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IN VITRO REGENERATION AND TRANSFORMATION OF APPLE (*Malus domestica* Borkh.) ROOTSTOCK MALLING 7 USING RICE CHITINASE GENE

SHARMA Parul*, MODGIL Manju, SHARMA Rajnish

Department of Biotechnology, Dr YS Parmar University of Horticulture & Forestry, Nauni, Solan (HP) 173 230 INDIA

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ABSTRACT

In most of the fruit crops, the low frequency of *Agrobacterium* mediated genetic transformation hampers the respective transgenics production. Hence, it is pre-requisite to understand and optimize the different parameters involved directly or indirectly in *Agrobacterium* mediated transformation. *A. tumefaciens* strain LBA4404 harboring the transforming vector pCAMBAR*chi11* containing the rice chitinase gene (*chi11*) and hygromycin phosphotransferase (*hpt*) and phosphinothricin acetyl transferase (*bar*) genes was used for carrying out genetic transformation of apple (*Malus x domestica* Borkh.) rootstock M7. Two explants viz. leaf and internodal segments were involved in regeneration and resulted 24.37% and 60.58% shoot regeneration, respectively. The obtained putative transformants were selected on full strength MS medium containing 5 mg l⁻¹ hygromycin and 500 mg l⁻¹ cefotaxime. Two days pre-culturing and 96 hours co-cultivation was found effective for procuring maximum number of hygromycin resistant shoots. Putative transgenic shoots were multiplied separately and rooted on root induction medium containing 5 mg l⁻¹ hygromycin. Further, PCR analysis was done using chitinase gene specific primers and two apple transgenic lines confirmed the integration of *chi11* gene by yielding 237 bp and 584 bp amplified products, respectively.

* Corresponding author

E-mail: tulip.parul@yahoo.co.in (Sharma Parul)

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1 Introduction

The domesticated apple is one of the most important fruit crops of the colder and temperate parts of the world. It belongs to the family Rosaceae, subfamily Maloideae, and the farmed apple tree with a pomaceous fruit is known as *Malus x domestica* Borkhausen (Harris et al., 2002). The long juvenile period, high levels of heterozygosity, long hybrid evaluation time and the process of releasing new cultivars are the critical factors which slow down the pace of various conventional apple breeding programmes. So there is an urgent need to adopt modern biotechnological tools which could help to accelerate this process. Plant genetic transformation using desirable genes has geared up and facilitated various apple crop improvement activities by targeting resistance to fungal or bacterial diseases, improved fruit quality, or root stocks with better rooting or dwarfing ability (Igarashi et al., 2016). Among fruit crops, domesticated apple attains the distinctiveness because of the availability of a wide range of clonal rootstocks, thereby permitting the development of a 'designer tree size' appropriate for high density plantations. The inherent characteristics of these rootstocks especially disease resistance, growth and flowering habits etc. has remarkable impression on scion varieties. In Indian conditions, rootstock like Malling 7 (M7) is recommended as it is virus-free, and has proven itself to be one of the most popular rootstocks in the commercial industry, mostly due to its capability of producing a semi-dwarf tree i.e. approximately 50-60% of standard size. It is tolerant to collar rot, a major soil-borne disease of apple. While, it shows susceptibility to a serious soil borne disease 'white root rot' caused by the fungus *Dematophora necatrix* Hartig.

There are number of limiting factors which restricts various plant disease management practices to some extent. Plant genetic transformation has emerged as a powerful and attractive strategy for the genetic improvement of fruit trees constrained by their reproductive biology and high levels of heterozygosity. This strategy has emerged as a promising tool and addressed various problems while opening new avenues for crop modification (Rao et al., 2009). *Agrobacterium*- mediated transformation is widely used gene transfer method for introducing foreign genes into dicotyledonous plants. In this process, type of *Agrobacterium* strain, disarmed binary vector and explant (leaf, hypocotyl or callus cultures) are the limiting factors for an efficient transformation. Reliable and highly efficient regeneration system is pre-requisite base for the development of successful transformation protocol in any crop. Hence, these biotechnological based strategies can be considered as an important supplement along with existing technologies to speed up various fruit crop improvement programmes.

Innate defense mechanisms assist plants to respond towards

various biotic stresses. Several researchers have demonstrated that many defense-related (DR) proteins, such as pathogenesis-related proteins (PR) and anti-microbial peptides (AMP) have been identified in different plants during biotic stress. Chitinases are the enzymes that rupture chitin i.e. the primary component of cell walls of several types of fungi and exoskeletons of invertebrates by hydrolyzing the β -1,4-linkage among N-acetylglucosamine units (Datta et al., 1999).

For enhancing disease resistance in apple, a chitinase gene from different sources was introduced for the first time against apple scab causal organism (Wong et al., 1999; Bolar et al., 2000). An efficient plant regeneration system along with gene transfer and selection system guarantees the genetic advancement of apple through transgenic production. However, low regeneration frequency as compared to those of herbaceous species keeps genetic transformation studies in apple lacked behind. Therefore, considering such kind of key limiting factors, we aimed at developing a simple and reliable genetic transformation protocol using antifungal chitinase gene in commercially important apple rootstock M7 rootstock.

2 Materials and Methods

2.1 Explants used

4-5 weeks old *in vitro* proliferating cultures (raised from axillary buds) of apple rootstock M7 were used for procuring two explants i.e. leaves and internodal segments.

2.2 Shoot regeneration experiments

The selected explants i.e. leaves and internodal segments were excised, wounded and then inoculated on solid shoot regeneration medium (RM) containing MS salts and vitamins (Murashige & Skoog, 1962) fortified with varying concentrations (mg l^{-1}) of cytokinins as mentioned in Tables 1 and 2. Further, regenerated shoots were multiplied on shoot multiplication medium (MM) i.e. MS salts and vitamins supplemented with benzyl adenine (BA) 0.5 mg l^{-1} , gibberellic acid (GA_3) 0.5 mg l^{-1} , indole-3-butyric acid (IBA) 0.1 mg l^{-1} for multiplication. 2.0-3.0 cm long regenerants were cut and induced to root by dipping in half strength MS liquid medium containing 0.5 mg l^{-1} thiamine, 20 g l^{-1} sucrose and 0.3 mg l^{-1} IBA. These were incubated in dark for initial first week and then transferred to basal solidified medium and then kept in light. For hardening, rooted shoots were washed gently. After four weeks, well established plantlets were transferred to earthen pots containing sand, soil and FYM (1:1:1 w/w). The plants were acclimatized by gradually exposing to natural conditions.

Table 1 Effect of growth regulators on leaf explants for callus induction and adventitious shoot regeneration

S. No.	Medium code	Plant growth regulators (mg l ⁻¹)				Frequency of callus induction (%)	Frequency of shoot regeneration (%)
		BA	TDZ	NAA	IAA		
1	RM1	2.0	-	0.5	-	64.72(53.56)*	0.00 (1.00)**
2	RM2	3.0	-	0.5	-	62.85(52.44)*	0.00 (1.00) **
3	RM3	4.0	-	0.5	-	60.7 (51.22) *	0.00 (1.00) **
4	RM4	5.0	-	0.5	-	60.58(51.11)*	0.00 (1.00) **
5	RM5	2.0	-	1.0	-	70.19(56.91)*	0.00 (1.00) **
6	RM6	3.0	-	1.0	-	72.46(58.35)*	0.00 (1.00) **
7	RM7	4.0	-	1.0	-	74.71(59.81)*	4.16 (2.27) **
8	RM8	5.0	-	1.0	-	73.53(59.04)*	0.00 (1.00) **
9	RM9	2.0	-	-	0.5	0.00 (0.00) *	0.00 (1.00) **
10	RM10	3.0	-	-	0.5	0.00 (0.00) *	0.00 (1.00) **
11	RM11	4.0	-	-	0.5	0.00 (0.00) *	0.00 (1.00) **
12	RM12	5.0	-	-	0.5	0.00 (0.00) *	0.00 (1.00) **
13	RM13	2.0	-	-	1.0	0.00 (0.00) *	0.00 (1.00) **
14	RM14	3.0	-	-	1.0	0.00 (0.00) *	0.00 (1.00) **
15	RM15	4.0	-	-	1.0	0.00 (0.00) *	0.00 (1.00) **
16	RM16	5.0	-	-	1.0	0.00 (0.00) *	1.38 (1.54) **
17	RM17	-	0.2	0.5	-	82.00(64.90)*	0.00 (1.00) **
18	RM18	-	0.4	0.5	-	82.27(65.10)*	0.00 (1.00) **
19	RM19	-	0.6	0.5	-	83.63(66.14)*	9.71 (3.27) **
20	RM20	-	0.8	0.5	-	85.41(67.54)*	18.05 (4.35) **
21	RM21	-	1.0	0.5	-	84.81(67.06)*	0.00 (1.00) **
22	RM22	-	0.2	1.0	-	88.05(69.78)*	0.00 (1.00) **
23	RM23	-	0.4	1.0	-	88.09(69.81)*	0.00 (1.00) **
24	RM24	-	0.6	1.0	-	88.51(70.19)*	0.00 (1.00) **
25	RM25	-	0.8	1.0	-	89.50(71.09)*	24.37 (5.03) **
26	RM26	-	1.0	1.0	-	87.85(69.60)*	0.00 (1.00) **
27	RM27	-	0.2	-	0.5	37.17(37.56)*	0.00 (1.00) **
28	RM28	-	0.4	-	0.5	37.20(37.58)*	0.00 (1.00) **
29	RM29	-	0.6	-	0.5	37.58(37.81)*	0.00 (1.00) **
30	RM30	-	0.8	-	0.5	37.59(38.02)*	0.00 (1.00) **
31	RM31	-	1.0	-	0.5	36.61(37.23)*	0.00 (1.00) **
32	RM32	-	0.2	-	1.0	41.00(39.81)*	0.00 (1.00) **
33	RM33	-	0.4	-	1.0	42.03(40.41)*	0.00 (1.00) **
34	RM34	-	0.6	-	1.0	41.63(40.18)*	0.00 (1.00) **
35	RM35	-	0.8	-	1.0	41.66(40.20)*	0.00 (1.00) **
36	RM36	-	1.0	-	1.0	40.86(39.73)*	0.00 (1.00) **
CD _{0.05}						3.36 (2.34) *	8.40 (0.65) **
SE (m)						1.18 (0.83)*	2.97 (0.23)**

(Values in parentheses are - *angular and **square root transformed values)

Table 2 Effect of growth regulators on internodal explants for callus induction and adventitious shoot regeneration

Sr. No.	Medium code	Plant growth regulators (mg l ⁻¹)		Frequency of callus induction (%)	Frequency of shoot regeneration (%)
		TDZ	NAA		
1	RM1	0.2	0.5	24.33(29.52)	12.75 (20.89)
2	RM2	0.4	0.5	20.50(26.90)	28.17 (32.04)
3	RM3	0.6	0.5	17.83(24.96)	39.67 (39.02)
4	RM4	0.8	0.5	15.33(23.04)	60.58 (51.09)
5	RM5	1.0	0.5	12.50(20.68)	41.83 (40.27)
6	RM6	0.2	1.0	40.50(39.50)	8.33 (16.75)
7	RM7	0.4	1.0	37.75(37.89)	19.75 (26.37)
8	RM8	0.6	1.0	31.50(34.12)	27.67 (31.72)
9	RM9	0.8	1.0	29.00(32.56)	29.42 (32.83)
10	RM10	1.0	1.0	21.33(27.47)	15.17 (22.90)
CD _{0.05}				2.67 (1.80)*	2.33 (1.52)*
SE (m)				0.89 (0.60)*	0.78 (0.51)*

(Values in parentheses are angular transformed values)

Figure 1 Map of pCAMBIA bar-ubi-chi11 transforming vector

2.3 Genetic transformation experiments

The chitinase gene construct harboring plasmid pCAMBIA bar-ubi-chi II (13.8 kb, Figure 1) with chitinase (chi-II, 1.1 kb) gene driven by ubiquitin-I (Ubi-I) promoter and two selectable marker genes i.e., hygromycin phosphotransferase (hpt) &

phosphinothricin acetyltransferase (bar) under the control of CaMV35S was obtained from Dr S. Muthukrishnan, KSU, USA. Further, plasmid DNA was transferred into *A. tumefaciens* strain LBA4404 via triparental mating by Sharma et al. (2012) for carrying out co-cultivation experiments.

2.4 Strain maintenance

The *A. tumefaciens* strain LBA4404 was regularly maintained on YMB (Yeast extract 1.0 g l⁻¹, Mannitol 10.0 g l⁻¹, Dipotassium phosphate 0.5 g l⁻¹, Magnesium sulphate 0.2 g l⁻¹, Sodium chloride 0.1 g l⁻¹, Calcium carbonate 1.0 g l⁻¹, Final pH 7.0) medium containing filter sterilized streptomycin (25 mg l⁻¹) and kanamycin (50 mg l⁻¹) (Hi-Media) respectively. To achieve proper growth, incubation at 28°C under dark conditions for 3-4 days and further storage at low temperature (4 ± 2°C) was followed.

2.5 Bacterial suspension preparation

For co-cultivation, fresh cultures of *Agrobacterium* strain LBA4404 were prepared in 25 ml liquid YMB medium containing 50 mg l⁻¹ kanamycin (filter sterilized) and incubated overnight at 28 °C in an orbital shaker at 200 rpm. Bacterial suspension was centrifuged at 6,000 rpm for 5 minutes and the pellet was re-suspended in MS basal liquid medium to a O.D. = 0.520 at 540 nm pertaining to 5 × 10⁸ cells per ml.

2.6 Antibiotics used

Two antibiotics i.e. hygromycin for transformants selection and cefotaxime for controlling *A. tumefaciens* growth were used. 5mg l⁻¹ Hygromycin and 500 mg l⁻¹ cefotaxime were added to pre-sterilized molten MS regeneration medium (RM) fortified with 0.8 mg l⁻¹ TDZ and 1.0 mg l⁻¹ NAA by filter sterilization through 0.22µm pore size membrane filter to carry out the respective transformation experiments.

2.7 Pre-culturing and co-cultivation experiments

Both types of explants were pre-cultured for 2 days in light prior to infection on RM and then further, co-cultivated on selective RM supplemented with 5mg l⁻¹ Hygromycin and 500 mg l⁻¹ cefotaxime. The pre-cultured explants were immersed in bacterial suspension for 15 minutes with gentle pricking and stirring for infection with *Agrobacterium*. Explants were further blotted on sterilized filter paper to remove the excess of bacteria and placed on basal MS medium after *Agrobacterium* infection. The cultures were co-cultivated/incubated for 96 hours in dark and observations were recorded on the basis of morphological evidences i.e. *Agrobacterium* growth and the average number of calli or shoots formed from survived explants after four weeks, respectively. Both types of explants were transferred to selective RM medium containing 500 mg l⁻¹ cefotaxime to eliminate the bacteria and 5 mg l⁻¹ hygromycin to select the putative transformed shoots following 96 hours (dark) of co-cultivation. Explants were washed with sterile distilled water containing 250 mg l⁻¹ cefotaxime, blotted on sterilized filter paper and then transferred on fresh selective RM to check the excessive bacterial growth. The performance of putative transformed cells or shoots

was compared with control on selective medium as well as on non-selective medium. Depending upon the growth of *Agrobacterium*, the concentration of cefotaxime was decreased gradually from 500 to 250 mg l⁻¹. 26 putative regenerants of length (≥ 0.5 cm) were separated off from the explant and multiplied on selective MM supplemented with 5 mg l⁻¹ hygromycin and 250 mg l⁻¹ cefotaxime. Of these, only 18 selected putative shoots (about 0.5 - 1.0 cm) were rooted and hardened.

2.8 Molecular level confirmation

Genomic DNA from leaves of 2 untransformed control shoots and 18 hygromycin resistant shoots were isolated following CTAB method and further quantitative and qualitative analysis was done using UV spectrophotometer (Bio-Rad). Two sets of specific primers for the amplification of rice endochitinase class I (*chil1*) gene were designed with their product size 237 bp (forward primer- GGACGCAGTCTCCTTCAAGA, reverse primer- ATGTCGCAGTAGCGCTTGTA) and 584 bp (forward primer- GCTTCTACACCTACGACGCCTT, reverse primer- GTAGCGCTTGTAACCCGATC), respectively to check the integration of T-DNA in the genome of putative transformants. PCR conditions were standardized for these primers by using isolated DNA along with two PCR controls i.e. positive control (plasmid DNA) and negative control (reaction mix with water except template DNA) in a thermal cycler (Eppendorf). 25 µl PCR cocktail consisted of 3U/reaction Taq DNA polymerase, 10x Taq DNA polymerase buffer, 1.5 mM MgCl₂, 10 pmol/reaction each specific chitinase primers (forward and reverse), 2.5 mM each dNTPs (deoxynucleotide triphosphate) and 50 ng/reaction template DNA respectively. Amplified PCR products were visualized and photographed under UV light using alpha imager® EC gel documentation system (Biovis) after electrophoresis on a 1.2 % (w/v) agarose gel containing 0.5 µg ml⁻¹ ethidium bromide.

2.9 Data analysis

The statistical analysis based on mean values per treatment was made using ANOVA technique for completely randomized design (CRD).

3 Results

3.1 Adventitious shoot regeneration studies

Instead of various other auxin and cytokinin combinations, adventitious shoots emerged only in TDZ and NAA combinations in case of both the explants (leaf and internodal segments). In case of leaf explants, from 16 different combinations of BA and NAA/IAA used, only RM7 and RM16 combinations produced adventitious shoots with a frequency of 4.16% and 1.38% respectively (Table 1). Likewise, out of 20 different combinations of TDZ and NAA/IAA, an average frequency of adventitious

shoot regeneration of 24.37%, 18.05% and 9.71% was achieved in RM25 (0.8 mg l⁻¹ TDZ and 1.0 mg l⁻¹ NAA), RM20 (0.8 mg l⁻¹ TDZ and 0.5 mg l⁻¹ NAA) and RM19 (0.6 mg l⁻¹ TDZ and 0.5 mg l⁻¹ NAA) respectively (Table 1). On the other hand, TDZ and IAA combinations failed to produce adventitious shoots. Only RM25 (0.8 mg l⁻¹ TDZ and 1.0 mg l⁻¹ NAA) showed both direct and indirect organogenesis (Figure 2). While assessing the frequency of adventitious shoot regeneration, internodal segments were found better than the leaf explants. The most significant treatment was 0.8 mg l⁻¹ TDZ and 0.5 mg l⁻¹ NAA with a maximum of 60.58% regeneration in case of internodal segments (Table 2, Figure 3). Although both leaf and internodal explants showed good regeneration rates with TDZ and NAA combinations but later showed relatively higher regeneration rate. Regenerated shoots were propagated on shoot multiplication medium (MM). About 70-75% rooting was noted in the regenerants with 50-60% hardening rate.

3.2 Effect of antibiotics

Optimization of antibiotics dosage is compulsory as it determines the basic pre-requisites like normal regeneration of putative transgenic shoots and controlled *Agrobacterium* growth.

Cefotaxime @ 500 mg l⁻¹ controlled the excessive *Agrobacterium* growth effectively after co-cultivation along with less regeneration frequency. While lower concentrations of cefotaxime i.e. 200-300 mg l⁻¹ triggered the regeneration. On the other hand, 5 mg l⁻¹ hygromycin proved effective for the selection of putative transformants after co-cultivation. In this study, data with respect to antibiotics standardization have not shown.

3.3 Genetic transformation experiments

Following 15 minutes infection of explants with *Agrobacterium* cell suspension (5x10⁸ cells/ml) with gentle stirring, co-cultivation for 96 hours in all the precultured leaf and internodal explants (Tables 3 & 4) had a strong impact on the regeneration frequency i.e. 1.75 & 7.66 as well as the number of putative transformed shoots i.e. 1.33 & 3.33, respectively. A total of 26 putative transgenic shoots from both the explants i.e. leaves and internodes (Figure 4) were obtained and maintained separately on selective (5 mg l⁻¹ hygromycin and 250 mg l⁻¹ cefotaxime) MM. Upon subculturing, the non-transformed tissues or the escapes shoots, which became necrotic or white, were removed. The putative transformed shoots revealed a steady growth pattern as compared to control. In present study, leaf explants predominantly generated

Figure 2 Shoot regeneration in leaf explants; (a-c) Direct & (d-f) Indirect regeneration on MS salts & vitamins + 0.8 mg/l TDZ + 1.0 mg/l NAA (RM 25).

Figure 3 Shoot regeneration in internodes (a-c) on MS salts & vitamins + 0.8 mg/l TDZ+0.5 mg/l NAA (RM 4).

Table 3 Effect of pre-culturing and co-cultivation durations on regeneration of leaf explants

Sr.No.	Pre-culturing (Days)	Co-cultivation (Hours)	Total no. of explants cultured	Frequency of shoot regeneration (%)	Average no. of shoots/ explant
1	0	24	90	0.00 (1.00)	0.00 (1.00)
		48	90	0.00 (1.00)	0.00 (1.00)
		72	90	0.00 (1.00)*	0.00 (1.00)*
		96	90	0.00 (1.00)*	0.00 (1.00)*
2	1	24	100	0.00 (1.00)	0.00 (1.00)
		48	100	0.00 (1.00)*	0.00 (1.00)*
		72	100	0.00 (1.00)*	0.00 (1.00)*
		96	100	1.00 (1.41)	1.00 (1.41)
3	2	24	95	0.00 (1.00)	0.00 (1.00)
		48	95	0.00 (1.00)	0.00 (1.00)
		72	95	0.70 (1.28)	0.66 (1.27)
		96	95	1.75 (1.65)	1.33 (1.52)
4	3	24	100	0.00 (1.00)	0.00 (1.00)
		48	100	0.00 (1.00)*	0.00 (1.00)*
		72	100	0.00 (1.00)*	0.00 (1.00)*
		96	100	0.74 (1.30)	0.66 (1.27)
5	4	24	90	0.00 (1.00)	0.00 (1.00)
		48	90	0.00 (1.00)	0.00 (1.00)
		72	90	0.00 (1.00)*	0.00 (1.00)*
		96	90	0.00 (1.00)*	0.00 (1.00)*
CD _{0.05}				0.19 (0.06)*	0.15 (0.05)*
SE(m)				0.06 (0.02)*	0.05 (0.01)*

(Values in parentheses are square root transformed values)

Figure 4 Putative transformed shoot regeneration on selective medium (a-c) through leaf explants (d-f) through internodes

Table 4 Effect of pre-culturing and co-cultivation durations on regeneration of internodal segments

Sr.No.	Pre-culturing (Days)	Co-cultivation (Hours)	Total no. of explants cultured	Frequency of shoot regeneration (%)	Average no. of shoots/ explant
1	0	24	70	0.00 (1.00)	0.00 (1.00)
		48	70	0.00 (1.00)	0.00 (1.00)
		72	70	1.17 (1.43)	1.00 (1.38)
		96	70	1.95 (1.65)	1.33 (1.48)
2	1	24	55	0.00 (1.00)	0.00 (1.00)
		48	55	0.00 (1.00)	0.00 (1.00)
		72	55	2.33 (1.82)	1.66 (1.62)
		96	55	4.33 (2.30)	1.66 (1.62)
3	2	24	60	0.00 (1.00)	0.00 (1.00)
		48	60	0.00 (1.00)	0.00 (1.00)
		72	60	4.00 (2.22)	2.33 (1.82)
		96	60	7.66 (2.92)	3.33 (2.07)
4	3	24	50	0.00 (1.00)	0.00 (1.00)
		48	50	0.00 (1.00)	0.00 (1.00)
		72	50	2.35 (1.81)	1.66 (1.62)
		96	50	5.87 (2.61)	2.00 (1.71)
5	4	24	55	0.00 (1.00)	0.00 (1.00)
		48	55	0.00 (1.00)	0.00 (1.00)
		72	55	1.11 (1.45)	1.00 (1.41)
		96	55	0.74 (1.30)	0.66 (1.27)
CD _{0.05}				0.93 (0.26)*	0.46 (0.13)*
SE (m)				0.32 (0.09)*	0.16 (0.04)*

(Values in parentheses are square root transformed values)

the putative shoots directly i.e. without callus phase. On the other hand, internodal segments generated the putative shoots indirectly i.e. through callus phase.

3.4 Molecular level confirmation

Integration of the chitinase gene in 18 putative transgenic shoots was checked by PCR analysis. Only two lines (T3 and T9) out of 18 were proved PCR positive by amplifying 237 bp and 584 bp fragments (Figure 5a & 5b) respectively. Consequently, the presence and integration of chitinase gene in the genome of apple rootstock M7 was demonstrated by PCR analysis. Hardening was attempted in case of rooted transgenic lines (Figure 6).

3.5 Transformation efficiency

In the present studies, the observed transformation efficiency with respect to the type of explants viz. leaf and internode was 0.32% and 1.70%. Out of two transgenic lines, one arose from leaf explants and another from internodes. Correspondingly, the actual transformation frequency was 0.05% and 0.08% in leaf and internodal explants.

4 Discussion

With the emergence of genetic transformation method the chances for the development of resistant cultivars have been increased in

Figure 5 (a) 237 bp & (b) 584 bp amplification in T3 and T9 transgenic lines.

Figure 6 Rooting in (a) control (b) transgenic line.

case of important commercial apple cultivars and rootstocks vulnerable to various fungal diseases. Genetic engineering widens the swiftness of genes by transferring them to plants from any other source like plants, animals or microorganisms which is usually limited in conventional breeding programs (Polanco et al., 2010). Many of the species and cultivars of apple perform poorly towards the development of transgenic tissues because of inefficient regeneration system (Akdemir et al., 2012). Increased leaf regeneration efficiency remains to be the most imperative factor for development of a transformation system in apple while using *A. tumefaciens* vectors. Axenically raised shoot tissue cultures serves as a perfect source for explants like expanded leaves or stems. For adventitious shoot bud/shoots induction from leaves and internodes TDZ was preferred over BA in our experiments. Regarding remarkable effect of TDZ in shoot bud induction our results are in concordance with Gercheva et al., 2000; Dobranszki et al., 2002; Qin et al., 2002; Ou et al., 2008.

Fundamentally, the transfer of bacterial T-DNA [positioned on its tumor-inducing (Ti) plasmid] into the plant nuclear genome is plant genetic transformation. For many fruit species *Agrobacterium* mediated transformation relies chiefly upon the type of genotype. Conversely to all other strains, *A. tumefaciens* LBA4404 strain could be adequately removed off from apple explants post co-cultivation with low amounts of antibiotics (Komori et al., 2009; Modgil & Sharma 2009).

Attributes of antibiotics like vast dimension for bacterial infection and low eukaryotic toxicity allows the widespread use of cefotaxime and carbenicillin to eliminate *A. tumefaciens* from plant cultures after co-cultivation. Findings of present study are coherent with Modgil & Sharma (2009) regarding remarkable effect of cefotaxime on bacterial growth elimination (@ 500 mg l⁻¹) and its prompt response towards shoot regeneration (@ 200-300 mg l⁻¹). The most significant step in any transformation procedure

is the discrimination of transformed regenerants from non transformed ones. Miki & McHugh (2004) reported broad spectrum activity of hygromycin against prokaryotes and eukaryotes and toxicity in plants. Nevertheless, the screening for transgenic regenerated shoots is often partial and difficult due to regeneration of escapes and chimeras. In this study 5 mg l⁻¹ hygromycin was used as a selection pressure for the co-cultivated explants which strengthened the previous findings (Kumar et al., 2004; Modgil & Sharma 2009, Sharma et al., 2012).

Pre-treatment allows the conditioning of the explants prior to *A. tumefaciens* infection which particularly improves the porosity of cell walls thereby enhancing the transformation rate (Gill et al., 2004). Further, it was reported that 15 minutes *Agrobacterium* cell suspension infection of explants is quite productive. On the contrary, many reports showed that the infection time and temperature are genotype dependent, while gentle shaking of leaves for different durations at room temperature was preferred to obtain maximum transformation efficiency in *M. domestica* cv. 'Jonagold' and rootstock MM106 respectively (De Bondt et al., 1994; Sharma et al., 2012).

Developed organogenesis protocols or regeneration systems are the backbone of competent genetic transformation methods. Presence of escapes (non-transformed tissues) in this studies is consistent with Radchuck & Korkhovoy 2005; Flachowsky et al., 2008; Sharma et al., 2012 in case of apple. Usually, the participation of few cells in the origin of new adventitious shoots enhances the likelihood of chimera development (George et al., 2008). Among the developed putative transformed regenerants, few can be chimeras i.e. a mix of transformed and non-transformed cells in the tissues (Hanke et al., 2007). In most transformation systems, the generation of a number of escapes is expected. Regeneration of escapes and chimerical shoots at high frequencies has been reported in some species, although their importance and frequency have been underestimated. The problem of chimerism seems to be more frequent than originally thought and it has been reported in several herbaceous species and woody fruit trees (Padilla & Burgos, 2010).

Though the adventitious shoot regeneration from wounded leaf segments was reported by Norelli et al., 1996 and Sharma et al., 2012, however, a proficient system of transgenic shoots production via apical internodal explants from etiolated 'Royal Gala' apple shoots was cited by Liu et al., 1998.

PCR has many potentialities in the various fields of biology. Among these, it is one of the diagnostic tool for transformed cells detection. Likewise, in the present investigations the presence of rice chitinase gene (*chiII*) in transformed plants was confirmed by amplifying 584 bp and 237 bp size fragments through PCR as previously reported by Ganesan et al., 2009; Sharma et al., 2012

who also used the same construct in case of cotton and apple rootstock 'MM106' respectively.

Though the genetic transformation efficiency is significantly lower in present study but first time it helps in developing a simple and reliable chitinase gene transfer protocol for the apple rootstock M7 by using *A. tumefaciens*. For the improvement of existing trusted rootstocks, transgenic technology can be applied by incorporating the useful foreign genes of interest. Further studies are required for the validation of transgene performance towards resistance to white root rot (*Dematophora necatrix*) in apple rootstock M7. The results obtained from this research would contribute for possible development of fungal resistant transgenic lines.

Conflict of Interest

Authors would hereby like to declare that there is no conflict of interest that could possibly arise.

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