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Full Length Research Paper

Factor affecting *Agrobacterium*-mediated transformation of rice chitinase gene in *Solanum tuberosum* L.

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Potato is a very important food crop and is adversely affected by fungus. *Agrobacterium*-mediated transformation can play an important role in the improvement of potato. The present study was conducted to optimize the different factors affecting *Agrobacterium*-mediated transformation of chitinase gene. Nodes were used as explant of potato cultivars Desiree and Sh-5. *Agrobacterium tumefacien* strain LBA4404 harboring a pB1333-EN4-RCG3 plasmid having chitinase gene and selectable marker hygromycin gene under the control of the CaMV 35S promoter was used. The parameters optimized for the potato transformation includes co-cultivation time, cefotaxime concentration, and days to pre-selection. The results show that the infection time (2 min) gave best mean value of transformation efficiency, that is, 2.9 and 2.1 in Desiree and Sh-5, respectively. The over growth of *Agrobacterium* were controlled with a concentration of 200 mg/l of cefotaxime and obtained maximum mean value of transformation efficiency of 3.38 and 3.10 in Desiree and Sh-5, respectively. The pre-selection period seven days prior to selection were considered effective for regeneration of explants and high transformation efficiency. The high mean value of regeneration (3.08 and 2.82) and transformation efficiency (3.00 and 2.60) was observed in Desiree and Sh-5, respectively. The putative transgenic plants were analyzed through PCR by using RCG-3 specific primers.

Key words: *Agrobacterium,* potato, transformation, rice chitinase gene, factors.

INTRODUCTION

Potato is one of the important cash crops of Pakistan. The average yield is very low when compared with other potato growing countries. The low yield can be attributed mainly to lack of availability of certified disease free healthy seeds. More than 95% of the seed requirement is met from the locally produced seed which suffers from many diseases caused by fungi (Abbas, 2006). Potato improvement through conventional breeding is very difficult due to male sterility, incompatibility, and autotetraploid genome makes it difficult to improve by conventional breeding.

It is important to develop a genetic transformation system for introduction of exogenous genes into plants in order to improve the plants quality and develop new varieties. Genetic transformation can bring improvement and is used as a tool for functional genomics in plants. There are various methods available for gene transformation in crops. These include electroporation, polyethelene glycol (PEG), biolistic method and *Agrobacterium*-mediated transformation. Among these, the most dominant technology used for the development of genetically modified crops is *Agrobacterium*-mediated transformation due to its ability to transfer large segments of DNA with minimal rearrangement, the precise insertion of transgenes resulting in fewer copies of inserted genes, simple technology with lower cost, and higher

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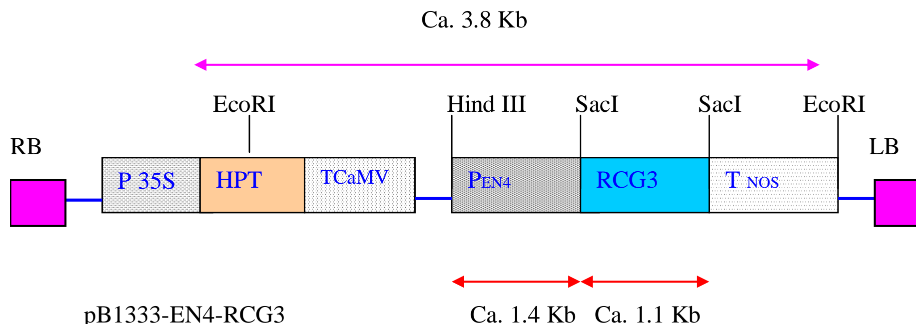

Figure 1. Plasmid pB1333-EN4-RCG3 used in the study.

transformation efficiency (Romano et al., 2003).

The frequency of regeneration and transformation in potato is relatively low (Park et al., 1996). It depends on many factors like bacterial strain, bacterial concentration, pre-culture period, co-cultivation period, immersion time, acetosyringone concentration, mannitol treated time, temperature, explant type, pH, etc. (Uranbey et al., 2005). Potato is its natural host and was transformed successfully firstly through Ti-DNA or Ri-DNA. This transformation brings many morphological and developmental changes in transgenic potato plants (Ooms et al., 1983). There are many protocols available for the *Agrobacterium* transformation because efficiency of transformation in potato is highly dependent on genotype (Cingel et al., 2010). Research efforts have also been performed to identify genes responsible for the growth and development of potato from diverse habitats.

Transformation of chitinase gene in different crop species has been reported by several researchers against various strains of fungus and other pathogens (Tabei et al., 1998; Kishimoto et al., 2002). Different types of chitinases like rice chitinase gene (CHI11) (Hong et al., 2005), insect (*Phaedon cochleariae*: coleoptera, chrysomelidae) chitinase gene (Saguez et al., 2005), and wild spinach chitinase gene (Hirai et al., 2004) were transformed in different varieties of potato. The objective of this study was to optimize various factors that affect *Agrobacterium*-mediated transformation of RCG-3 gene in potato cultivars (cv.) Desiree and Sh-5.

MATERIALS AND METHODS

Genetic transformation

Agrobacterium-mediated genetic transformation was used for the integration of rice chitinase gene (RCG-3) into two potato cv. Desiree and Sh-5.

Binary vector

Binary vector having the plasmid pB1333-EN4-RCG3 (Figure 1) was used in this study. It is encoded by Cht-3 from a genomic rice clone (RCG3) and hygromycin resistance gene under the CaMV 35S promoter, possessing (EN4) tandemly repeated enhancer region (-290 to -90) of its own. Bacterial strain LBA4404 acquiring this plasmid was used for transformation.

Plant transformation

Nodes of potato varieties Desiree and SH-5 were used as explant in transformation studies. The optimized media for shoot multiplication was used for transformation purpose. The following steps were carried out for efficient transformation.

Culture of *Agrobacterium tumefaciens*

Yeast extract peptone (YEP) media (15 ml) containing kanamycin and hygromycin at 50 mg/l each for pB1333-EN4-RCG3 plasmid was taken in an autoclaved falcon tube and 5 to 8 µl of *A. tumefaciens* strain LBA4404 from the glycerol stock was added. The culture was placed on the shaker with 90 to100 rpm at 28°C overnight, to enable it to grow up to the OD_{600} 0.5 to 0.6.

Infection of explants

The nodes of potato, about 5 to 6 mm length, were cut from the two weeks old *in vitro* plants of two varieties Desiree and SH-5 and dipped in bacterial suspension having OD_{600} 0.5 to 0.6 for 2, 4 and 6 min. After infection for different time period, the explants were blot-dried with the help of sterilized filter paper.

Co-cultivation of explants

The infected nodes were shifted to Petri plates containing solid Murashige and Skoog (MS) plant medium. The media in plates were covered with sterilized Whatman filter paper. The plates were sealed with parafilm and placed at 28°C in the dark for three days. The non-infected explants with *Agrobacterium* were also used as control.

Washing of explants

The intact *Agrobacterium* with explants for long period decreased the transformation efficiency. To get rid of excess *Agrobacterium,* they must be removed from explants, and for this purpose, washing was done. The explants were washed three times with autoclaved water for 5 min. Then the cefotaxime in liquid MS media was added

Table 1. Different treatments used in the transformation of potato.

Infection time	(2, 4, and 6 min)				
Cefotaxime concentrtion (mg/l)	0	100	200	400	500
Pre-selection (days)	0 3 5 7	0 3 5 7	0 3 5 7	0 3 5 7	0 3 5 7

and the nodes were washed two times for five minutes each. The different concentrations (0, 100, 200, 400 and 500 mg/l) of cefotaxime were used to optimize the lethal dose for *Agrobacterium*. After washing, nodes were shifted on autoclaved paper towel for drying.

Pre-selection

After washing and drying, the explants were transferred to regeneration medium for 0, three, five and seven days of pre-selection to optimize the best pre-selection period on which maximum explants were regenerated.

Selection

After pre-selection, the explants were shifted to selection media containing 200 mg/l cefotaxime and 20 mg/l hygromycin. The cultures were transferred after every 10 to 15 days interval on fresh media. The transformed cells were selected and regenerated in the presence of hygromycin (Table 1).

Molecular analysis

Isolation of plant genomic DNA

The healthy leaf from transformed and control potato plants were taken and their genomic DNA was extracted through cetyltrimethylammnonium bromide (CTAB) method (Iqbal et al., 1997).

PCR analysis for transformed plants

The extracted DNA was run in 1% agarose gel for the confirmation of its presence. Then, PCR analysis was used to confirm the transformation of RCG-3 gene in the potato genome. The RCG-3 specific forward 5-GGTGACATCGTCCGCTACAC-3 and reverse 5-GGTGTTCCAGTACCACAGCG-3 primers were used for the amplification of transformed gene. The PCR amplified 750 bp bands of chitinase gene. The PCR amplification was done under the following conditions; the pre-denaturation was done at a temperature of 94°C for 5 min and the next 35 cycles was done as the denaturing at 94°C for 45 s, annealing at 52°C for 45 s and extension at 72°C for 90 s. The post extension was done at 72°C for 20 min. Confirmation of amplified DNA fragments were done by running in 1% agarose gel; detection was done through ethidium bromide staining and viewed in gel documentation.

Statistical analysis

Two factors were considered in this study - varieties and treatments (infection time, cefotaxime concentration and days to pre-selection). Data were collected and analyzed. Two factorial analysis of variance (ANOVA) was applied with the help of computer software MSTATC. Their significant means were compared by using Duncan's multiple range test (DMRT) through MSTATC.

RESULTS AND DISCUSSION

Effect of infection time

Co-infection also played an important role on *Agrobacterium* growth and transformation efficiency. Significant differences were observed among the three treatments but no significant differences were observed between two genotypes for bacterial overgrowth. Statistical analysis showed that regeneration of nodes on selection medium and transformation efficiency was genotype dependent. Minimum mean value of overgrowth (10 and 11), maximum mean value of regeneration of nodes on selection medium (3.2 and 2.4) and maximum mean value of transformation (2.9 and 2.1) for Desiree and Sh-5, respectively was observed on 2 min infection time. As the infection time increased, the undesirable bacterial overgrowth also increased and the regeneration of nodes and transformation efficiency decreased. The maximum mean value of overgrowth (14.75 and 15.16), minimum mean value of regeneration of nodes on selection medium (1.2 and 1.0), and minimum mean value of transformation (1.1 and 0.9) for Desiree and Sh-5, respectively was observed on 6 min infection time (Table 2). The co-infection time (2 min) was most suitable for the stable integration of RCG-3 gene into potato genome and to control the *Agrobacterium* overgrowth. These results are confirmatory with those of Jabeen et al. (2009) who reported the co-infection time in tomato was 2 min. Our results deviated from Terakawa et al. (1997) who obtained transgenic tobacco plant by immersing the leaf disc in bacterial suspension for 5 min. Jun et al., (2007) also found best result in sweet potato when co-infected the embryogenic callus with *Agrobacterium* for 10 min. The difference in the time was due to type of explant, varieties, crop and *Agrobacterium* strain.

Elimination of *Agrobacterium* overgrowth through cefotaxime

For successful *Agrobacterium* transformation, the overgrowth of bacteria from culture is needed to be controlled during pre-selection and selection period. Antibiotic which is commonly used to eliminate *A. tumefaciens* from plant tissues is cefotaxime which has

Table 2. Effect of different co-infection time on over growth of *Agrobacterium tumefaciens* LBA4404 and transformation efficiency.

Co-infection time (min)	Variety	Explant showed overgrowth	Regeneration	Transformation efficiency
2	Desiree	10 ± 2.20[c]	3.2 ± 0.71[a]	2.9 ± 0.42[a]
	Sh-5	11 ± 1.50[bc]	2.4 ± 0.49[b]	2.1 ± 0.52[c]
4	Desiree	12.5 ± 0.76[abc]	1.5 ± 0.17[c]	1.5 ± 0.17[bc]
	Sh-5	14 ± 1.04[ab]	1.2 ± 0.10[c]	1.0 ± 0.00[c]
6	Desiree	14.75 ± 1.95[a]	1.2 ± 0.07[c]	1.1 ± 0.08[c]
	Sh-5	15.16 ± 1.45[a]	1.0 ± 0.00[c]	0.9 ± 0.10[c]
LSD		3.416	0.796	0.627

LSD, Least significant difference.

Table 3. Effect of different doses of cefotaxime to control the growth of *Agrobacterium tumefaciens* LBA4404 and transformation efficiency.

Cefotaxime concentration (mg/l)	Variety	Overgrowth control	Regeneration	Transformation efficiency
0	Desiree	0 ± 0.00[b]	0 ± 0.00[d]	0 ± 0.00[e]
	Sh-5	0 ± 0.00[b]	0 ± 0.00[d]	0 ± 0.00[e]
100	Desiree	0 ± 0.00[b]	0 ± 0.00[d]	0 ± 0.00[e]
	Sh-5	0 ± 0.00[b]	0 ± 0.00[d]	0 ± 0.00[e]
200	Desiree	40 ± 2.91[a]	4.00 ± 1.53[a]	3.38 ± 0.47 [a]
	Sh-5	42.17 ± 1.45[a]	3.10 ± 1.53[ab]	3.10 ± 0.66[a]
400	Desiree	38.59 ± 3.18[a]	3.00 ± 0.32[ab]	1.75 ± 0.14[b]
	Sh-5	41.25 ± 3.53[a]	2.50 ± 0.52[bc]	1.50 ± 0.08[bc]
500	Desiree	40 ± 1.15[a]	2.50 ± 1.00[bc]	1.10 ± 0.25[cd]
	Sh-5	42.42 ± 2.66[a]	1.65 ± 0.76[c]	1.00 ± 0.30[d]
LSD		9.00	1.315	0.471

LSD, Least significant difference.

influenced morphogenesis either positively or negatively (Ling et al., 1998). During washing and pre-selection of potato varieties Desiree and Sh-5, different doses of cefotaxime were used. There were significant differences observed among the cefotaxime concentrations to control the bacterial overgrowth. Statistical analyses showed that there were no significant differences between lower doses (0 and 100 mg/l) and among higher doses (200, 400, and 500 mg/l) of cefotaxime to control the bacterial overgrowth. The concentration (400 and 500 mg/l) of cefotaxime showed maximum control on the overgrowth of *Agrobacterium* but the mean value of regeneration of explants and transformation efficiency were lower on these concentration and most of the explants died (Table 3). Cefotaxime concentration (200 mg/l) during washing and pre-selection showed complete control on over-growth of *A. tumefaciens* and also had no diverse effect on the nodal explants. It gave maximum mean value of regeneration of nodes (4.00 and 3.10) and high transformation efficiency (3.38 and 3.10) for Desiree and Sh-5, respectively (Figure 2 and Table 3). Our results correlate with the findings of Jun et al. (2008) who reported that 200 mg/l of cefotaxime in washing media as well as in pre-selection and selection media showed best results for controlling *Agrobacterium* growth. Banerjee et al. (2006) obtained the transgenic potato by using 250 mg/l cefotaxime in the media. Kishimoto et al. (2002), and Liu et al. (2004) reported that cefotaxime at 500 mg/l was found to be effective for the control of *Agrobacterium* growth. These results correlate to that of our findings but deviates at the regeneration point of explant because in this study, the regeneration rate of explant is very low. There are reports that the addition of antibiotics may exert an effect, either positively or negatively on the

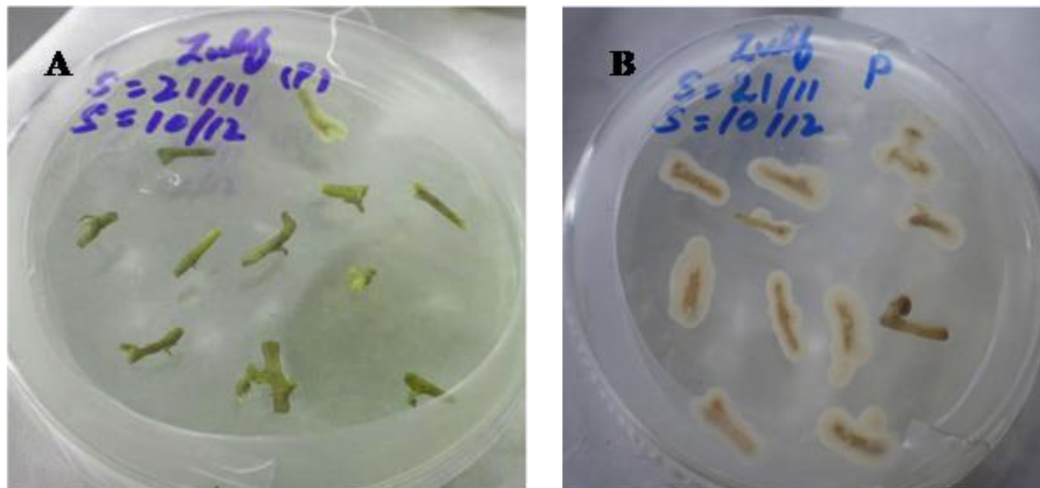

Figure 2. Cefotaxime treated explants showing control on overgrowth of *Agrobacterium* (a); control explants showing the overgrowth of *Agrobacterium* (b).

Table 4. Effect of pre-selection days on regeneration of explants and transformation efficiency.

Pre-selection period (days)	Variety	Regeneration	Transformation efficiency
0	Desiree	0.4 ± 0.30^{de}	0.26 ± 0.24^{de}
	Sh-5	0.0 ± 0.00^{e}	0.0 ± 0.00^{e}
3	Desiree	0.8 ± 0.30^{cd}	0.8 ± 0.30^{cd}
	Sh-5	0.94 ± 0.06^{cd}	0.94 ± 0.06^{c}
5	Desiree	1.75 ± 0.34^{b}	1.60 ± 0.25^{b}
	Sh-5	1.38 ± 0.21^{bc}	1.15 ± 0.15^{bc}
7	Desiree	3.08 ± 0.44^{a}	3.00 ± 0.00^{a}
	Sh-5	2.82 ± 0.28^{a}	2.60 ± 0.17^{a}
LSD		0.708	0.623

LSD, Least significant difference.

growth and regeneration of explants during transformation. Sensitivity of plants to antibiotics is species specific and depends on plant growth conditions (Lin et al., 1995; Joersbo and Okkels, 1996; Chauvin et al., 1999). Cefotaxime had negative effect on the regeneration of camellia (Tosca et al., 1996), and tomato (Ling et al., 1998), but had positive effect on callus growth and organogenesis in wheat (Mathias and Boyd, 1986) and *Pinus pinea* (Humara et al., 1999).

Pre-selection period

After co-cultivation, the days to pre-selection are very important steps for efficient transformation. Statistical analyses showed that significant differences were observed among the different period of pre-selection for regeneration of nodes and transformation efficiency. When explants were taken directly on the selection stage with hygromycin at 20 mg/l, minimum regeneration (0.4 and 0.00) and transformation efficiency (0.26 and 0.00) for Desiree and Sh-5, respectively was observed. Result shows that with the increase of pre-selection period, the mean value of regeneration of nodes and transformation frequency also increased, which indicated the effect of pre-selection period. The maximum mean value of regeneration of nodes (3.08 and 2.82) and transformation frequency (3.00 and 2.60) for Desiree and Sh-5, respectively, were observed seven days to pre-selection (Table 4). Similar results were found by Khan et al. (2003) on canola. Rashid et al. (1996) used different pre-selection period (0 to 10 days) during transformation of *Moricandia arvensis* and seven days of pre-selection period was found suitable for successful transformation.

Figure 3. *Agrobacterium* treated explants on selection media showing regeneration (a); control explants on selection media showing death (b); plants regenerated on selection media (c).

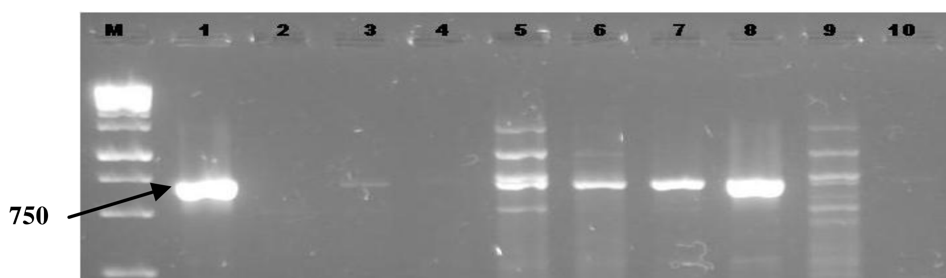

Figure 4a. PCR amplification of rice chitinase gene of Desiree variety; lane M, 1 kb marker (Fermentas Lithuania); lane 1, positive control; lane 2, control (non-transformed plant DNA), lanes 3 to 10, transgenic plants that survive on selection media.

Jabeen et al. (2009) found that seven days to pre-selection significantly increased the transformation efficiency in tomato. Banerjee et al. (2006) obtained the transgenic potato on eight days pre-selection period.

Selection

The plantlets that survived during pre-selection treatment were transferred on selection media containing 20 mg/l hygromycin and 200 mg/l of cefotaxime. This selection pressure was given to the nodes for the confirmation of RCG-3 gene and to control the bacterial growth. During transformation phase, the plants were transferred 10 to 15 days interval on the fresh media (Figure 3c). The same treatment of antibiotic was also applied on the control plants. Non-transformed plants died on the selection pressure (Figure 3b). The *Agrobacterium* treated explant that survived on this selection pressure (Figure 3a) was analyzed for the presence of RCG3 gene by using PCR.

Molecular analysis

The molecular analysis of transformed plants was done by using PCR for the confirmation of RCG3 gene in potato varieties. The genomic DNA from the putative plants was extracted through CTAB method. The PCR analysis was done using the RCG3 specific primers. The transformed plants showed 750 bp amplification product that was absent in negative control but present in positive controlled one (Figures 4a and b). PCR analysis showed that the combination of 2 min infection time, seven days pre-selection and 200 mg/l cefotaxime gave maximum RCG-3 gene positive plants; transformation frequency were 28 and 18% for Desiree and Sh-5, respectively (Table 5). Zhang et al. (2009) obtained the transformation efficiency of 67% in two potato varieties - Favorite and Shepody. Bin et al. (2009) transformed the ODREB2B gene in Huangmazi and Zhongshu No.3, and obtained 48 and 49% transformation efficiencies. The difference in transformation efficiency was due to bacterial strain, media, explants, etc. Cinegel et al. (2010) found that regeneration and transformation efficiency was strongly genotype dependent in potato.

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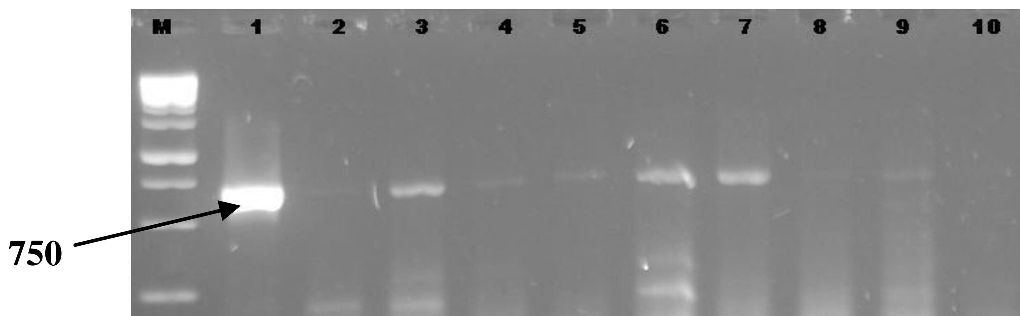

Figure 4b. PCR amplification of rice chitinase gene of Sh-5 variety; lane M, 1 kb marker (Fermentas Lithuania); lane 1, positive control; lanes 2 to 9, transgenic plants that survive on selection media; lane 10, control (non-transformed plant DNA).

Table 5. Transformation efficiency of both varieties after different factors.

Factor			Varieties	Over growth	Regeneration (%)	Transformation (%)
Co-infection time (min)	Pre-selection period (days)	Cefotaxime concentration (mg/l)				
2	7	200	Desiree	14	30	28
			Sh-5	14	20	18

Resources (NIAR), Tasukuba, Ibaraki, Japan on providing chitinase gene constract.

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