TRANSFER OF *GLUCANASE* GENE TO RESIST LATE BLIGHT DISEASE IN POTATO (*Solanum tuberosum* L.)

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Potato (*Solanum tuberosum* L.) is one of the world's most economically important tuber crops belonging to the family *Solanaceae*. It plays an important role in the food chain, as it ranks the 4th in importance after rice, wheat and maize (Solomon-Blackburn and Barker, 2001). Potato is a good and cheap source of carbohydrates, vitamins, minerals and proteins. It has multipurpose use in daily consumption and also industrial purpose (Hoque, 2010). Successful *in vitro* plant regeneration of potato has been achieved from explants of different organs and tissues of potato such as leaf, stem, tuber discs and unripe zygotic embryos (Srivastava *et al.*, 2012).

Bacterial and fungal diseases are currently one of the major factors impacting crop production. For example, nearly 20% of potatoes (*Solanum tuberosum* L.) are now lost due to diseases. Conventional practice to overcome this problem used the chemical fungicides which have adverse environmental effects causing health hazards to humans and other non-target organisms, including beneficial life forms. In advance, molecular biology techniques have developed transgenic plants conferring pathogenic resistance, with few reports of fungal resistance (Schickler and Chet, 1997).

Basically, recognition of plant defense mechanism has initiated by isolation and characterization of plant genes and proteins. The plant response to pathogen attack is a complex defense mechanism (Bowles, 1990), which comprises the synthesis of pathogenesis related proteins (PR) (Broglie *et al.*, 1986), phytoalexins and cell wall modifications (callose formation and lignifications) (Lozovaya *et al.*, 1998). The PR proteins include ß-1, 3-glucanases (PR-2) and chitinases (PR-3) (Fanta *et al.*, 2003). β-1, 3-glucanases was identified among PR proteins which presented in many higher plants and considered to be one of broad generalized defense mechanisms against pathogen attack (Cornelissen and Melchers, 1993).

The major goal of the present study are: 1) to produce transgenic potato cultivars resistant to late blight, 2) Introduce *glucanase* gene into potato cultivars through genetic engineering techniques by

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Agrobacterium mediated transformation and 3) Confirming the integration of the introduced genes in the genomic DNA of the putatively transformed potato plants using molecular analysis on DNA level (Polymerase chain reaction, PCR) and mRNA level (Reverse transcription PCR, RT- PCR).

MATERIALS AND METHODS

This study was performed in Micro propagation Technology Lab., Agriculture Genetic Engineering Research Institute, Cairo, Egypt, during the period from 2011 to 2016.

1-Regeneration of potato

Three potato cultivars (Diamond, Desiree and Spunta) were used as source of explants in the subsequent regeneration for initiation and maintenance of callus tissue. Each plate was contained five embryonic calli and the experiment was performed with 10 replicates according to the method which was described by Visser (1991). Explants were then dissected and cultured on two different regeneration media. The first medium contained Murashige and Skoog salts (MS) with vitamins (Murashige and Skoog, 1962) and supplemented with a 1 mg/l of IAA, a 10 mg/L of GA3 and a 1 mg/l of BA). While the second medium contained MS salts with vitamins and supplemented with a 3 mg/L of 2,4-D for 5 days in a dark. Subsequently, embryogenic calli were transferred into fresh media which contained a 5 mg/L of NAA and a 4 mg/L of KIN in a light.

2- Gene cloning

The target DNA sequences were amplified by using specific primer for *glucanase* gene, (GLN/F 5-'CAT ATG ATG GCT TTT CTA AGT TCT CTT GTA GC-3'), (GLN/R 5'- GTC GAC TTA ATT GAA ATT GAG TTG ATA CTT T -3'). The PCR program was as follows: denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension for a total of 35 cycles at 72°C for 1 min. Following amplification, PCR products were analyzed by agarose gel electrophoresis as described by Spychalla and Bevan (1993).

2.1- Cloning of glucanase gene into pGEM®-T Easy Vectors

PCR amplified β-1, 3 glucanase (*GLN*) gene was purified and ligated in pGEM-T Easy vector as shown in Fig. (1). The sequence of the multiple cloning sites is shown with a PCR product inserted by TA cloning strategy. The insert PCR product is flanked on each side by EcoR1 site (Sreeramanan *et al.*, 2006). Following ligation, recombinant plasmids were transformed into *E. coli* competent cells, and cultured in liquid media for plasmid miniprep.

2.2- Cloning of glucanase gene inserted in pRI 201-AN plant expression vector

The *ß-1, 3-glucanase* gene coding sequence was sub cloned in the plant expression vector pRI (Fig. 2), under 35S as a strong promoter and kanamycin gene (*NPTII* gene) as a selectable marker using

digested construct 1 by Sal I and Nde I restriction enzymes to obtain clone 2 (Matzke and Matzke, 1998).

2.3- Transformation of potato using Agrobacterium tumefaciens

One of the most spectacular achievements in the area of plant biology is the elucidation of the mechanism by which *A. tumefaciens* delivers the transferred DNA (T-DNA) to the plant cell through a cascade of events, which include induction *vir* genes formation of the T-DNA intermediates and T strand and the transfer of the latter to the plant cell.

The present protocol described the methodology for transformation of potato using LBA4404 *A. tumefaciens* strain which carrying a disarmed pGV2260 Ti plasmid in a pRI201-AN binary vector with a selectable marker gene that conferred resistance against the kanamycin antibiotic to the transformed cells. Transformed potato leaves were incubated on MS basal media supplemented with a 1 mg/l of IAA, a 10 mg/L of GA3, a 1 mg/l of BA, a 100 mg/l of kanamycin monosulfate and a 200 mg/l of cefatoxcen. The leaves were transferred every 10 days to fresh media.

2.4- Detection by PCR and RT-PCR procedures

Total RNA from potato leaves was isolated and the amount and purity of isolated RNA was determined using the nano-drop. For first strand cDNA synthesized a 2 µg of total RNA which was immediately reverse transcribed after the RNA extraction.

RESULTS AND DISCUSSION

Plant regeneration capacity among the three tested potato cultivars (Spunta, Diamant and Desiree) was compared on two different types of regeneration media with two different types of explants (inter-node and leaf) and cultured for induction and regeneration on regeneration media. The data were analyzed after seven weeks from culturing. The percentage of explants that induced to develop shoot in culture media are shown in Table (1).

The callusing response of the both types of explants on MS media containing a 3 mg/l of 2.4,D showed the best result for the leaf explants of Desiree cultivar compared with that of Spunta and Diamant cultivars. The calli were transferred on a fresh medium containing NAA and KIN but only the inter-node explant was more effective to introduce shoot proliferation than leaf explant from all cultivars. In addition, when the calli were transferred on a fresh medium containing BA with IAA and GA3, the leaf explant was more effective to introduce the shoot proliferation (Table 1).

Data was analyzed by the SAS System, the GLM Procedure and Duncan's Multiple Range Test for the average percentage of the number of shooted plants. This test controls the Type I comparison wise error rate, not the experiment wise error rate (Tam and Lang, 2003) (Table 1).

Cloning of glucanase gene PCR product into pGEM®-T Easy Vectors

PCR amplified *β-1, 3-glucanase* (*GLN*) gene was purified and ligated into pGEM-T Easy vector as shown in Fig. (1). Following ligation, recombinant plasmids were transformed into *E. coli* competent cells, and cultured in liquid media for plasmid DNA miniprep. Sreeramanan *et al.* (2006) who used antibiotic as a selection agent for stable integration. The white selected colonies which were first subjected to PCR screening using the GLN-F/GLN-R primer pair indicated the expected clone fragment size at 1020 bp as shown in Fig. (3). Screening of clone 1 for *glucanase* gene using *EcoR*1 restriction enzyme for orientation, resulted in a release of GLN - coding sequence from the pGEM -T Easy cloning vector. A DNA fragment at about 1020 bp was released and separated by agarose gel electrophoresis.

Cloning of glucanase gene inserted into PRI 201-AN plant expression vector

The *ß-1, 3-glucanase* gene coding sequence was sub cloned in pRI201-AN plant expression vector under 35S as a strong promoter and kanamycin gene (*NPTII* gene) as a selectable marker as shown in Fig. (2). Harboring 35S promoter for constitutive over expression of *glucanase* gene should be done to increase glucanase protein resistance to the late blight fungus, *P. infestans* (Matzke and Matzke, 1998). For detection the integrated *glucanase* gene, DNA fragment at 1020 bp coding was amplified by polymerase chain reaction (PCR) using the GLN Forward and GLN Reveres primer pair as shown in Fig .(4).

Transformation in Agrobacterium tumefaciens

Since both physical and biological methods are easy for transfer the important traits to interest plants, this technique is a helpful for a lot of crops improvement for those which increase resistance against biotic and abiotic stresses or for those which improve the quality of food. Transgenic plants have been produced plants that are resistant to herbicides, insects, and diseases. These qualities transferred to plants through a lot of application for genetic transformation protocols using discs and tuber discs (Beaujean *et al.,* 1998). The amplified fragment at 1020 bp of *glucanase* gene was transformed in competent *Agrobacterium tumefaciens* cells and PCR was utilized in this investigation for the rapid screening of *glucanase* gene using specific primer as shown in Fig. (5).

Shoots proliferation of potato plants cultivar Desiree

Plant transformation using *Agrobacterium* is affected by both host specificity and the ability of *Agrobacterium* to reach the cells in the target tissue. So a new and efficient *Agrobacterium* based transformation method that overcomes these limitations and enhances of DNA transfer was required (Hussain *et al.*, 2007). The leaf explants isolated from Desiree cultivars were transformed by

LBA4404 *A. tumefaciens* strain which harboring the PRI plasmid. After exposure to 5 min, the explants; were transferred into MS media and incubated at 28°C over night, then transferred into callus induction media (MS media containing a 3 mg/l of 2.4,D). The leaf explants derived calli were placed on regenerating media containing a 1 mg/l of BA with a 1 mg/l of IAA and a 10 mg/l of GA3 for two weeks. The selected transformed explants were sub cultured into freshly prepared media containing a 25 mg/ml of kanamycin and a 200 mg/l of cefatoxcen. During the selection process successfully transformed calli were continued to vigorously grow to produce shoot initiations, whereas the non-transformed ones failed to form shoot and eventually bleached and became necrotic within three weeks (Fig. 6). Transformation efficiency produced a positive PCR result with the *glucanase* gene primer (Fig. 7).

PCR detection for transformed potato using specific primer

PCR using specific primer to *glucanase* gene was used to confirm the stable integration of *glucanase* gene in the T-DNA on putative transgenic plantlets genomes. Figure (6) showed a clear fragment at 1020 bp in the transgenic plants only, however, no such fragment was seen in the non-transformed controls and this result agree with (Yamamoto *et al*., 2000) who observed that in two transgenic grapevine lines (out of 9) no positive bands for the rice *chitinase* gene (RCC2) were obtained.

RT-PCR detection for transformed potato using specific primer

To confirm the integration and expression of *glucanase* gene in the positive transgenic potato plants; RT- PCR reaction was applied using c-DNA of the isolated RNA from putatively positive PCR transgenic plants using a specific primer to *glucanase* gene (Nie and Singh, 2001). The results of RT-PCR reaction confirmed the integration and expression of *glucanase* gene in the positive transgenic plant as shown in Fig. (8).

SUMMARY

Potato late blight is a pandemic disease caused by the highly virulent (*Phytophthora infestans*) fungus. The regeneration capacity was done among three tested potato cultivars (Spunta, Diamont and Desiree). Two different types of explants (inter-node and leaf) were cultured for calli induction and plant regeneration .The highest value of leaf explants was for Desiree cultivar (80%), which induced from developed callus in the 2,4-D medium. The best value of maximum shoot regeneration was also for Desiree cultivar in the BA, IAA and GA3 media from leaf which proved to be more effective. Then cloning of *glucanase* gene in pRI plasmid which carrying kanamycin resistance (*nptII*) gene was perfomed and followed by transformation in *Agrobacterium tumefaciens* LBA4404 strain which used for plant transfection. Nucleotide and amino acid sequences of transformed *Agrobacterium* were analyzed. The putative

transgenic plantlets genomes and cDNA of the isolated RNA for glucanase gene.

REFERENCES

Beaujean, A., R. Sangwan, A. Lecardonnel and B. Sangwan-Norreel (1998). *Agrobacterium*-mediated transformation of three economically important potato cultivars using sliced internodal explants: an efficient protocol of transformation. Journal of Experimental Botany, 49: 1589-1595.

Bowles, D. J. (1990). Defense-related proteins in higher plants. Annual Review of Biochemistry, 59: 873-907.

Broglie, K. E., J. J. Gaynor and R. M. Broglie (1986). Ethylene-regulated gene expression: molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. Proceedings of the National Academy of Sciences, 83: 6820-6824.

Cornelissen, B. J. and L. S. Melchers (1993). Strategies for control of fungal diseases with transgenic plants. Plant Physiology, 101: 709.

Fanta, N., X. Ortega and L. M. Perez (2003). The development of *Alternaria alternata* is prevented by chitinases and ß-1, 3-glucanases from Citrus limon seedlings. Biological Research, 36: 411-420.

Hoque, M. (2010). *In vitro* tuberization in potato (*Solanum tuberosum* L.). Plant Omics, 3: 7.

Hussain, J., C. Millman, J. Scott, P. Hannon and H. Matlay (2007). Ethnic minority graduate entrepreneurs in the UK characteristics, motivation and access to finance. Industry and Higher Education, 21: 455-463.

Lozovaya, V. V., A. Waranyuwat and J. M. Widholm (1998). β-l, 3-glucanase and resistance to *Aspergillus flavus* infection in maize. Crop Science, 38: 1255-1260.

Matzke, M. and A. Matzke (1998). Epigenetic silencing of plant transgenes as a consequence of diverse cellular defence responses. Cellular and Molecular Life Sciences CMLS, 54: 94-103.

Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia plantarum, 15: 473-497.

Nie, X. and R. Singh (2001). A novel usage of random primers for multiplex RT-PCR detection of virus and viroid in aphids, leaves,

and tubers. Journal of Virological Methods, 91: 37-49.

Schickler, H. and I. Chet (1997). Heterologous chitinase gene expression to improve plant defense against phytopathogenic fungi. Journal of Industrial Microbiology and Biotechnology, 19: 196-201.

Solomon-Blackburn, R. M. and H. Barker (2001). Breeding virus resistant potatoes (*Solanum tuberosum*): a review of traditional and molecular approaches. Heredity, 86: 17-35.

Spychalla, J. P. and M. W. Bevan (1993). *Agrobacterium*-mediated transformation of potato stem and tuber tissue, regeneration and PCR screening for transformation. Plant Tissue Culture Manual. Springer, p: 379-396.

Sreeramanan, S., M. Maziah, N. Rosli, M. Sariah and R. Xavier (2006). Particle bombardment-mediated co-transformation of *chitinase* and *β-1, 3 glucanase* genes in banana. Biotechnology, 5: 203-216.

Srivastava, A., L. Diengdoh, R. Rai and T. Bag (2012). Tissue culture-technology harnessed for potato seed production. Keanean J. Sci., 1: 80-86.

Tam, D. M. and N. T. Lang (2003). *In vitro* selection for salt tolerance in rice. OmonRice, 11: 68-73.

Visser, R. G. (1991). Regeneration and transformation of potato by *Agrobacterium tumefaciens*. Plant Tissue Culture Manual. Springer, p: 301-309.

Yamamoto, T., H. Iketani, H. Ieki, Y. Nishizawa, K. Notsuka, T. Hibi, T. Hayashi and N. Matsuta (2000). Transgenic grapevine plants expressing a rice chitinase with enhanced resistance to fungal pathogens. Plant Cell Reports, 19: 639-646.

Table (1): Regeneration of three tested potato (*Solanum tuberosum* L.) cultivars (Desiree, Spunta and Diamond).

Potato Cultivars	Media (I) (BA with IAA and GA3)			Media (II) (NAA and KIN)		
	% of shooted plants from inter-node	% of roots per callus	No. of shoots/ callus	% of shooted plants from inter-node	% of roots/ callus	No. of shoots/ callus
Desiree	30	0	50.0[c]	5	5	77.2[a]
Spunta	18	0	30.5[d]	20	6	90.3[a]
Diamond	31	1	66.3[b]	1	1	25.0[d]
Potato Cultivars	% of shooted plants from leaf	% of roots/ callus	No. of shoots/ callus	% of shooted plants from leaf	% of roots/ callus	No. of shoots/ callus
Desiree	38	0	88.4[a]	13	30	40.9[c]
Spunta	21	0	38.4[c]	11	26	38.8[c]
Diamond	14	0	39.7[c]	7	7	27.5[d]

Means with the same letters are not significantly different Duncan's at 0, 05

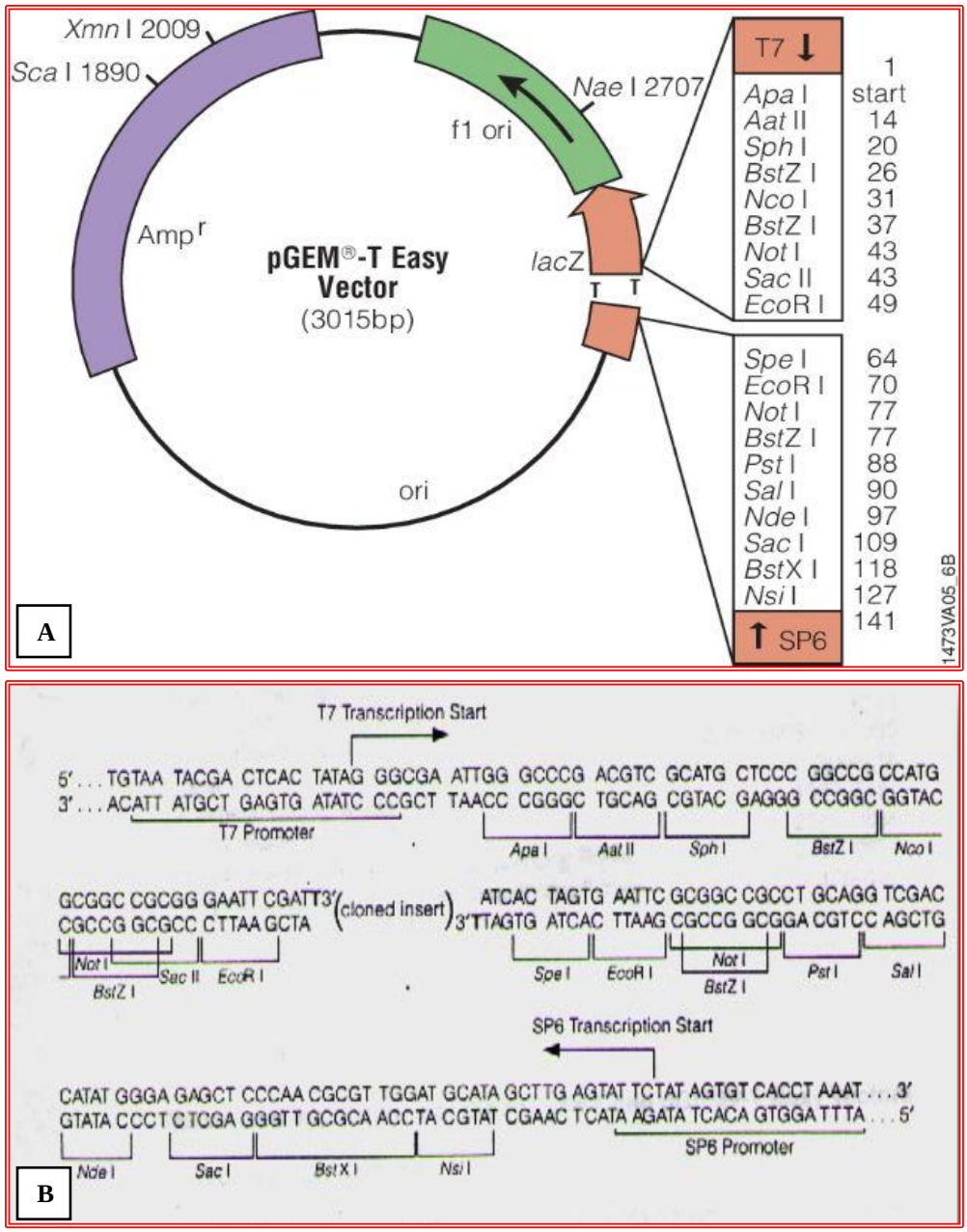

Fig. (1): (A) Restriction map and general specification of pGEM-T easy vector. (B) Sequence of restriction enzyme site of pGEM-T easy vector, the arrow indicates the start of the transcription of the T7 RNA polymerase.

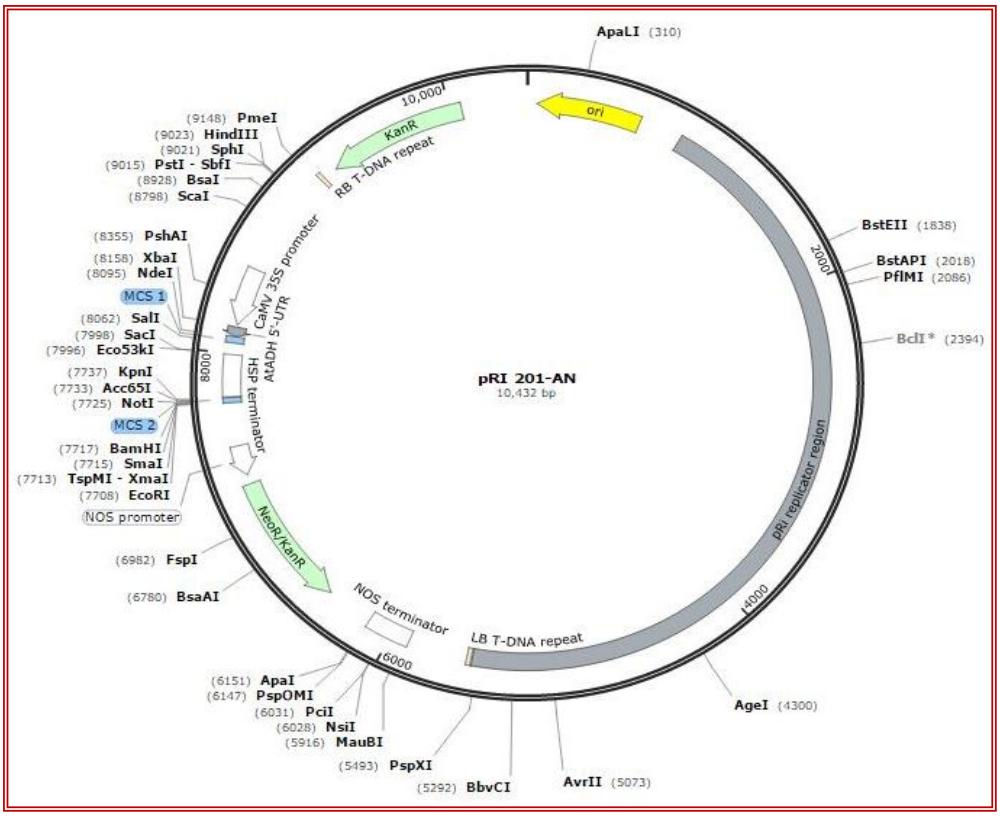

Fig. (2): Restriction map and general specification of pRI201-AN Plasmid.

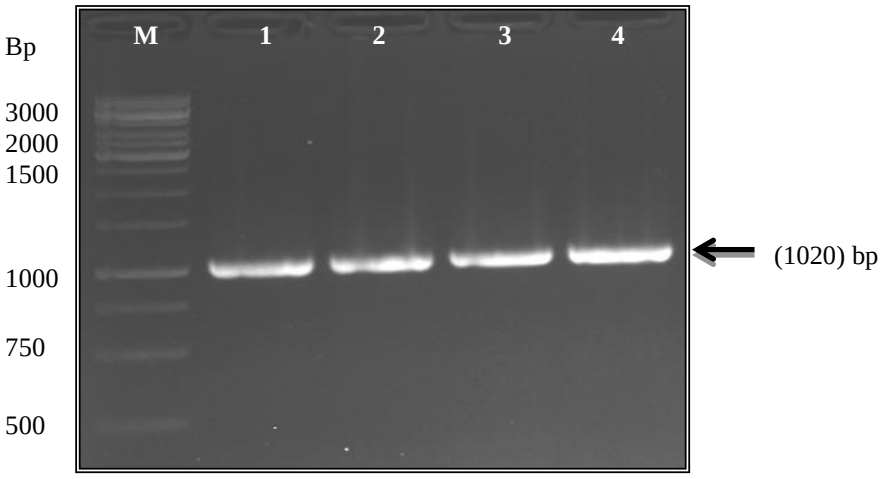

Fig. (3): Integration of *glucanase* gene into pGEM-T easy vector, as confirmed by the presence of the 1020 bp PCR-amplified glucanase fragment. M = 1 Kb ladder: Lane 1 to 4 = fragment of *glucanase* gene.

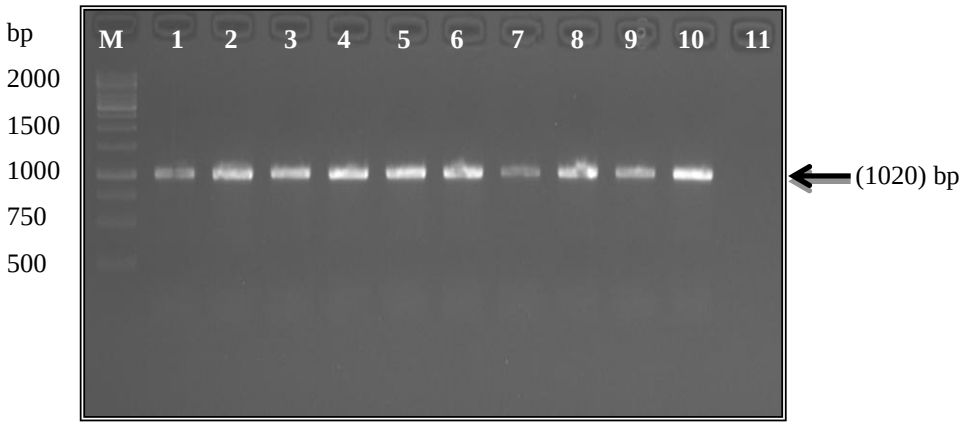

Fig. (4): Agarose gel for PCR product at1020 bp of *glucanase* gene from clone 2. M: 1 Kb ladder. Lanes from1 to 10 represent 10 different colonies. Lane11: negative control.

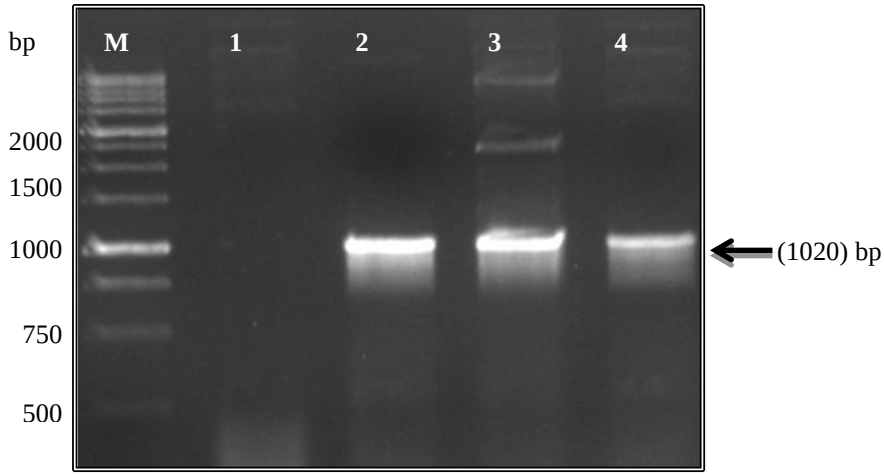

Fig. (5): Agarose gel for PCR product: at 1020 bp of *glucanase* gene from *Agrobacterium* M: 1 Kb ladder; Lane 1: negative control; Lane 2: clone2; Lane 3 to 4: transformeted *Agrobacterium* colonies.

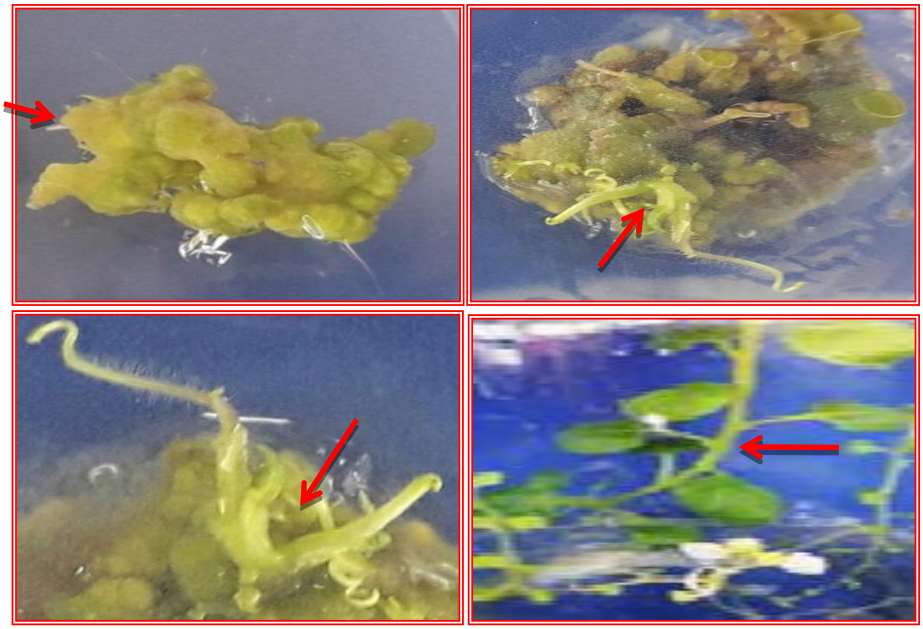

Fig. (6): Callus induction and plant regeneration for leaf explants in Desiree potato cultivar (a, b); Shoot regeneration after seven weeks of subculture (c). The arrows show shoots growing. Node cut elongation on MS media with a 200 mg/l of cefatoxcen (d).

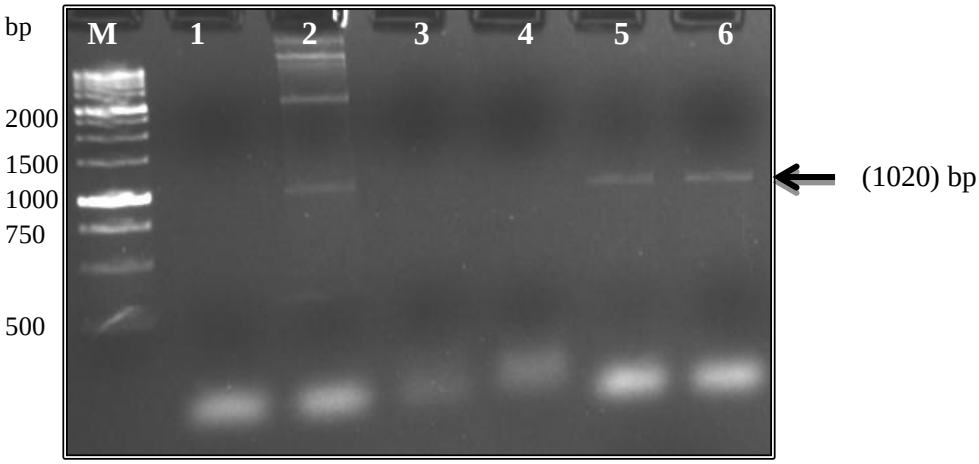

Fig. (7): Agarose gel of PCR product at 1020 bp of *glucanase* gene from the transgenic potato lines. M: 1 Kb ladder. Lane 1: non transgenic potato line. Lane 2: clone2. Lane 3 to 6: transformed potato lines.

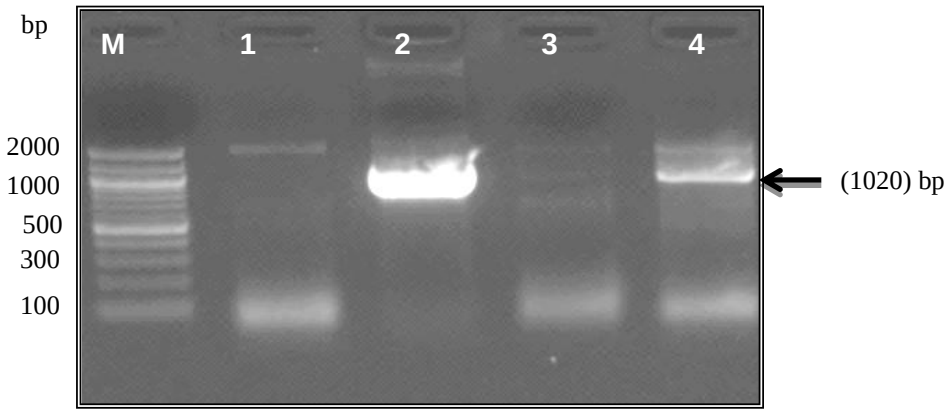

Fig. (8): Verification of *glucanase* gene into putatively transgenic potato plants via RT-PCR analysis. Screening plants showed the expected fragment at 1020 bp in several plant individuals. Lane M: 100 bp DNA ladder (gene ruler™Fermentas), Lane (1): negative control, Lane (2): represented putatively transgenic plants (positive), Lanes (3 & 4): transformed potato plants.