Pak. J. Bot., 41(5): 2605-2614, 2009.

STUDY OF THE FACTORS AFFECTING *AGROBACTERIUM* MEDIATED GENE TRANSFORMATION IN TOMATO (*LYCOPERSICON ESCULENTUM* MILL.) CV. RIOGRANDE USING RICE CHITINASE (*CHT-3)* GENE

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Abstract

The factors affecting the *Agrobacterium* mediated gene transformation of tomato cv. Riogrande are reported in detail. Shoot tips (explants) were transformed with pBI333 carrying rice chitinase *(cht-3)* gene. Approximately 49% transformation frequency was achieved. The optimum conditions for successful transformation were: infecting the explants with the *Agrobacterium* suspension for two minutes, co-cultivation period for two days, 50 µM of acetosyringone in the co-cultivation medium, pre- selection for seven days and 500 mg/l cefaxine in the pre- selection. The transgenic plants were selected on the medium containing 50 mg/l hygromycin. The presence of hygromycin and chitinase gene in the hygromycin resistant (T0) transgenic plants was evaluated by Polymerase Chain Reaction (PCR) analysis. A fragment of 500bp size for the hygromycin gene was amplified from the 100% hyg resistant plants while 250 bp long fragment for the *cht-3* gene was amplified from the 88% of the transgenic plants only.

Introduction

Agrobacterium mediated gene transformation is the only known example of natural inter kingdom genetic exchange. *Agrobacterium tumefaciens* is a gram-negative bacterium, which infects the dicots. Since the beginning of 20th century it was known to cause 'crown gall' disease in dicots. While from the last two decades it has been widely reported for the transformation of genes with desired characters in almost all the crops.

Agrobacterium mediated gene transformation is widely reported in dicotyledenous crops such as tobacco (Liu *et al*., 2004), potato, cucumber and tomato (Punja & Raharjo, 1996).

Tomato serves as genetic model for improving other dicotyledonous crop plants (Ling *et al*., 1998). For the improvement and practical studies of tomato successful transformation is essential. Although transformation in tomato is widely reported but it is still time consuming and needs some improvement (Frary & Earle, 1996) because the genotypes vary in their response to specific treatment. McCormick *et al*., (1986) first time reported the transformation in tomato. Since then there have been various reports on the *Agrobacterium* mediated gene transformation in different cultivars of tomato world wide (Qiu *et al*., 2007; Guerra *et al*., 2007).

In tomato regeneration capacity and transformation frequencies show variation among cultivars. Transformation frequencies have ranged from 6%-49% (Qiu *et al*., 2007), 40% (Su *et al*., 2002) to 49.5% (Raj *et al*., 2005). Efficiency of transformation is affected by many factors like *Agrobacterium* strain, inoculation duration, co-cultivation time, pre- selection period, concentration of acetosyringone or other antibiotics in the

media, type of explants and genotype (Wu *et al*., 2006). After successful transformation the stability and presence of inserted gene must be confirmed by molecular analysis of antibiotic resistant (selectable marker) plants, prior to further studies. The presence of foreign genes in the putative transformants are confirmed by polymerase chain reaction (PCR) analysis and expression of genes can be tested by Southern analysis and can be further analyzed at RNA level by Northern analysis. In tomato molecular analysis of the transformants by using PCR, Northern and Southern analysis is very well documented in number of studies (Roy *et al*., 2006; Wu *et al*., 2006). The objective of this study was to develop a protocol for the efficient and reliable *Agrobacterium* mediated gene transformation of a tomato variety cultivated in Pakistan.

Material and Methods

Plant material and media used: Shoot tips (1-2 cm pieces) of the tomato (*Lycopersicon esculentum* Mill.) cv. Riogrande were proved best for the *In vitro* regeneration of tomato in the earlier studies by Jabeen *et al*., (2005) so they were used as the explant source for gene transformation studies.

***Agrobacterium* strain and plasmid:** Transformation of tomato cultivar Riogrande was carried out by using *Agrobacterium tumefaciens* strain EHA101 harboring pBI333 plasmid. pBI333 is a binary vector consisting of 1.1- kb DNA fragment encoding *Cht-3* from a genomic rice clone (*RCG3*) and hygromycin resistant (*HPT*) gene driven by cauliflower mosaic virus (CaMV) 35S promoter, possessing (EN4) four tandemly repeated enhancer regions (Nishizawa *et al*., 1999).

***Agrobacterium* culture:** Glycerol stock of the *Agrobacterium* strain EHA101 harboring the plasmid pBI333-EN4-RCG3 was refreshed by streaking on LB medium containing 50 mg/l kanamycin and 50 mg/l hygromycin in Petriplates. These Petriplates were incubated at 28°C for 2-3 days. Single colonies were picked and were grown on LB liquid containing 50 mg/l kanamycin and 50 mg/l hygromycin overnight at 28°C. Liquid culture of *Agrobacterium* was used for transformation.

Determination of the lethal dose of hygromycin for selection: Explants were tested for their sensitivity to hygromycin in order to determine the lethal dose of hygromycin for the selection of transformants. Explants were subjected to the regeneration medium with 25 mg/l, 50 mg/l, 75 mg/l and 100 mg/l of hygromycin for four weeks. The cultures were incubated in culture room at 25±2ºC, 10h photoperiod at 48-µmol/m-²s².

Co-cultivation: In order to carry out co-cultivation *Agrobacterium* suspension was prepared. For this purpose overnight culture of *Agrobacterium* having OD_{600} of 1-1.5 was centrifuged at 3000 rpm for 15 min., in a falcon tube. Supernatant was discarded and pellet was resuspended in MS-liquid. The explants were soaked in bacterial suspension for 1 to 4 minutes in order to optimize the inoculation duration. Explants were then blotted on filter papers and were transferred to co-cultivation medium (MS solid+ 1mg/l zeatin+0.1 mg/l IAA, pH 5.8) in Petri plates. Petri plates were wrapped with parafilm and were incubated at 28°C in dark for 2 days. Explants not infected with *Agrobacterium* were also placed on the same conditions and were labeled as control. Co-cultivation period (days) was also optimized by placing the inoculated explants on co-cultivation medium for 1-4 days.

Determination of the lethal dose of cefaxime for *Agrobacterium*: Growth of the *Agrobacterium* after 2-3 days of co-cultivation reduces the efficiency of transformation so removal of *Agrobacterium* is must. Different concentrations of cefaxime (0, 100, 200, 300, 400, 500, 600, 700, 800 mg/l) were used in the regeneration medium (MS solid+1mg/l zeatin+0.1 mg/l IAA, pH 5.8) in order to determine the lethal dose of cefaxime for *Agrobacteriumi*.

Effect of Acetosyringone on transformation efficiency: In order to improve the efficiency of transformation different concentrations of acetosyringone 0, 25, 50, 75 and 100 µM of acetosyringone were added in the co-cultivation medium. These co-cultivated explants were then transferred on the selection medium (MS solid+ 1mg/l zeatin+0.1 mg/l IAA+50 mg/l hygromycin, pH 5.8) containing 500 mg/l cefaxime. The best co-cultivation medium was then used for production of transgenic plants. Efficiency of transformation was recorded after four weeks.

Pre-selection and selection: Co-cultivated explants were washed with washing medium (MS liquid, pH 5.8) and blotted on filter papers. Dried explants were then placed on regeneration medium containing 500 mg/l cefaxime for 3, 5 and 7 days prior to selection. Efficiency of transformation was recorded after the four weeks on the selection medium.

Molecular analysis: Presence of *Cht- 3* and *hygromycin* gene in the transformed plants was confirmed by polymerase chain reaction (PCR). PCR was carried out by using genomic DNA from plants, plasmid DNA and primers of rice chitinase (*Cht-3*) and *hygromycin (hpt*) *genes*. First of all genomic DNA of T0 plants and plasmid pBI333 was isolated.

PCR optimization: The primers pairs employed to detect *hpt* and *RCG3* genes were as designed and described by Nishizawa *et al*., (1999). For RCG3 the primer sequence was 5'-AAGCATGCCCTACCCGCCTTCCTAGTTG-3' (forward) and 5'-GTATAATTGCG GGACTCTAATC-3' (reverse) which amplified a 250 bp fragment. Similarly for ***hpt*** primer sequence was 5'-ATGAAAAAGCCTGAACTCACCGCGA-3' (forward) and 5'-TCCATCACAGTTTGCCAGTGATACA-3' (reverse), which amplified a 500 bp fragment. Different PCR conditions were selected as reported for a study such as Mg^{+2} concentration, primer concentration and annealing temperature were optimized for *RCG3* and *hpt* genes.

Statistical analysis: All experiments were carried out in completely randomized design (CRD). Each experiment was replicated thrice. Data was analysed by using Minitab 13, Statistica and MSTAT-C softwares. Significance of data was checked by using least significance (LSD) test.

Results and Discussion

Hygromycin resistance of normal explants: It was observed that when no hygromycin was added in the medium explants remained green and regeneration started in more than 80% of explants. Addition of hygromycin affected adversely the explants growth. Increase in the concentration of hygromycin stopped the regeneration of explants. When 25 mg/l of *hyg* was added in the medium only 50% explants had shown regeneration and rest of the explants became pale (Fig. 1a). As the concentration was increased up to 50 mg/l no regeneration was observed and explants became pale yellow (Fig. 1b). Further increase in concentrations resulted in the complete necrosis of the explants (Fig 1 c and d). Statistically

concentration x efficiency of regeneration interaction was significant at p= 0.01 (Table 1). Hygromycin has already been reported for the selection of transformed explants harboring rice chitinase gene and non-transformed explants with very low frequency of selection escape in many crops like Banana (Maziah *et al*., 2007), pigeon pea plants (Kumar *et al*., 2004) and American ginseng plants (Chen & Punja, 2002). Olhoft *et al*., (2003) developed hygromycin based selection system for transgenic shoots with low frequency of selection scape. Roy *et al*., (2006) used 40 mg/l hygromycin as the lethal dose for the selection of transgenic shoots containing gene against drought tolerance in tomato cv. Pusa Ruby. They tested the leaf explants on the medium containing 10, 20, 30, 40, 50 and 60 mg/l hygromycin and concluded that 40 mg/l hygromycin is the optimum concentration for the selection of transgenic shoots as the un-transformed explants turned brown within one week and no morphogenic response was observed.

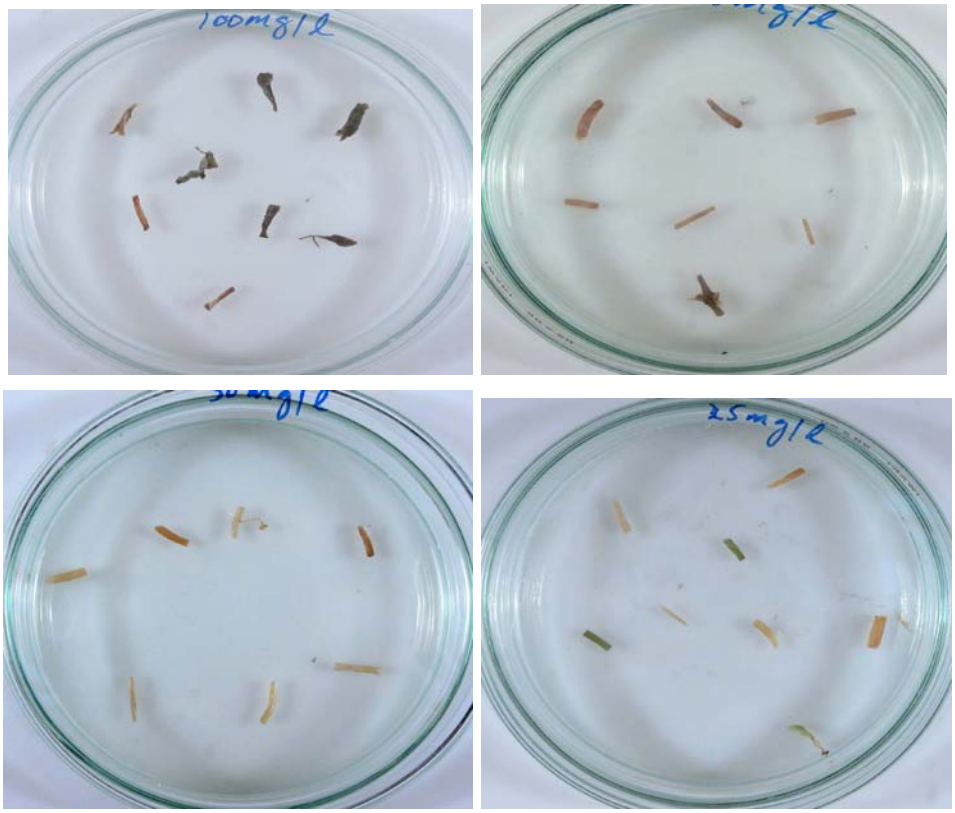

Fig 1. Selection of explants on different concentrations of hygromycin(a) 25mg/l (b) 50mg/l (c) 75mg/l (d) 100mg/l.

Table 1. Comparison of the average no of explants regenerated at different concentrations of hygromycin.

Concentration of Hyg (mg/l)	Average number of explants regenerated	Morphological characteristics of explants
0	81.0[a]	Explants were green and regenerated
25	50.0[b]	Half of the explants became pale yellow
50	0.00[c]	Explants became pale yellow
75	0.00[c]	Explants became pale yellow and black
100	0.00[c]	Explants became black

LSD for concentration x average number of explants regenerated interaction = 3.273
Values followed by the same letters are not significantly different at α = 0.01
Data is the average of three replicates

Factors affecting transformation: In this study various factors affecting the robust T-DNA delivery like co-cultivation period, co-cultivation time, effect of acetosyringone, lethal dose of cefaxime to terminate the co- cultivation phase are described in detail.

Effect of co-cultivation period: Co-cultivation was carried out at 28°C in dark for 1, 2, 3, 4 days for two minutes. It was observed that transformation efficiency was adversely affected by the co-cultivation period. Explants co-cultivated for one day died on selection medium whereas transformation efficiency was increased when the explants were co-cultivated for two days but further increase in co-cultivation period i.e., for 3 and 4 days resulted in excessive growth of *Agrobacterium.* Our results are in line with the number of reports on tomato transformation studies. Raj *et al*., (2005) regenerated 49.5% shoots on selection medium after two days of co-cultivation by using cotyledon as the explant source in tomato.

Effect of co-cultivation time: Co-cultivation time was optimized by keeping the co-cultivation period constant. It was observed that co-cultivation time also affected the transformation efficiency. Explants co-cultivated for one minute died on the selection medium while transformation efficiency of the explants co-cultivated for 3 and 4 minutes was zero due to the excessive growth of *Agrobacterium*. Roy *et al*., (2006) immersed the pre- cultured leaf discs of tomato in bacterial suspension for 3 minutes and reported the maximum efficiency of transformation. Wu *et al*., (2006) reported 10 minutes inoculation duration in tomato.

Determination of the optimum dose of cefaxime to terminate the co-cultivation phase after two days: Transformation efficiency is adversely affected by the growth of *Agrobacterium* in the medium after the two days of co-cultivation. In order to remove the *Agrobacterium* completely after the two days of co-cultivation from the medium different concentrations (0, 100, 200, 300, 400, 500, 600, 700, 800 mg/l) of cefaxime were added in the regeneration medium. After four weeks data was recorded. It was observed that addition of cefaxime in the medium increased the efficiency of transformation. Results showing the efficiency of transformation by using different concentrations of cefaxime are summarized in Table 2 and Table 3. When no cefaxime was added in the medium excess bacterial growth was observed in the cultures leading to the death of explants. It was observed that increase in the concentration of cefaxime up to 500 mg/l increased the efficiency of transformation gradually up to 34.33% but further increase in the concentration of cefaxime from 600 to 800 mg/l decreased the efficiency of transformation rapidly and at 800 mg/l the explants died only 2% transformation efficiency was observed (Table 3). At 500 mg/l *Agrobacterium* growth was not observed in any of the individual culture as well as in the medium and efficiency of transformation was maximum.

Determination of optimum concentration of acetosyringone: Addition of acetosyringone in the co-cultivation medium improves the efficiency of transformation. After optimizing the co-cultivation period, co-cultivation time and termination of co-cultivation phase different concentrations of acetosyringone (0, 25, 50, 75, 100 μM) were also tested in the co-cultivation medium. Explants were co- cultivated for two days on the medium containing these concentrations after the coinfection with *Agrobacterium* suspension for two minutes. After co-cultivation explants were transferred to the

Table 2. Analysis of variance of the effect of concentration of cefaxime on efficiency of transformation.

Source of variation	Degree of freedom	Mean square (MS)	F	P
Treatment	8	318.08**	373.40	00
Error	18	0.852		
Total	14			

** = Highly significant

Table 3. Comparison of the efficiernсy of transformation by using different concentrations of cefaxime in the medium.

Concentration of cefaxime (mg/l)	Efficiency of transformation (mean ± S.E)
0	9.33 ± 0.33^{g}
100	12.33 ± 0.33^{f}
200	15.33 ± 0.33^{e}
300	24.00 ± 0.57^{c}
400	26.66 ± 0.88^{b}
500	34.33 ± 0.33^{a}
600	21.00 ± 0.57^{d}
700	8.00 ± 0.57^{g}
800	2.00 ± 0.57^{h}

LSD 0.01 = 2.16; Values followed by the same letters are not significantly different at α = 0.01
Data is the average of three replicates

selection medium containing 500 mg/l cefaxime. Data was recorded after the four weeks. Results showing the efficiency of transformation after using different concentrations of acetosyringone in the co-cultivation medium are summarized in Table 4 and Table 5. Acetosyringone has already been reported effective to improve the efficiency of transformation in many crops like carrot (Guivarch *et al*., 1993), apple (James *et al*., 1993; Wier *et al*., 2001) and rice (Rashid *et al*., 1996a) including tomato (Joao & Brown, 1993). A concentration of 50- 200 µM acetosyringone in the co-cultivation medium has been reported in the earlier studies to improve the efficiency of transformation in tomato. Our results are similar to the study conducted by Amoah *et al.,* (2001). They reported that 50 µM acetosyringone gave optimum transient gene expression, with higher concentration being toxic and lower concentration less effective. Results of our study are also in agreement with the earlier report by Wu *et al.*, (2007). They reported 50 µM acetosyringone to be effective for the optimum efficiency of transformation in tomato cv. Lichan. Our results are contrary to the earlier studies conducted by Moghaieb *et al*. (2004) and Raj *et al*. (2007). They reported 100 µM and 200 µM respectively of acetosyringone for optimum efficiency of transformation in tomato. This difference in the efficiency of transformation at different concentrations of acetosyringone may be due to the difference in the genotype and bacterial strain.

Table 4. Analysis of variance of the effect of concentration of acetosyringone on efficiency of transformation.

Source of variation	Degree of freedom	Mean square (MS)	F	P
Treatment	4	614.77**	542.4	00
Error	10	1.13		
Total	14			

** = Highly significant

Table 5. Comparison of the efficiency of transformation by using different concentrations of acetosyringone in the medium.

Concentration of acetosyringone (µM)	Efficiency of transformation (mean ± S.E)
0	34.33 ± 0.33^{c}
25	38.00 ± 0.57^{b}
50	46.00 ± 0.57^{a}
75	23.66 ± 0.88^{d}
100	9.00 ± 0.57^{e}

LSD 0.01 = 2.75; Values followed by the same letters are not significantly different at α= 0.01
Data is the average of three replicates.

Table 6. Analysis of variance of the effect of pre-selection period on efficiency of transformation.

Source of variation	Degree of freedom	Mean square (MS)	F	P
Treatment	2	8.78**	15.80	00
Error	6	0.56		
Total	8			

** = Highly significant

Table 7. Comparison of the efficiency of transformation after the pre-selection periods.

Pre- selection period (days)	Efficiency of transformation (mean ± S.E)
3	46.00 ± 0.577^{b}
5	48.33 ± 0.33^{a}
7	49.33 ± 0.33^{a}

LSD 0.01 = 2.26; Values followed by the same letters are not significantly different at α= 0.01
Data is the average of three replicates.

Effect of pre-selection or direct selection: Prior to selection explants were placed on medium containing 500 mg/l cefaxime for 3, 5 and 7 days to study the effect of pre-selection period on the efficiency of transformation. After this treatment explants were transferred to the selection medium and data was recorded after four weeks. It was observed that pre- selection period increased the efficiency of transformation. Statistically the difference between the efficiency of transformation of the explants after pre selection period was highly significant (Table 6). As the pre- selection period was increased, efficiency of transformation also increased (Table 7) and after the pre-

selection period of seven days efficiency of transformation increased up to 49%. It was found that pre-selection period of seven days is optimum for the efficiency of transformation and these explants showed maximum efficiency after the pre- selection of seven days instead of direct selection.

Selection: Pre- selected explants were transferred to the selection medium supplemented with 50 mg/l hygromycin in addition to 500 mg/l cefotaxime. Results indicated that 49% of the tomato cv. Riogrande shoots were regenerated on the selection medium within three weeks which was less than untransformed control explants on the medium without hygromycin (79.1%). Our results are similar to the study conducted by Raj *et al*., (2005). They reported that 49.5% tomato cv. Pusa Rubby shoots regenerated on selection medium within three weeks when cotyledons were used as the explant source. Wu *et al*., (2006) reported 27% efficiency of transformation in tomato cv. Lichun. Qiu *et al*., (2006) reported 20.8 % efficiency of transformation in tomato. Our results and results of the previous studies revealed that this difference in efficiency of transformation is may be due to difference in bacterial strain, plasmid construct and cultivar used.

Polymerase chain reaction (PCR) for the presence of *Cht-3* and hygromycin gene: Integration of chitinase and hygromycin resistant gene in T0 putative hygromycin resistant transgenic plants was analysed by PCR. The PCR results of transgenic and control plant are displayed in Fig. 2 a & b. The expected 250 bp fragment for *Cht-3* was amplified from the 88% transgenic plants (Fig. 2a) whereas no fragment was obtained from non transgenic control plants and two transgenic plants (plant no 9 and 10, Fig. 2a) and 500 bp fragment for hygromycin was amplified from all the hygromycin resistant transgenic plants (Fig. 2b). Nishizawa *et al*., (1999) reported the presence of 250 bp fragment in some hygromycin resistant transgenic rice plants by using the same primer sequence. They also obtained some plants in which hygromycin fragment was present but *Cht-3* was absent. The absence of RCG3 fragment in the hygromycin resistant plants could be due to the deletion of RCG3 fragment during the process of transformation. Kishimoto *et al.,* (2002) detected the presence of rice chitinase gene in cucumber by PCR analysis. Chen & Punja (2002) amplified a DNA fragment of 947 bp for *hpt* and 584 bp for RCC2 in American ginseng. Yamamoto *et al.,* (2000) also confirmed the existence of 900 bp DNA fragment of rice chitinase (RCC2) gene by PCR analysis and reported that two transgenic lines (NM- Chi- 17 and NM- Chi- 20 had no positive band for RCC2

Conclusion

This study suggested that gene transformation in tomato is influenced by number of factors and rice chitinase gene (*Cht-3*) can be successfully transformed in tomato cv cultivated in Pakistan by controlling the co-cultivation period, inoculation duration and concentration of antibiotics.

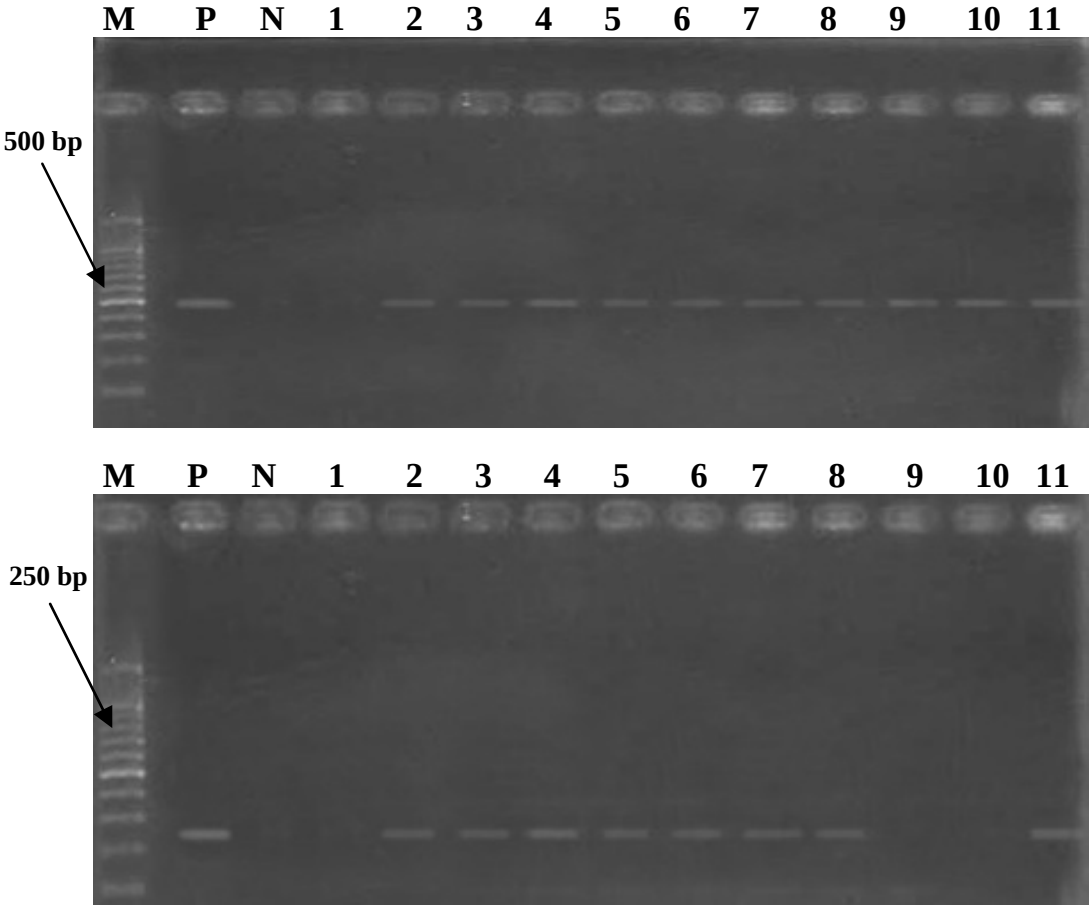

Fig. 2. PCR analysis to detect the presence of 500 hygramycin fragment (a) and 250 bp *Cht*3 fragment (b) in transgenic plants.
M= 100 bp molecular weight ladder, P= pBI333, N= Non-transgenic control plant, Lane 1= Empty, Lane (2-11) = Transgenic plants

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(Received for publication 25 June 2009)