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INTRODUCTION OF RICE CHITINASE GENE IN POTATO BY *AGROBACTERIUM*-MEDIATED TRANSFORMATION

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Potato is an important food crop of the world. Different viral, bacterial and fungal pathogens cause heavy economic losses of this crop every year. Potato has complex genetic makeup due to which induction of disease resistance through conventional breeding is difficult. Genetic manipulation through different transformation techniques is more precise and successful tool. In the present study different factors were investigated, which have an influence on potato transformation. The optimal dose of cefotaxime was found 500 mg/l which did not affected the growth of the potato tissues. The explants treated with *Agrobacterium* in the presence of acetosyringone resulted in higher frequency of transformation as compared to the explant without it. Two minutes time for co infection was found appropriate for optimum transformation efficiency. The two days co-cultivation period along with 7 days preselection was found suitable for potato transformation. The putative tranformants regenerated on MS medium supplemented with 20 mg/L hygromycine and 500 mg/L cefotaxime, from nodal explants while the non-transformed tissue turned brown and gradually died after two or three sub culturing on the selection media containing selective antibiotic hygromycin. The shoots obtained on selection media shifted on root induction media supplemented with similar concentrations of hygromycin and cefotaxime, resulted complete plantlet formation after 10 days. It was observed that all the hygromycin positive plants also exhibited positive bands of desired size of 823 bp for chitinase gene, suggesting co-transformation of both genes in transformed plants.
Keywords: *Solanum tuberosum*, chitin, hydrolytic enzyme, fungal pathogens.

INTRODUCTION

Potato is a significant, widely cultivated commercial cash crop around the world. In Pakistan, it is cultivated on an area of 178.22 thousand hectares with production of 4000.3 thousand tones (FAOSTAT, 2016) annually. It is a vegetatively propagated crop, therefore diseases can be easily transmitted from one infectious generation to other, rendering this crop prone to several, viral, bacterial and fungal diseases. Among the fungal diseases the most commonly are the late blight, early blight, Fusarium wilt, stem cankers and verticillium wilt. Economic loss due to these diseases, sometime occurs as high as 75% (Khalid *et al*., 2000).

Potato is a tetraploid and heterozygous in nature due to which breeding for disease resistance is difficult by conventional means. Therefore, it is genuinely needed that the disease resistant potato cultivars be produced by other advance tools such as genetic manipulation. Foreign gene incorporation into crop plants through different transformation techniques as particle bombardment and agrobacterium methods have made easy for desired agronomic characteristics and resistance against pathogens (Altpeter *et al*., 2005; Tzfira and Citovsky 2006).

Chitinases are pathogen related proteins, induced during fungal infection and found in wide variety of plants (Chai *et al.,* 2002; Singh *et al.,* 2015). Chitinase is considered as hydrolytic enzyme which degrades the chitin cell wall of majority of filamentous fungi. It hydrolyzes the chitin to N-acetyl glucosamine oligomers by cleaving β-1, 4 bonds. Many researchers have reported that transformation of chitinase genes in crops, may leads to resistance against different fungal pathogens. Nishizawa *et al.* (1999) reported higher level of resistance against rice blast pathogen *Magnaporthe grisea* by incorporation of class I rice chitinase gene described as rice chitinase cDNA clone (RCC2). While, Tabei *et al*. (1998) and Kishimoto *et al*. (2002) were successful to induce resistance in cucumber plants by incorporation of RCC2 genes against gray mold (*Botrytis cinerea*). Maximova *et al.* (2005) exploited antifungal activity of chitinase gene in transgenic *Theobroma cacao* containing cacao class I chitinase which enhanced the resistance against *Colletotrichum gloeosporioides* fungus. Kova *et al*. (2013) expressed rice chitinase gene in banana leading to resistance

against black leaf streak disease. Similarly, Jabeen *et al*. (2015) reported that expression of chitinase in tomato resulted in enhanced resistance to Fusarium Wilt and Early Blight. These studies point towards the usefulness of chitinase gene in controlling fungal disease, therefore, in current study transformation of chitinase gene has been carried out in an elite potato variety known as cardinal, which is very popular among the farmers for its high yield and good acceptability for household cooking and in chips industry. The cardinal is already resistant to bacterial diseases; adding resistance to fungal disease through transformation of RCC2 gene will make it more productive for farmers in Pakistan.

MATERIALS AND METHODS

***Plant materials*:** Promising cultivar of potato variety cardinal was selected for the study and cardinal seed tubers were obtained from Potato Research Program of National Agriculture Research Center, Islamabad, Pakistan. These tubers were given thermotherapy following Kaiser (1980) to remove any possible virus infection. Further, these tubers were grown in pots containing autoclaved peat moss in green house conditions. After approximately, one month of sowing, the most upper young tissue of 5-6 mm were taken to tissue culture laboratory, surface sterilized with 50% Clorox bleach for 10 minutes and then meristem tip tissue were excised under stereo-microscope (40X) with the help of sterilized needles and scalpel in the laminar air flow and cultured over sterilized filter paper in liquid MS medium (Murashige and Skoog, 1962). The virus free plantlets were maintained and multiplied on optimized MS solid media having 1.0 mg/L GA_3 and 100 mg/L each of KH_2PO_4, KNO_3 and NH_4NO_3. The cultures were kept at 25±2°C with 16 hrs photoperiod. The nodes were excised from these virus free *in vitro* stocks for the transformation of chitinase gene.

***Direct regeneration*:** Nodes (0.5-1.0 cm) were excised from 8-10 cm long two weeks old *in vitro* shoots and cultured on MS media supplemented with 0.5 mg/l IAA and 4.0 mg/l BAP. The cultures were placed at 25±2°C with16 hrs photoperiod (Hussain *et al.*, 2005).

***Plasmid and bacterial strain*:** The binary vector pBI333-EN4-RCG3 (Fig. 1) was acquired from National Institute of Agriculture Resources (NIAR) Tskuba, Ibaraki, Japan, through material transfer agreement. The vector consisted of 1.1-kb DNA fragment containing chitinase gene Cht-*3*, from a rice genomic clone (RCG3) and hygromycin resistance gene, both of which are under the expressional control of enhanced 35S promoter from cauliflower mosaic virus (CaMV) separately. It also possessed four tandemly repeated enhancer regions (EN4) (–290 to –90) of its own. For selection in bacteria, this binary plasmid also harbors kanamycin resistant genes as selectable marker. The vector was initially cloned in *E. coli* followed by transformation into *Agrobacterium* strain EHA-101 via electroporation.

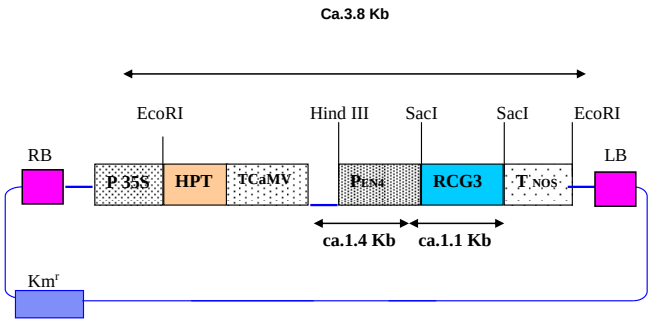

Figure 1. Schematic representation of plasmid pB1333-EN4-RCG3 used for potato transformation. The plasmid contained hygromycin phosphate transferase (HPT) gene, terminator of the nopaline synthase (TNOS) gene, CaMV 35S promoter (P35S), enhanced Ca MV 35S promoter (PEN4) and coding region of the *Cht*-3 genes (RCG-3). Km^r is Kanamycin resistance gene, while RB and LB represents right and left border. Sites of various restriction enzymes are also shown.

***Transformation*:** *Agrobacterium tumefacien* strain EHA101 containing binary vector pBI333-EN4- RCG3 having chitinase gene, kanamycin and hygromycin resistant genes was streaked onto YEP medium containing 50 mg/L hygromycin and kanamycin each. Culture was left for two days to form the colonies. A single colony was picked and cultured in liquid YEP medium containing 50 mg/L hygromycin and kanamycin each until to an $O.D._{.600}$=0.3. Following transformation treatments were given to acquire higher transformation efficiency.

***Effect of acetosyringone and co-infection time*:** Effect of acetosyringone and co-infection time on the transformation efficiency was observed by treating the explants with overnight grown *A. tumefaciens* strain EHA101 solution with and without addition of acetosyringone (10 µg/mL) for 2, 5, 10 or 15 minutes.

***Co-cultivation*:** After co-infection, the explants were left for co-cultivation (2, 3, 4 and 5 days) on solid (CCM) medium (Table 1). Explants were washed three times with liquid regeneration (RM) medium supplemented with 500 mg/L cefotaxime (*Aventis*) for which lethal dose was optimized using 0, 100, 250, 500 and 1000 mg/L. The explants were dried with sterilized filter papers every time.

***Pre-selection and selection of putative transformants*:** Following co-cultivation, the explants were transferred on pre-selection medium (PSM) containing 500 mg /L cefotaxime for 7, 8 or 10 days (Table 2). After pre-selection they were transferred to selection (SM) medium having 20 mg/L hygromycin and 500 mg/L cefotaxime. The regenerated plants were shifted on fresh selection medium after every two weeks. After six weeks of sub-culturing, the putative transformed tissues were shifted on selection media.

***Transgenic analysis*:** Approximately 2-3 leaves were taken from the plantlets produced on selection media, surface

Table 1. Medium used in different steps of potato transformation.

Media	Stages of regeneration					
MS (1962)	**RM**	**CCM**	**WM**	**PSM**	**SM**	**RtM**
Vitamins mg/l						
Thiamine HCl	0.1	0.1	0.1	0.1	0.1	0.1
Nicotinic acid	0.5	0.5	0.5	0.5	0.5	0.5
Pyridoxine	0.5	0.5	0.5	0.5	0.5	0.5
Myoinositol	100	100	100	100	100	100
Hormones mg/l						
BAP	4.0	4.0	4.0	4.0	4.0	0.0
IAA	0.5	0.5	0.5	0.5	0.5	0.0
NAA	0.0	0.0	0.0	0.0	0.0	0.1
Acetosyringone	0.0		0.0			
Antibiotics mg/l						
Cefotaxime	0.0	0.0	500	500	500	0.0
Kanamycin	0.0	0.0	0.0	0.0	100	100
Hygromycin	0.0	0.0	0.0	0.0	10.0	10.0

RM: Regeneration medium CCM: Cocultivation medium, PSM: Pre Selection Medium, SM: Selection medium, RtM. Rooting Medium WM: Washing media in liquid form only and MS: Murashige and Skoog 1962.

Table 2. Different treatments used in the transformation of potato.

	Infection time (2, 5, 10 and 15 min)											
Co-Cultivation (days)	2			3			4			5		
Pre-Selection. (Days)	7	8	10	7	8	10	7	8	10	7	8	10
Explant cultured	100	100	100	100	100	100	100	100	100	100	100	100

washed with distilled water, blotted and quickly frozen in liquid nitrogen to avoid release of phenolic compound and DNAases. The DNA was isolated using CTAB method as described by Hyder *et al*. (2007).

***PCR amplification of hygromycin resistant gene*:** Isolated genomic DNA of plantlets produced from selection media were confirmed by PCR amplification for the presence of hygromycin resistant gene. The gene was amplified by using two primers; Forward primer 5'-GCTCCATACAAGCCAACCAC-3' (20bp) and reverse primer 5'- CGAAAAGTTCGACAGCGTCTC-3' (21 bp). Expected size of the hygromycin gene amplicon was 683 bp. The typical PCR reaction was spiked with about 50 ng DNA template, *Taq* buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 % Nonidet-P40) 200 µM of each dNTPs, 1.5 units *Taq* DNA Polymerase (recombinant) (Fermentas UAB Lithuania), and 25 pM of each primer. Pre-PCR cycling conditions were: 3 minutes of denaturation at 94°C, 2 minutes of annealing at 52°C and extension at 94°C for 3 minutes. Then 35 cycles of denaturing of 30 seconds at 94°C, 30 seconds of annealing at 52°C and 40 seconds of extension at 72°C, and final extension cycle of 20 minutes at 72°C.

***PCR amplification of chitinase gene*:** The RCG3 gene was detected by PCR using specific primers; forward primer 5'-CTCCACCTCCGATTACTGC- 3' (19 bp) and reverse primer 5'-GTAGGGCCTCTGGTTGTAGC-3' (20 bp), corresponding to position 129 at 5'end and 951 at 3'end to the sequence of RCG-3. The PCR conditions were similar to those described earlier for hygrmycine gene amplification, except nonidet-P40, which was supplemented to a final concentration of 0.5 percent. PCR was performed with the following conditions: 35 cycles of denaturation of 20 seconds at 96°C, annealing of 20 seconds at 57°C, while 40 seconds of extension at 72°C followed by a final extension cycle of 20 minutes at 72°C. Amplified DNA fragments were electrophoresed in 1.5 percent agarose gel and stained with ethidium bromide for documentation. Expected product size of chitinase gene was 823 bp.

RESULTS AND DISCUSSION

***Transformation*:** Various factor including determination of lethal dose of cefotaxime, effect of acetosyringone on transformation, co-cultivation period, and pre-selection and selection conditions were investigated in this study for the successful transformation of potato variety cardinal.

***Optimization of lethal dose of cefotaxime*:** Different doses of cefotaxime 0-1000 mg/l were tested. The optimal dose of cefotaxime to inhibit the excessive growth of *Agrobacterium* was found 500 mg/l, which have not affected the growth of the potato tissues, while were effective in controlling the excessive *Agrobacterium* growth (Table 3).

***Effect of acetosyringone*:** The explants co-cultivated with *Agrobacterium* supplemented with acetosyringone has

resulted higher frequency of transformation as compared to the explant treated without acetosyringone. It was observed that the explants treated with *Agrobacterium* without washing in the presence of acetosyringone, did not produce transformed plants and died in selection stage. The explants infected with *Agrobacterium* after washing without acetosyringone showed 5% transformation efficiency, while the explants infected with *Agrobacterium* after washing buffer supplemented with the acetosyringone resulted in 15% transformation efficiency.

Table 3. Comparison of mean areas of bacterial growth inhibition at different cefotaxime levels.

Antibiotic Dose (mg/l)	Area of bacterial growth inhibition (mm)
0.0	0.0
100	2.3
250	4.6
500	10.4
1000	13.8

Acetosyringone, a phenolic compound is released at wounded sites of the dicotyledonous plants and absent in monocotyledonous plants. This compound attracts the *Agrobacterium* and enhanced their transformation efficiency as reported by Veluthambi *et al.* (1989) and Hiei *et al.* (1994). Rashid *et al.* (1996a) had also obtained high transformation efficiency when acetosyringone was added during induction and co-cultivation for transformation of rice. In the present study, it was found that washing assisted *Agrobacterium* mediated transformation and addition of acetosyringone during co-infection enhanced the transformation efficiency in potato.

***Co-infection*:** The two minutes time span was found appropriate for optimum transformation efficiency of potato variety cardinal as also reported for potato by Ahmad *et al.* (2012) and for tomato by Jabeen *et al.* (2015). During this study the period of 5, 10 and 15 minutes co-infection caused higher rate of *Agrobacterium* contamination around the tissues which was difficult to control in proceeding stages.

Terakawa *et al*. (1997) produced transgenic tobacco by immersion of the leaf discs in agrobacterial suspension for 5 minutes. Similarly, Yamamoto *et al.* (2000) obtained transgenic grapevine plants expressing rice chitinase gene by immersion of somatic embryos in bacterial suspension for 15 minutes. Chang *et al*. (2002) transformed potato (c.v. Russet Burbank) with pea β-1, 3-glucanse and chitinase genes by co-infection of nodal explants with *Agrobacterium* solution for 10 minutes. Banerjee *et al.* (2006) has reported 15 minutes co-infection time for transformation of potato using the leaf discs as an explant source.

***Co-cultivation*:** The two days co-cultivation period was found suitable for potato transformation. It was observed that with an increase in co-cultivation period there was an excessive *Agrobacterium* growth. The explants co-cultivated for 3, 4 and 5 days showed over growth of *Agrobacterium* during the co-cultivation stage. These explants did not survive on subsequent stages. The explants co-cultivated for 2 days remained fresh and there was no bacterial growth on the tissues. The results showed that tissues were adversely affected due to prolonged co-cultivation periods. The optimal co-cultivation time for potato explant with *Agrobacterium* was 2 days to produce higher number of transgenic plants. Always there is variable response for co-cultivation for different periods to obtain transgenic plants of different crops. Two days of co-cultivation for potato *S. andigena* (Banerjee *et al*., 2006) transformation, while three days for potato cultivar Russet Burbank (Chang *et al*., 2002) were reported. Similar results were reported by Rashid *et al.* (1996), Ahmad *et al.* (2013) and Jabeen *et al.* (2015) during the transformation studies of rice, potato and tomato respectively. three days co-cultivation for rice transformation was reported by Yamamoto *et al*. (2000), while Liu *et al*. (2004) has described efficient transformation system in soyabean by using five days co-cultivation of embryonic tips and obtained 15.8 percent transformation efficiency. Kim *et al.* (2004) during transformation of *Perilla fructescen* obtained 1.4 percent transgenic plants derived from hypocotyle tissue from 3 days co-cultivated explants. The difference of transformation efficiencies in this study was due to genetic complexity of potato with in the potato cultivars and the target explants used for transformation despite the claim of De Block (1988) who has reported that transformation efficiency is genotypically independent. There were different reports by different researchers on co-cultivation period ranging from two day to five days. The most of the researchers were agreed on two days of co-cultivation duration because with an increase in co-cultivation period the overgrowth of *Agrobacterium* occurred and it becomes difficult to control the *Agrobacterium* overgrowth in later stages which causes complete wilting of explants due to infection.

***Pre-selection*:** The pre-selection of tissue for seven days was sufficient to control the bacterial growth and revival of transformed tissues. The survival percentage of explants on pre-selection duration of seven days was higher as compared to 8 or 10 days. This survival percentage of the explants decreased with an increase in pre-selection duration, indicating the effect of pre-selection duration. All the explants without infection (control) survived on pre- selection media and there was no contamination. Rashid *et al.* (1996b) had also used different pre-selection treatment (0-10 days) to control the excessive *Agrobacterium* growth during the transformation of *Moricandia arvensis* and found that 7 days of pre-selection period was quite suitable for efficient transformation. Ahmad *et al.* (2013) and Jabeen *et al.* (2015) reported similar results for potato and tomato respectively. Pre-selection for 7 days was found appropriate for the regeneration of transformed plants (Philip and Hampson,

1995; Tabei *et al*., 1998) despite a report that transformation efficiency is genotypically independent (De Block, 1988).

***Selection*:** When tissues were shifted on selection media the transgenic plants regenerated from the nodal explants while the non-transformed tissue turned brown and gradually died after two or three sub culturing on the selection media containing hygromycin (Fig. 2). The shoots obtained on selection media were shifted on root induction media containing 20 mg/l hygromycin and 500 mg/l cefotaxime (Table 3) resulted complete plantlet formation after 10 days (Fig. 3).

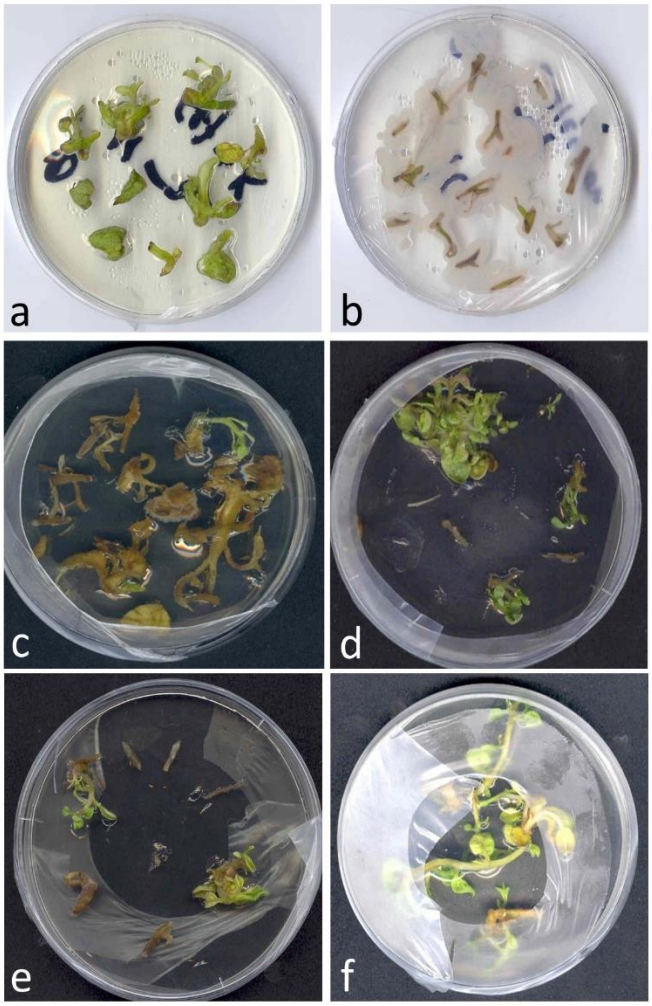

Figure 2. Selection of transformed cardinal variety plants on regeneration media. Various stages of transgene production are shown. (a) Transformed plants on selection, (b) Non-transformed plants on selection medium, (c) browning of non-transformed plants on selection medium, (d) transformed shoot multiplication on selection medium, (e) regeneration of transformed shoots on selection media and (f) transformed shoot enlargement on selection medium.

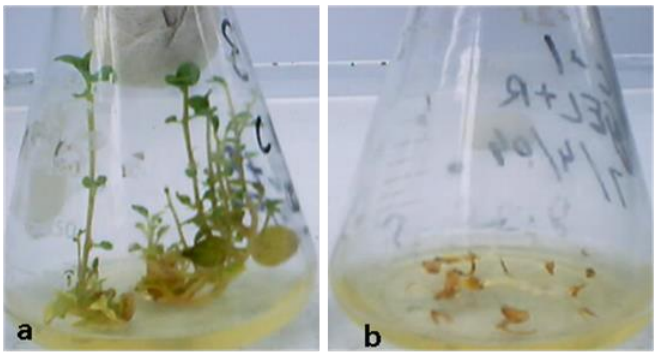

Figure 3. Production of transgenic potato plantlets of Cardinal variety in media containing hygromycin. The effect of hybromycin on the growth of (a) transformed and (b) non-transformed plants.

***Transgenic analysis*:** Twenty-four transgenic plantlets obtained through selection were analyzed using PCR, and out of 24 plants 15 (62.5%) were found hygromycin positive. The DNA fragment of approximately 680 bp was also amplified from these plants. It is noteworthy that no specific PCR product could be amplified from control plants (Fig. 4).

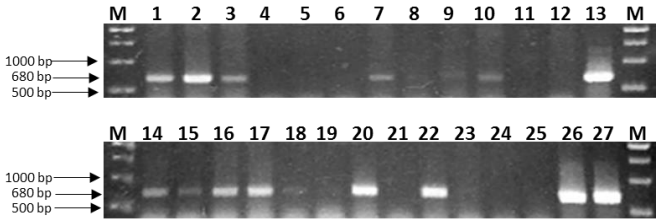

Figure 4. Transformation confirmation of transformed cardinal variety plants by PCR amplification of hygromycin gene. Confirmation of transformation of cardinal potato variety plants by PCR amplification of hygromycin, which produced a specific band of 680 bp in transformed plants and in non-transformed plants there is no specific product. Lane 12 non-transformed plant as PCR negative control, while lane 13 is PCR using pB1333-EN4-RCG3 plasmid DNA as positive control. Lane 1-3, 7, 9-10, 14-18, 20, 22, 26 and 27 are positive for transformation, while others are negative. M is 500 bp Marker (Fermentas Lithuania). **Lane 2-12, 17-30:** PCR +ve lines of potato tarnsformnats for hygromycin. **Lane13:** DNA of non-transformed plant (Control)

After confirmation with hygromycin primers the same genomic DNA was used for amplification with chitinase primers. When these plants were tested for the presence of chitinase gene with specific primers, a PCR amplified product of approximately 823 bp size corresponding to chitinase gene (Fig. 5) was obtained from transgenic plants. It was observed that all the hygromycin plants also exhibited positive bands of desired size of 823 bp of chitinase gene.

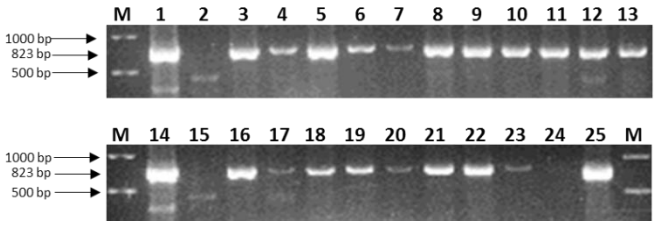

Figure 5. Transformation confirmation of transformed cardinal variety plants by PCR amplification of chitinase gene. Confirmation of transformation of cardinal potato variety plants by PCR amplification of chitinase, which produced a specific band of 823 bp in transformed plants and in non-transformed plants there is no specific product. Lane 24 non-transformed plant as PCR negative control, while lane 25 is PCR using RCG primes with pB1333-EN4-RCG3 plasmid DNA as positive control. Lane 1, 3-13, 14, and 16-23 are positive for transformation, while others are negative. M is 500 bp Marker (Fermentas Lithuania).

The PCR profile conditions for hygromycin gene amplification were different because with the same condition amplification of chitinase gene was not obtained. For chitinase amplification, the reaction mixture was supplemented with 0.5% Nonidet-P40, as chitinase gene has higher GC contents due to which secondary structure could not be fully denatured. Addition of Nonidet-P40 a detergent had the capability to break the secondary structure and thus the desired bands were amplified with the approximate size of 823 bp. Chang *et al.* (2002) has also used 0.08% Nonidet in PCR reaction mixture during the amplification of pea chitinase gene.

Nishizawa *et al.* (1999a) has also reported the transformation of hygromycin phosphotransferase gene (HPT) along with chitinase gene in transformed plants of rice by PCR. In the present study PCR results were obtained by amplifying 823 bp fragment. This was possible because of the addition of the Nonidet-P40 in the PCR reaction mixture. Kishimoto *et al.* (2002) reported the presence of rice chitinase gene from transformed cucumber (RCC2) plants. Also, Mitani *et al.* (2006) has reported the PCR amplification of rice chitinase gene (RCC2) from transformed trifoliate orange and obtained about 330 bp band of chitinase gene.

The conditions optimized for transformation of an elite potato variety; the cardinal, can be used to transform other potato varieties and could lay a foundation for genetic improvement of potato for various biotic and abiotic factors.

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