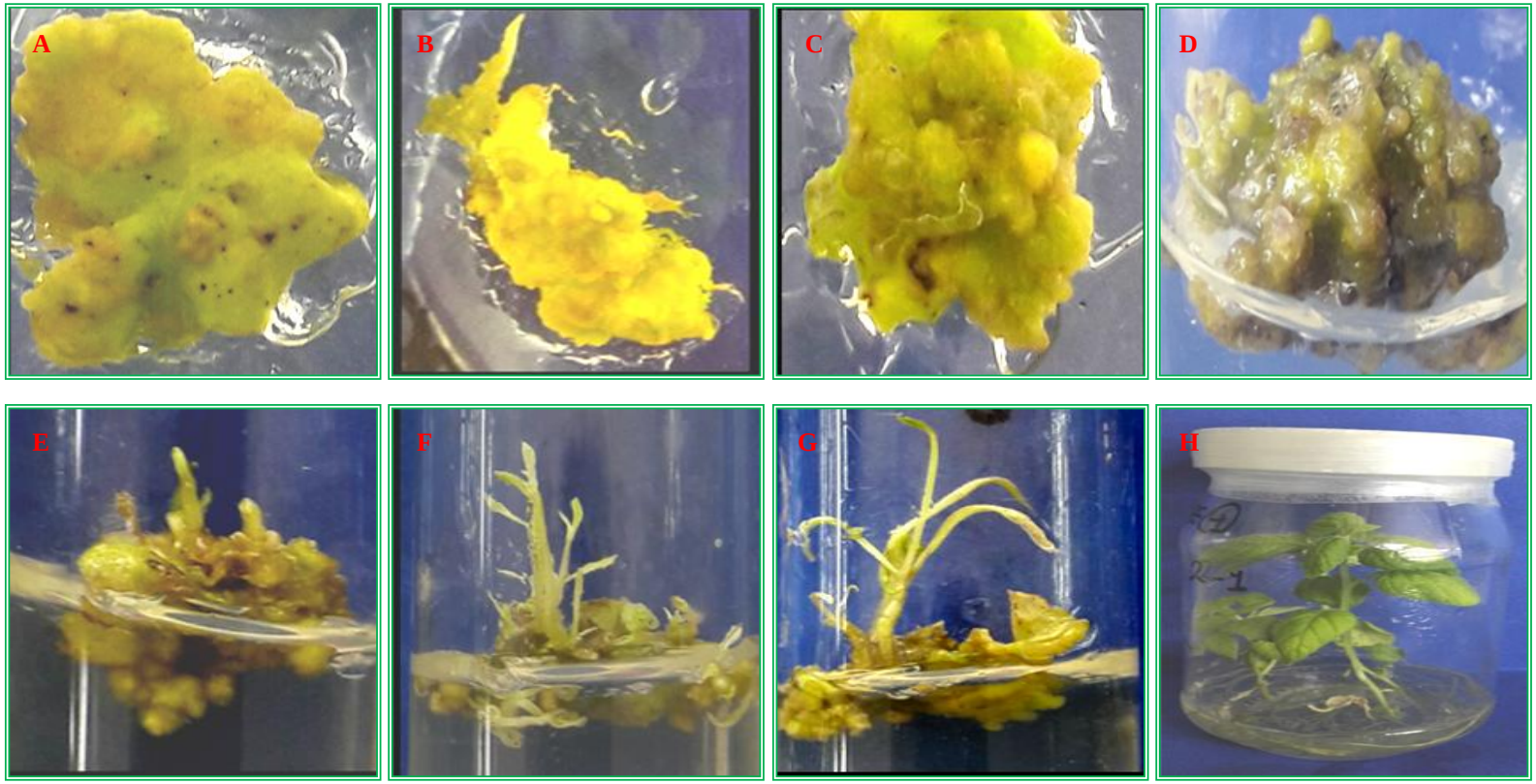

Fig. (5: A- H): Stages of callus induction, regeneration and elongation.

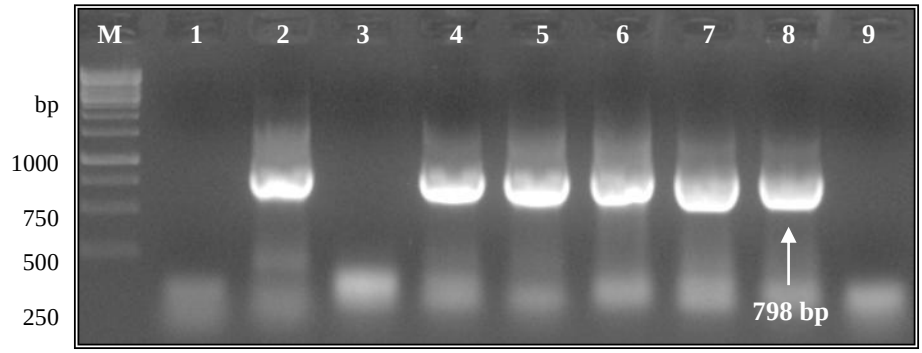

Fig. (6): Screening of transgenic plants of Desiree cultivar by using *CHI* primers and expected size was 798 bp. 1: Negative control, 2: Positive control, 3: Desiree control, 4 to 8: Transgenic plants, 9: Negative plant.

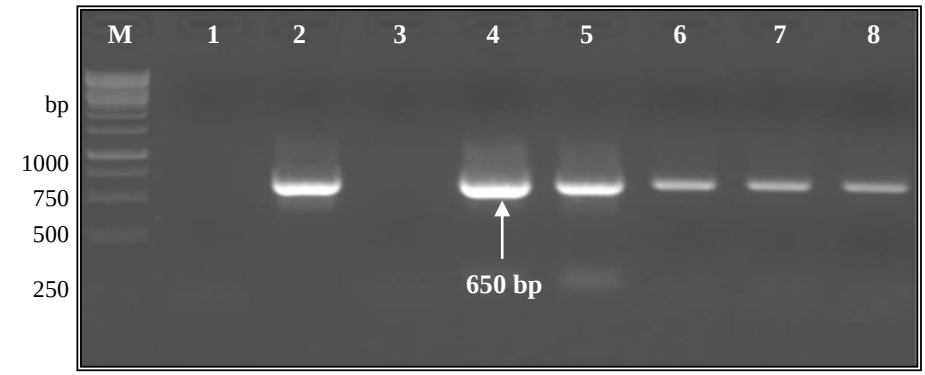

Fig. (7): Detection of transgenic plants Desiree cultivar by using NPTII primer and expected size was 650 bp. 1: Negative control, 2: Positive control, 3: Desiree control, 4 to 8: Transgenic plants.

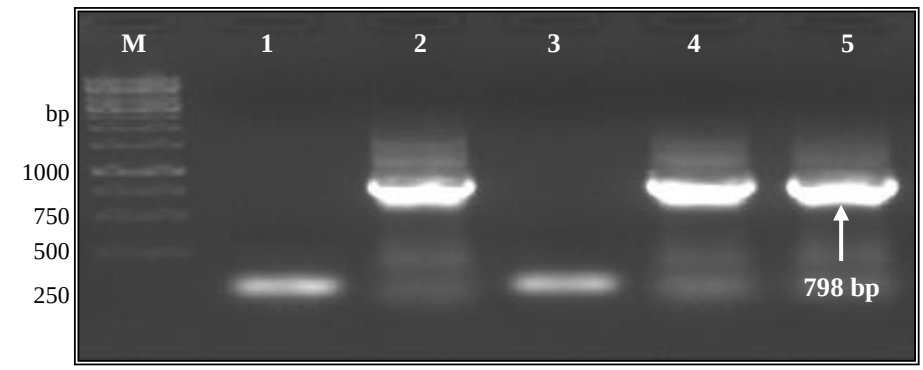

Fig. (8): Evaluation of transgenic plants of PVY 5 and PVY15 expected size was 798 bp, 1: Negative control, 2: Positive control, 3: Desiree control, 4: Transgenic PVY5 plants and 5: Transgenic PVY15 plants.