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Agrobacterium-mediated transfer of chitinase gene in apple (*Malus* x *domestica* Borkh.) rootstock MM106

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ABSTRACT

***Agrobacterium tumefaciens* strain LBA4404 harbouring the transforming vector pCAMBAR *chi11* containing chitinase gene was used in transformation studies using leaf as an explant in apple rootstock MM106. Putative shoots were selected on regeneration medium containing 5 mg/l hygromycin and 500 mg/l cefotaxime. Two-day preculturing and 96 h co-cultivation incubation were found effective to obtain maximum number of hygromycin-resistant shoots. Two transgenic lines were confirmed after amplifying the integrated *chi11* gene. The amplified fragments were further analysed for their authenticity using sequencing analysis. The obtained sequences were subjected to BLAST analysis, and showed 100 per cent homology with the rice endochitinase class I gene thus proves its integration. The transformation efficiency of putative transgenic shoots was observed to be 1.57 per cent.**

Key words: *Agrobacterium*-mediated transformation, apple rootstock, fungal resistance, rice chitinase gene.

INTRODUCTION

Clonal rootstocks preserve special characteristics and special influence on scion varieties such as disease resistance, growth and flowering habit. For high density plantations in Himachal Pradesh, semi-dwarf rootstocks like Malling Merton (MM) 106 are needed. Trees on MM106 are very productive, well anchored and do not sucker, which is a problem with trees on other apple rootstocks like Malling 7. Fruit bearing starts early on MM106 and the tree gives good crop consistently. It is resistant to wooly aphid and seedling blight, but is susceptible to powdery mildew, collar rot etc. Because of the limitations of the conventional breeding programme like heterozygosity, long juvenile phase and various inbreeding depressions, thereby leading to delay and difficulty in the relation of genotypes with improved characteristics.

The most widely used strategy to engineer fungal disease resistance has been over expression of chitinases and glucanases. Chitinases such as poly [1,4-N-acetyl-β-D-glucosamine] glycan hydrolase (EC 3.2.1.14) are low molecular weight pathogenesis-related (PR) proteins that are often extracellular, acid soluble and protease resistant. They catalyse hydrolysis of β-1,4 linkages of N-acetyl-D-glucosamine chitin polymer of fungal mycelial walls into N-acetyl glucosamine oligomers. Chitinolytic enzymes from rice have *in vitro* activity against several plant pathogenic fungi. Rice chitinase gene encoding different types of hydrolytic enzymes have been transformed in several crops like chrysanthemum (against *Botrytis cinerea*, Takatsu *et al.*, 13), cucumber (against *Botrytis cinerea*, Tabei *et al.*, 12), grape (against *Uncinula nectator*, Yamamato *et al*., 14) to reduce disease severity. The major limiting factor preventing the development of gene transfer techniques for perennial crops is the lack of efficient regeneration systems (Dandekar, 1). Increasing leaf regeneration efficiency is critical for the development of a transformation system in apple using *Agrobacterium tumefaciens* vector (Klee *et al.*, 5). The first report of direct and reproducible regeneration of adventitious shoots from leaf explants of apple rootstock MM106 was developed by Modgil *et al.* (7). Furthermore, Modgil *et al.* (8) revealed several factors affecting high regeneration frequency in leaves of MM106. The present effort was to develop fungal resistance in commercially important rootstock MM106 using genetic engineering with a rice chitinase gene.

MATERIALS AND METHODS

Plasmid DNA of rice chitinase gene construct (pCAMBIA 1300-bar-ubi-*chi11*) contained two selectable marker genes, *i.e.*, hygromycin phosphotransferase (*hpt)* and phosphinothrycin acetyltransferase (*bar*) under the control of CaMV35S and a desirable gene chitinase (1.1 kb) under the control of ubiquitin promoter. The plasmid DNA of chitinase gene construct was introduced into DH5α *E. coli* strain by electroporation and to *Agrobacterium tumefaciens* strain LBA4404 by triparental mating method.

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Four-five young expanded apical leaves used for apple transformation studies were excised from four-week old proliferated shoot cultures of apple rootstock MM106 maintained *in vitro* as previously described (Modgil *et al*., 7,8). The leaf explants with cut margins were pricked using injection needle and pre-cultured on shoot regeneration medium containing basal salts and vitamins of MS medium with 30 g/l sucrose, 8.0 g/l Difco Bacto agar and supplemented with 5.0 mg/l benzyl adenine (BA) and 1.0 mg/l naphthalene acetic acid (NAA). Pre-culturing was done for different durations [0 (fresh), 1, 2, 3 and 4 days] prior to infection and co-cultivation to achieve highest regeneration frequency. Leaves were infected with *Agrobacterium* cell suspension (10^8 cells/ml) for 20 min. with gentle stirring and co-cultivated for different durations of time (24, 48, 72 and 96 h) on shoot regeneration medium in dark, at 24 ± 2°C. After co-cultivation, explants showing bacterial growth on wounded surface as well as at their cut leaf margins were washed with sterile distilled water containing 250 mg/l cefotaxime, blotted dry on sterile filter papers and cultured on selective shoot regeneration medium supplemented with 500 mg/l cefotaxime and 5 mg/l hygromycin to control the bacterium growth and selection of transformed tissues (Modgil and Sharma, 9).

After 8-9 weeks, the shoots grown to about 6-8 mm in size, were excised and transferred to shoot proliferation medium containing 0.5 mg/l BA, 0.5 mg/l GA_3 and 0.1 mg/l IBA, 30 g/l sucrose, 0.7 g/l agar supplemented with 5 mg/l hygromycin and 250 mg/l cefotaxime, respectively. To check the effect of enhanced selection pressure, a few lines were also attempted to 1.5-fold concentration of hygromycin, *i.e.*, 7.5 mg/l. Randomly selected few lines were also subjected to selective proliferation medium supplemented with different concentrations (1-5 mg/l) of phosphinothrycin (PPT) herbicide for further selection of putative transform ants. The putative transgenic lines were rooted on rooting medium containing half-strength MS basal salts, 0.5 mg/l thiamine and 0.5 g/l agar supplemented with 5 mg/l hygromycin, after 3 h dip in 30 mg/l IBA. Rooted shoots were transferred to pots containing coco-peat for hardening. The statistical analysis on mean values per treatment was made using analysis of variance (ANOVA) techniques for completely randomized design (CRD).

Genomic DNA from three untransformed control shoots and fourteen hygromycin resistant shoots after random sampling, were isolated according to standard CTAB method with some modifications. The isolated DNA were subjected to polymerase chain reaction (PCR) to confirm the integration of chitinase gene into apple genome using negative control (water) and positive control (plasmid DNA) respectively. Expression primers (Forward Primer - GGACGCAGTCTCCTTCAAGA and Reverse primer - ATGTCGCAGTAGCGCTTGTA) for the rice endochitinase class I (*chi11*) gene were used with their product size 237 bp. Amplified products of PCR positive transgenic lines and plasmid (positive control) on the agarose gel after electrophoresis were excised, eluted and subjected to sequencing PCR. The amplified products were loaded in the sequencer, 3130_{xL} Genetic analyzer (16 capillary automated DNA sequencer) based on the principle of Sanger's dideoxy method for sequencing. The sequences thus obtained were analysed using BLAST (Basic Local Alignment Search Tool) analysis to check their homology with rice endochitinase class I gene.

RESULTS AND DISCUSSION

Pricked leaves with cut margins promoted high regeneration frequency with maximum number of shoots per explant, which was a positive characteristic for using these explants in gene transfer studies. Growth parameters of leaf explant were recorded to determine the effect of different preculturing and co-cultivation duration on shoot regeneration frequency (Table 1). Leaves precultured for two days prior to co-cultivation for 96 h showed highest shoot regeneration frequency (5.75%) with maximum number of shoots (Fig. 1). All the explants co-cultivated for 24 and 48 h turned brown and died without showing any resistance to selection pressure.

As competence for transformation events may also require differentiation of cells for shoot induction, a pre-culture period of two days was found effective (Sangwan *et al*., 11). In apples, a pre-culture of leaf explants prior to co-cultivation slightly increased the number of GUS expressing zones in *M. domestica* cv. Jonagold (De-Bondt *et al*., 2). Pre-culturing allows the conditioning of the explant prior to *Agrobacterium* infection and has been found to increase transformation events in some fruit species, such as apple, grape, kiwifruit and peach. The conditioning of leaves particularly improved the porosity of cell walls and thus enhances the transformation rate. Hence, the increased porosity may aid the insertion of T-DNA through the cell wall. In the present results, co-cultivation for 96 h in all pre-cultured leaves had a strong effect on the regeneration frequency as well as the number of putative transformants.

The total 34 lines transgenic shoots were obtained *in vitro* and maintained separately in the selective shoot proliferation medium supplemented with respective antibiotics. A few hygromycin resistant shoots were further attempted for selection on 1.5-

Table 1. Effect of pre-culturing and *Agrobacterium* co-cultivation time on shoot regeneration frequency in apple rootstock MM106.

Pre-culturing (day)	Co-cultivation (h)	Total No. of explants cultured	Shoot regeneration (%)	No. of shoots per explant
	24	110	0.00 (1.00)	0.00
0	48	110	Callusing	Callusing
	72	110	3.02 (2.00)	1.00
	96	110	3.94 (2.22)	1.00
	24	105	0.00 (1.00)	0.00
1	48	105	Callusing	Callusing
	72	105	3.17 (2.04)	1.00
	96	105	4.76 (2.40)	1.33
	24	110	0.00 (1.00)	0.00
2	48	110	Callusing	Callusing
	72	110	4.85 (2.42)	1.17
	96	110	5.75 (2.60)	1.83
	24	115	0.00 (1.00)	0.00
3	48	115	Callusing	Callusing
	72	115	2.03 (1.74)	1.00
	96	115	2.61 (1.90)	1.00
	24	110	0.00 (1.00)	0.00
4	48	100	0.00 (1.00)	0.00
	72	100	Callusing	Callusing
	96	100	1.17 (1.47)	1.00
$CD_{0.05}$			0.12	0.28

Values in parentheses are Arc sine transformed values.

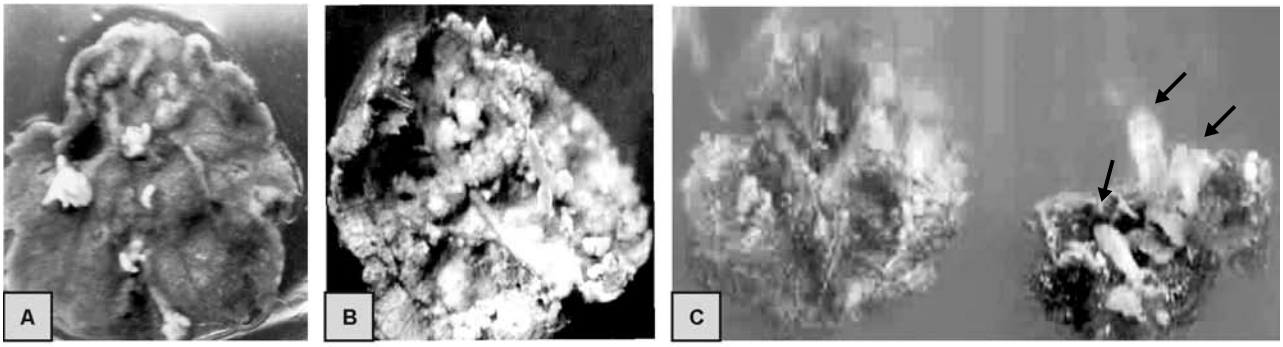

Fig. 1. Leaf explants pre-cultured for 2 days and co-cultivated for 96 h with *Agrobacterium* showing regeneration.

fold concentration of hygromycin (7.5 mg/l) but they showed necrosis and later died. Whereas, Dolgov *et al.* (3) further selected hygromycin resistant (5 mg/l) shoots of commercial cultivar 'Melba' and clonal rootstock 'N545' in fresh medium supplemented with 10 mg/l hygromycin, where they grew well. However, rests of the putative shoots were multiplied on selective shoot proliferation medium, but their growth was slow as compared to control shoots growing in the antibiotic-free medium. Multiplication rate of control shoots was 6-10 times more than that of putative transgenic shoots and the shoot length was almost double (*i.e.*, 3 cm) in former as compared to latter (*i.e.*, 1.5 cm). All the control shoots died at 5.0 mg/l PPT, while some of the putative transgenic lines exhibited resistance against the toxic residues of the respective herbicide.

While studying the comparative growth behaviour of antibiotic resistant shoots in the present investigation, it has been found that the shoots without *Agrobacterium* infection died in the selective shoot proliferation medium supplemented with selection pressure due to the toxic effect of hygromycin and PPT whereas, the putative shoots transformed with *Agrobacterium* proliferated successfully. This could be due to the integration of chimaeric gene in the apple genome which resists it for hygromycin toxicity. Similarly, in apple cultivar 'Florina' shoots transformed with pCMB-B: GUS, showed a reduced growth *in vitro*, as compared to the control plants (Radchuk and Korkhovoy, 10). However, some putative transgenic shoots showed necrosis and died after further transfer to selective shoot proliferation medium indicating that they were escapes. The deep cell layers inaccessible to *Agrobacterium* infection within the explant might be responsible for these escapes (Draper *et al*., 4). Similar type of escapes were found in 'Florina' and apple rootstock 'MM106', while growing on kanamycin supplemented medium, few shoots became colourless or white and some other shoots died because of vitrification or chimeras (Radchuk and Korkhovoy, 10).

Out of total 14 (T1-T14) randomly selected putative transformants, rooting was observed in only two lines (T4 and T12) after 45-60 days. Some of the putative shoots did not root and died due to necrosis, thus assumed to be escapes. Root emergence was also slow as compared to control (Fig. 2). Similar type of escapes has been identified by Draper *et al.* (4) in apple rootstock 'Alnarp2', where, two lines of transformants showed 100 per cent rooting in 5 mg/l hygromycin but the rooting was delayed and root number was lesser than the untransformed shoots. It has been stated that when transformed shoots were able to be rooted in selective medium, T-DNA insertion appeared to be stable (Klee *et al*., 5). Phosphinothricin herbicide was not found so much effective during the present studies. Since, the numbers of escapes were higher than the selection pressure containing hygromycin antibiotic. Similar findings have been reported in *Agrobacterium*-mediated β-glucuronidase gene transfer studies in apple rootstock 'N545' (Dolgov *et al*., 3).

Integration of rice chitinase gene in apple genome was confirmed by performing PCR analysis. PCR has been reported to be useful as a rapid check for transformants (Lassner *et al*., 6). In the present investigations, only two (T4 and T12) hygromycin resistant lines were found PCR positive by yielding 237 bp size of amplification in total 14

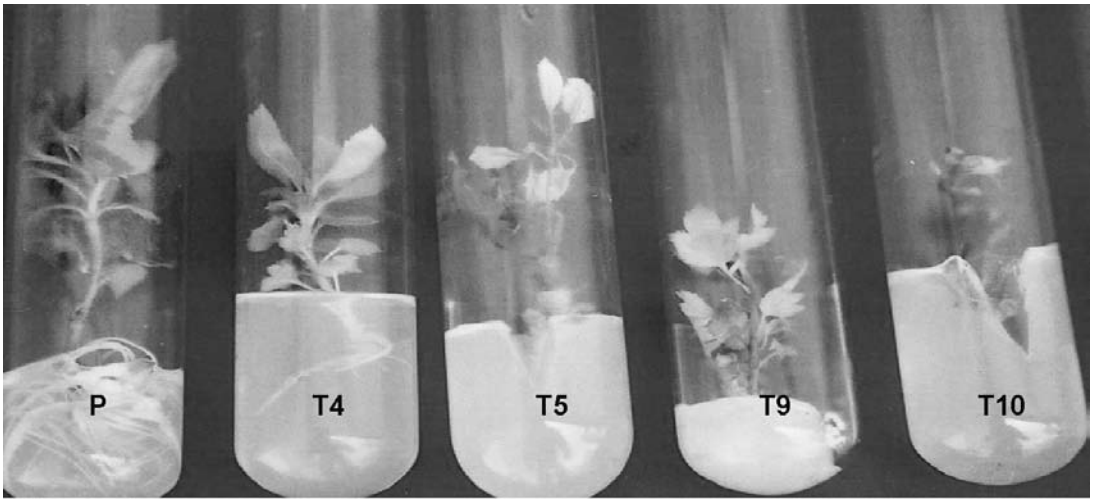

Fig. 2. Comparative growth behaviour of regenerants on root induction medium where; P = Positive control, T4 = Transgenic line, T5-T10 = Escapes.

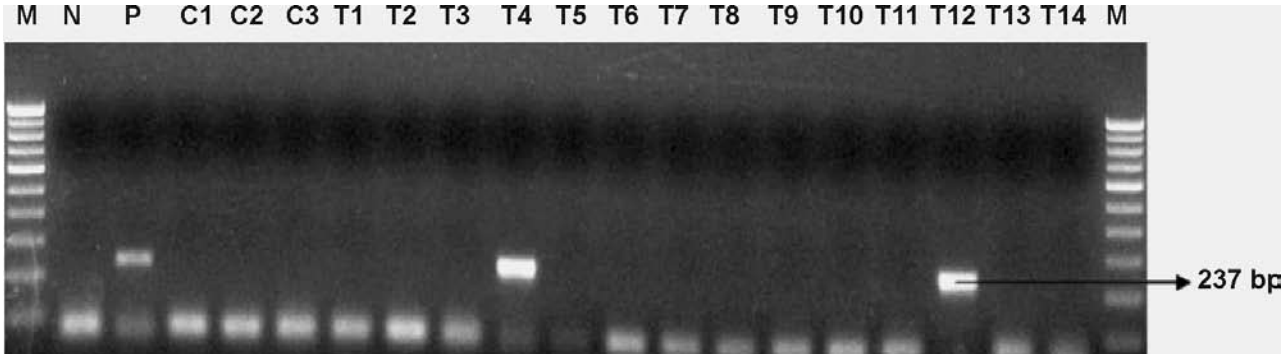

Fig. 3. Gel showing 237 bp amplification in T4 and T12 transgenic lines. Where; M = Marker, N = Negative control, P = Positive control (plasmid), C1-C3 = Control DNA, T1-T14 = Putative DNA.

putative transformants (T1-T14). The integration of rice chitinase gene against gray mold (*Botrytis cinerarea*) in cucumber was revealed by Tabei *et al.* (12) through PCR by amplifying 310 bp fragments of rice chitinase cDNA (*RCC3*) gene. Yamamato *et al.* (14) reported integration of *RCC2* gene in grapevine cv. Neo Muscut against powdery mildew caused by *Uncinula nectator* through PCR analysis and the integration was confirmed by getting amplification of 900 bp with *npt*II gene, harboured in the same gene construct.

After analysis of the sequencing peaks by an automated analyzer, the obtained sequences were subjected to BLAST analysis. BLAST analysis showed 100 per cent homology of these sequences with rice endochitinase class I gene sequence. Similarly, sequencing analysis was used by Zhu *et al.* (15) for further confirmation of the PCR products of Dapple apple viroid (DAVd) gene construct in apple and pear using fluorescently labeled primers. Hence, after doing sequencing of the amplified products, it was confirmed that the rice endochitinase class I gene has been integrated into the genome of two transgenic lines (T4 and T12) of apple rootstock MM106. Transgenic lines (T4 and T12) were being maintained in the selective shoot proliferation medium and the four plantlets of these two respective transformants have been hardened successfully. The transformation efficiency of putative transgenic shoots was observed to be 1.57 per cent.

The results presented here provide strong evidence for the integration of chitinase gene (*chi11*) in apple rootstock MM106. To our knowledge, this is the first report using chitinase gene in apple rootstock MM106 in order to attempt for conferring fungal resistance. Although, the frequency of transformation was low, the protocol is reliable and can be used to mobilize genes of horticultural importance in this valuable rootstock.

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