Journal of Applied Horticulture, 25(2): 166-172, 2023
DOI: https://doi.org/10.37855/jah.2023.v25i02.29
Journal of Applied Horticulture
ISSN: 0972-1045

Efficient genetic transformation of papaya using RNAi CP gene against papaya ringspot virus

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Abstract

Papaya Ring Spot Virus disease is wide spread across papaya growing countries of the world and is one of the major impediments in successful papaya cultivation. Genetically engineered papaya varieties *viz.*, SunUp and Rainbow have already been developed and commercialized in USA using *coat protein* mediated resistance. However, transgenic papaya conferring resistance to papaya ringspot virus has not been developed in India till date due to lack of suitable genetic transformation protocol for Indian papaya varieties and unavailability of *coat protein* gene construct for harbouring broad-spectrum resistance. The current study reports an efficient gene delivery mechanism in papaya. Young globular embryos infected for 30 minutes with *Agrobacterium tumefaciens* strain LBA 4404 harbouring hairpin loop of truncated *coat protein* gene and subsequently co-cultivated in presence of 100 pM acetosyringone and 1mM spermidine in dark for 72 hours gave rise to independent transgenic events characterized by PCR, dot blot hybridisation and RT-PCR.

Key words: *Agrobacterium, coat protein,* genetic transformation, papaya

Introduction

India is the world's largest producer of papaya, accounting for 25% of total global production. Currently, papaya is being cultivated over an area of 142 thousand ha with production of 6011 thousand mt (Kalleshwaraswamy and Kumar, 2008, Anonymous, 2020). The prevalence of papaya ringspot virus (PRSV) causes massive losses in papaya growing countries including India. This viral disease is caused by a potyvirus that spreads *via* aphids and affects tree vigour, fruit quality, flavour, and growth.

Pathogen derived resistance (PDR) has proven to be an effective tool in combating plant viruses. Genetic engineering for virus resistance has been found effective whereby transgenic plants expressing virus genome sequence resist attack by corresponding viruses (Patil *et al.,* 2011 and Mishra *et al.,* 2016). It is believed that resistance is RNA mediated *via* post transcriptional gene silencing. PRSV resistant transgenic papaya has been developed and commercialized in 1998 in Hawaii, USA by Dr. Dennis Gonsalves and team using the biolistic approach to transform embryogenic papaya cultures (Gonsalves, 1998 and Tripathi *et al.,* 2007). SunUp and Rainbow cultivars of transgenic papaya have been developed by cloning *cp* gene of PRSV HA 5-1 (Tennant *et al.,* 2001).

In terms of isolate variability of PRSV, India is diverse (Mishra and Patil, 2018; Patil *et al.,* 2017). Jain *et al.* (2004) discovered 11% diversity in Indian PRSV isolates. Somatic embryogenesis-based *In vitro* regeneration systems have already been developed (Bhattacharya *et al.,* 2002; Mishra *et al.,* 2008). Except for the report of Mishra *et al.* (2008), who devised shoot tip transformation in papaya, a robust genetic transformation system for Indian papaya cultivars is lacking, although shoot tip transformation is usually not preferred due to the development of chimaera. Therefore, this paper reports an efficient *Agrobacterium* mediated genetic transformation system of papaya using RNAi-CP (PRSV).

Materials and methods

Development of gene construct: Marker-free hairpin (RNAi) construct of PRSV Tr-*CP* (341bp) gene driven by CaMV S35 promoter was prepared in a binary vector pCAMBIA 2300 (Fig. 1) after making the vector marker free by removing *nptII* gene and placing GUS expression cassette of pBI121 binary vector in the MCS of pCAMBIA 2300 binary vector. Before cloning in binary vector, the sense and antisense Tr-CP (341bp) inserts were cloned in pGEM-T Easy cloning vector separately and subsequently

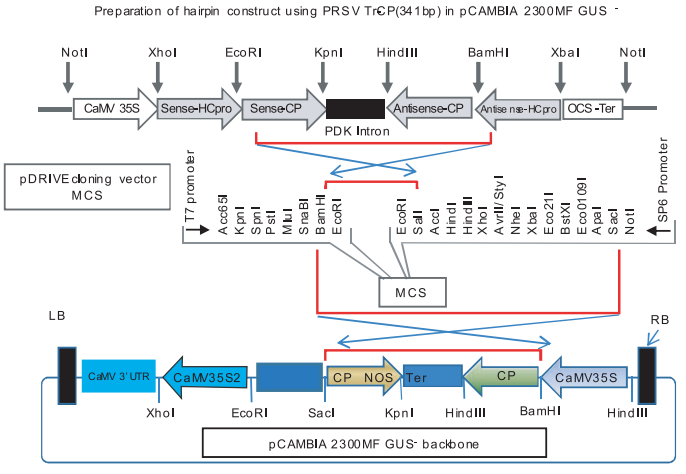

Fig. 1. Preparation of transformation vector construct using hairpin loop of PRSV Tr-cp gene (341 bp) in pCAMBIA 2300 MF GUS

in pHANNIBAL, a hairpin construction vector. The sense and antisense inserts along with the introns were then released and sub-cloned in binary vector pCAMBIA 2300 MF in the place of GUS gene driven by CaMV 35S promoter by triparental mating.

Development of gene delivery system

Bacterial colony: Five hundred µL glycerol stock of *Agrobacterium tumefaciens* strain LBA4404 harbouring the *tr-hp-cp* gene construct in pBINAR binary vector was inoculated in 20 mL of Luria Broth (LB) containing 25 mg L^{-1} kanamycin and 12.5 mg L^{-1} rifampicin. It was kept in incubator shaker for 36 hours at 26±2°C. After 36 hours, growth was checked and 500 µL of the liquid culture was spread onto LB agar media with kanamycin and rifampicin 25 mg L^{-1} and 12.5 mg L^{-1}, respectively. The culture broth was placed in incubator shaker for 24 hours at 26±2°C. Pin head colonies were used to inoculate 50 mL LB growth supplements with kanamycin and rifampicin and kept in incubator shaker for 24 hours at 230 rpm. Growth was recorded by taking O.D. at 600 nm using spectrophotometer (Chemito UV 2010).

Optical density (OD): Fifty mL of overnight grown *Agrobacterium* culture was poured in autoclaved centrifuge tube and cells were harvested by centrifugation at 6000 rpm for 5 minutes. The pellet containing *Agrobacterium* culture cells were washed thrice using sterile Murashige and Skoog (MS) salt solution. This suspension was used for determination of O.D. and agro-infection. The harvested *Agrobacterium* culture was resuspended in MS salt solution and mixed well. One mL culture was kept in cuvettes to measure the O.D. at 600 nm. The O.D. of the culture was adjusted to 0.5, 1 and 1.5 by serially diluting the culture using MS salt solution.

Agro-infection and co-cultivation: Globular somatic embryos of papaya were infected with *A. tumefaciens* strain LBA4404 in MS salt solution. This suspension was used for determination of O.D. and agro-infection. The explants were placed in a petriplate and mixed with *Agrobacterium* culture of different O.D. and was kept in incubator shaker at 26°C for 30, 45 and 60 minutes. The explants were inoculated on co'cultivation medium (MS + L-Cysteine 100 mg L^{-1} with 5.2 pH) at 26±2°C under dark conditions for 24, 48 and 72 hrs, in order to standardize the co' cultivation time.

Use of acetosyringone for enhancing transformation efficiency: In order to assess role of acetosyringone fortification on transformation efficiency, the infected embryos were subjected to 50, 100 and 200 µ M acetosyringone, and no acetosyringone as control, in MS medium supplemented with 100 mg L^{-1} LCysteine having pH of 5.2) acetosyringone in cocultivation media under dark conditions.

Role of polyamine in augmenting efficient transformation: The experiment was carried out using polyamines as pre'conditioners for explants prior to infection with *Agrobacterium* harbouring *tr-hp-cp* gene. In the experiment, the somatic embryos were pre' cultured with 50, 100 and 200 pM polyamine, and no polyamine as control, 48 hr prior to infection.

Use of antibiotics for removing *Agrobacterium* muck: After co-cultivation, the explants were washed (100 and 500 mg L^{-1} cefotaxime sodium solution; 100 and 500 mg L^{-1} streptomycin solution and cocktails of antibiotics) in MS salt solution for removing the *Agrobacterium* muck. The explants were placed in an autoclaved bottle and the washing solution (MS salt + antibiotic solution) was added to it. It was placed in incubator shaker at 26±2°C for 15 minutes at 260 rpm. After 15 minutes, the explants were washed three times with MS salt solution and were blotted dry on sterile blotting paper and inoculated onto MS basal media.

Molecular analysis

PCR based detection of transgene integration: Leaves from putative transformants were ground to fine powder using liquid nitrogen and total genomic DNA was extracted using the CTAB method (Dellaporta *et al.,* 1983). The agarose gel electrophoresis (0.8 %) of the total plant genomic DNA was carried out using a submarine horizontal agarose slab gel as described by Sambrook *et al.* (1989). PCR was performed on the total DNA of putative transformant plant leaves (having *tr-hp-cp* gene), isolated by Dellaporta's method. Transformed plants were diagnosed using forward and reverse gene specific primers (**F:** 5''GGG AAT TCC CGC AGC AAA TTG AC'3'; **R:** 5''AGG GTA CCC TCT TGA TTC CAT AC'3'). PCR reaction was conducted using commercially available PCR Kit (Fermentas) with *Taq* DNA polymerase. Amplification procedures of putative transgenic papaya PCR conditions are shown in Table 1. Thermocycler device 'My Cycler PCR system' (Bio-Rad) was used for amplification. To recover the PCR amplified products, the final volume of the reaction mixture was made 25 µl. PCR run mixture of 25 µl contained 2-6 µl of sample DNA solution, dNTPs concentration of 10 mM for each nucleotide and 25 pMol of each primer. The Mg^{++} concentration was 25 mM. *Taq* DNA polymerase and *Taq* DNA polymerase buffer were used according to manufacturer's instructions. The components were added sequentially in a thin wall PCR microfuge tube. The amplified products were resolved on 1.5 % agarose gel and visualized in the presence of ethidium bromide under UV transilluminator.

RNA extraction and Real Time-PCR of transformants: Total RNA was extracted from papaya leaves using Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) following manufacturer's instructions. The 2 µg of total RNA was reverse transcribed using a cDNA synthesis kit (Verso cDNA synthesis kit, Thermo, India) with Oligo (dT) as primer, following manufacturer's instructions in a Bio-Rad S1000 Thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The resulting cDNA was diluted 1:10 and 1 µL of the diluted cDNA was used for PCR amplification as described in Patil *et al.* (2017). The RT-PCR amplified products were resolved on 1 % agarose gel prepared with TAE (Tris-acetate-EDTA), stained with ethidium bromide and visualized under ultraviolet (UV) light.

Dot blot genomic hybridisation: Sap extracts were prepared by vortexing ground plant tissue (0.1 g) in 0.5 mL of TE buffer

Table 1. PCR conditions for detection of *tr-hp-cp*

Name of gene	I (1 cycle)	II (35 cycle)			III (1 cycle)	
	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Amplicon size
Hairpin loop of truncated *Coat protein*	95 °C for 5 minutes	95 °C for 30 seconds	52 °C for 45 seconds	72 °C for 1 minutes	72 °C for 5 minutes	341bp

(pH 8.0) containing 1 % Sodium dodecyl sulphate (SDS), 0.2 % DIECA'Na and 0.5 mL of phenol/chloroform (1:1), followed by clarification with chloroform. Extracts were precipitated with ethanol and resuspended in 1 mL of TE (pH 8.0). Aliquots of 100 pL were loaded onto Zeta-Probe membrane using a BioDot blotting manifold (Bio-Rad Laboratories). The agarose gel-isolated RT-PCR product of transgenic papaya containing *cp* gene was ^{32}P-labelled as a probe using the random primed DNA labelling kit. Zeta-Probe membranes were prehybridized and hybridized following the procedures.

Results and discussion

Optical density of the bacterial culture: Young globular somatic embryos of papaya were infected with overnight grown bacterial colony at three different optical densities at 600 nm *viz.,* 0.5, 1.0 and 1.5. An optical density ($OD_{600\ nm}$) of 1.0 of bacterial colony favoured survival of embryos. Only 2.3 *%* embryos died after a week with 1.0 OD (Fig. 2). However, 7.8 to 14 % mortality of transformed embryos was registered with lower or higher OD (0.5 and 1.5) of bacterial culture. Optimum optical density of *Agrobacterium* is very important for causing infection. Maximum bacterial population has been reported when OD_{600} is between 0.6 to 1. The results are also in conformity with other workers. It has been found that optical density (600 nm) of 1.0 is good for infecting large number of tissues. This result is also in accordance with Azad and Rabbani (2005 who found that 0.8 OD at OD_{550} is optimum for the infection of the explant.

Effect of infection time and co-cultivation periods on

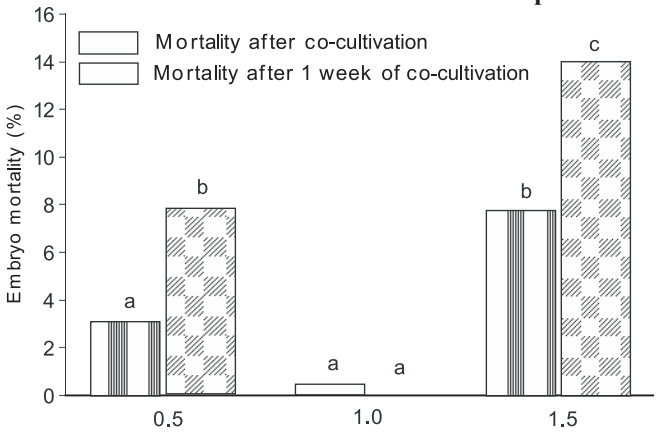

Fig. 2. Effect of optical density of the bacterial culture during, *Agrobacterium* infection

transformation efficiency: Time of exposure of *Agrobacterium tumefaciens* strain LBA4404 with plant tissue is extremely crucial step. The plant tissues were infected for 30, 45 and 60 minutes with *Agrobacterium* under agitated condition. Data (Fig. 3) revealed that *Agrobacterium* infection for 45 minutes was found optimum for efficient gene integration. A maximum of 6.39 % putative transformed embryos were recorded when explants were infected for 45 minutes. Higher exposure (60 minutes) was lethal to explants whereas lower exposure (30 minutes) was insufficient for gene integration. Explants need to be infected with *Agrobacterium* carrying gene of interest for delivery of gene. 45 minutes exposure of explant to *Agrobacterium* under agitated condition gave better transformation efficiency. Over exposure (60 minutes) of *Agrobacterium* led to overlapping of explant with bacterial colony which caused anaerobic condition for the tissue ultimately causing death of explant. The density of bacterium used for infection is adjusted either by monitoring the time of overnight cultures during incubation or by diluting the overnight bacterial cultures. The bacterial density and inoculation time is directly correlated to each other. The optimum bacterial culture densities vary in different fruit crops. In general, MS basal liquid medium is used to dilute overnight cultures to achieve the appropriate OD for infecting explants. In strawberry, overnight culture OD_{600} nm at 0.8 was found optimum (James *et al.,* 1990) to infect explants for 20-30 minutes while in case of leaf disks (El Mansouri *et al.,* 1996) overnight culture was diluted to 1:10 with MS and gently shaken for 20 minutes to infect the explants. In apple cultivar Royal Gala, overnight culture (OD_{600} nm) was adjusted to 0.814 and in *cv.* Golden Delicious (OD_{600} nm) to 0.30.5, to infect explants for 4-6 minutes (Puite and Schaart, 1996). In almond, OD of 0.6 and a 30 minutes infection time was found to be fair for transformation (Archilletti *et al.,* 1995). Same has been reported by Fitch *et al.* (1993) but Azad and Rabbani (2005) reported 1 min infection is sufficient for the transfer of T-DNA.

The results (Fig. 3) clearly showed 45 minutes infection of *Agrobacterium* with the tissue was most optimum which produced significantly higher putative transformants (8.8 %).

Infected embryos were co-cultivated for 24, 48 and 72 hours

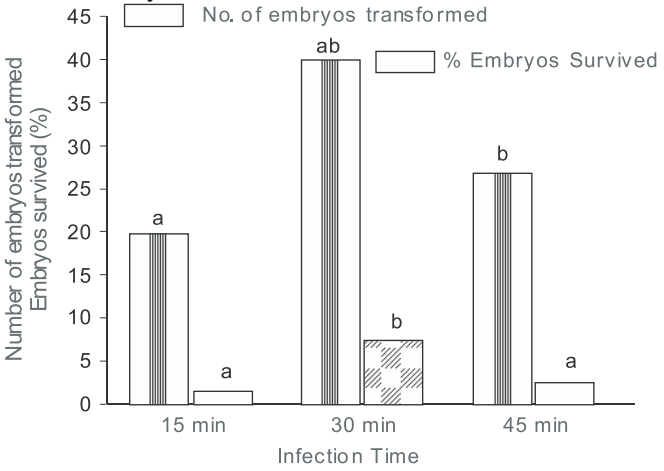

Fig. 3. Effect of infection time on survival of embryos

under dark for integration of gene. Result (Fig. 4) revealed that co-cultivation for 72 hours produced significantly higher survival of putative transgenic embryos (8.8 %) whereas when the time was reduced to 48 hours then the survival of putative transgenic

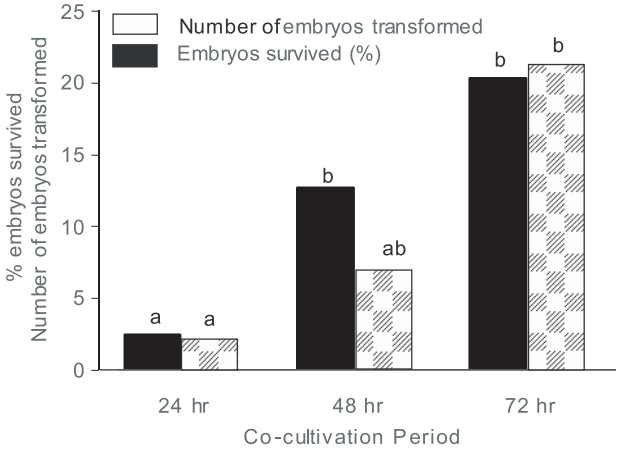

Fig. 4. Effect of co-cultivation time on survival of embryos

embryos decreased significantly (4.8 %) whereas co-cultivation time of 24 hours was insignificant for introgressing the gene into host genome. The co-cultivation of explants with *Agrobacterium* is necessary to allow the bacterial cell to infect explant cells. The explants after infection with *Agrobacterium* were blotted dry on sterile filter papers and then transferred to agrified regeneration medium for co-cultivation for different time regime (24, 48 and 72 hours). However, co-cultivation time varies among different crops. The data clearly revealed that tissues co-cultivated for 72 hours in dark with *Agrobacterium*, gave higher percentage of putative transformants (8.80 %) while 48 hours co-cultivation period reduced (4.80 %) number of putative transformants. Tissues co-cultivated for 24 hours did not produce any transformants. In citrus, explants after *Agro-infection* were co-cultivated for 3 days (Cervera *et al.,* 1998, Perez *et al.,* 1998 and Han *et al.,* 1999), *Clonorchis sinensis* and *C. reticulate* (3 days) and sweet orange (2 days) for transgene integration. In case of papaya shoot tip transformation, it was observed when co-cultivation period is increased; it causes the overgrowth of bacterium which leads to rotting of explant. In grapes, co-cultivation period of 1-5 days has been reported (Dutt *et al.,* 2008). In *Vitis vinifera,* co-cultivation period of 1 day (Baribault *et al.,* 1990), 2 days (Harst *et al.,* 2000), 3 days (Nakano *et al.,* 1994) and 5 days (Hoshino *et al.,* 2000) gave the best results.

Role of acetosyringone and spermidine on transformation efficiency: It is evident from the data that fortification of acetosyringone during co-cultivation period is extremely important for attachment of *A. tumefaciens* containing the gene of interest with explant. It is clear from the result that 100 µM acetosyringone was ideal for enhancing transformation efficiency. Acetosyringone was fortified with bacterial culture 12 hour prior to co-cultivation and further its supplementation in co-cultivation medium enhanced integration of gene. The use of phenolics (acetosyringone) may elevate the expression of the *vir* region and the transformation rate of the explants (Bolton *et al.,* 1986). Acetosyringone has been routinely used in transformation experiments. In Tamarillo, addition of acetosyringone to bacterial culture and co-cultivation medium increased transformation efficiency (35 %) significantly (Atkinson and Gardner, 1993). The data clearly revealed that acetosyringone (100 pM) augmented the process of transformation leading to production of higher putative transformants (11.20 %). However, several workers have reported sharp increase in transformation frequency by the use of acetosyringone (20-100 pM) in co-cultivation medium *e.g.* in walnut (McGranahan *et al.,* 1988 and McGranahan *et al.,* 1990), apple (Dandekar *et al.,* 1990), grapes (Nakano *et al.,* 1994 and Scorza *et al.,* 1996) banana (May *et al.,* 1995) and citrus (Han *et al.,* 1999 and Piestun *et al.,* 2000). The acetosyringone concentration of 12.5 µM was found optimum in liquid suspension cultures. In solid medium, the acetosyringone concentration ranged from 50-100 pM while in the latter, it was routinely used in transformation experiments and was found to be more effective than lower used concentrations (Cancino *et al.,* 2000 and Fitch *et al.,* 1993). Their data suggested that when overnight grown bacterial cultures were induced for 4 hrs with 50 µM acetosyringone prior to co-cultivation, it improved the transformation efficiency.

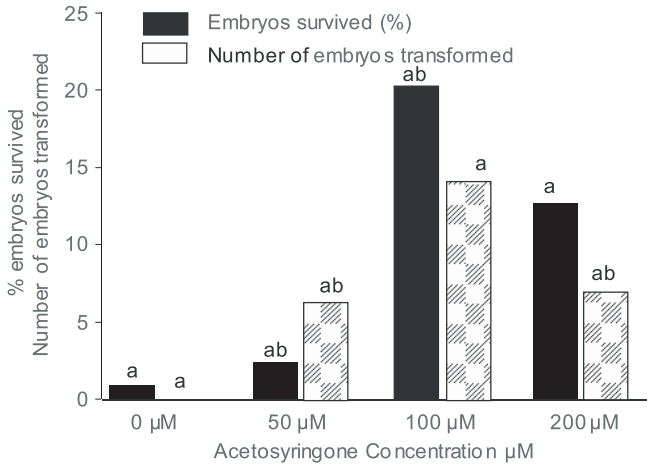

Fig. 5. Effect of acetosyringone on survival of embryos

Inclusion of polyamine particularly spermidine before infection in the *A. tumefaciens* colony has been found to be extremely important for increasing transformation efficiency. It has been observed from the result (Table 2) that 100 µM spermidine augmented the process of transformation. However, decreasing or increasing concentration of spermidine (50 or 200 µM) further reduced the transformation efficiency. Long exposure or higher concentration of spermidine was not helpful in enhancing transformation. The data clearly revealed that spermidine at 100 µM concentration used as pre-conditioner increased the recovery of transformants, however with lower or higher concentration (50 to 200 pM) it got significantly reduced (7.20 and 4.80, respectively). It is also necessary to understand the possible role of host polyamines influencing *Agrobacterium* process leading to transformation (Kumaria and Rajam, 2003) and it has been suggested that the optimum cellular polyamine level in the host plant through the modulation of polyamine metabolism by using exogenous polyamine may be helpful in enhancing transformation frequency. The supplementation of spermidine in the selection medium has led to the enhancement of transformation frequency in wheat. Immature embryo-derived calli of spring wheat (*Triticum aestivum* L.) *cv.* Veery 5 were transformed using *A. tumefaciens*. Regeneration media supplemented with 0.1 mM spermidine improved the recovery of transformants from pHK21/LBA4404-infected calli from 7 to 24.2 %, resulting in an increase in the overall transformation frequency from 1.2 % to 3.9 %. The results suggested that two important factors that could lead to an improved transformation frequency of cereals (Khanna and Daggard, 2003). Higher transformation could be achieved with spermidine (Kumar and Rajam, 2006) in long term callus culture in rice (Bajaj and Rajam, 1996). Spermidine (1 mM) enhanced the *vir* gene induction when *Agrobacterium* cells were treated prior to acetosyringone addition (Kumar and Rajam, 2005). *Agrobacterium* mediated genetic transformation

Table 2. Control of *Agro*-infection using antibiotic washing

Antibiotics	Concentration (mg L^{-1})	*Agrobacterium* colony		
		After 24 hours	After 48 hours	After 72 hours
Cefotaxime	100	-	++	+++
	500	-	-	+
Streptomycin	100	-	-	++
	500	-	-	+
Cefotaxime + streptomycin	250 cefotaxime + 250 streptomycin	-	-	+
	300 cefotaxime + 500 streptomycin	-	-	-

- no control, + slight control, ++, moderate control, +++

and the regeneration of transgenic plants were achieved in *Hevea brasiliensis.* Immature anther-derived calli were used to develop transgenic plants. Somatic embryos were then regenerated from these transgenic calli on MS medium containing 2.0 mg L^{-1} spermine and 0.1 mg L^{-1} abscisic acid. Mature embryos were germinated and developed into plantlets on MS medium supplemented with 0.2 mg L^{-1} gibberellic acid, 0.2 mg L^{-1} kinetin (KIN) and 0.1 mg L^{-1} indole-3-acetic acid. A transformation frequency of 4 *%* was achieved (Jayashree *et al.,* 2003).

Control of *Agro*-infection using antibiotic: The best control of Agro-infection (8.80 %) was observed (Table 3) when the cocultivated explants were washed by MS salt solution supplemented with 100 mg L^{-1} cefotaxime. When the concentrations of cefotaxime were increased to 300 or 500 mg L^{-1}, 100 % removal of *Agrobacterium* was observed but it also caused severe mortality to smaller and younger explant. Lin *et al.* (1995) used 10 mg L^{-1} carbenicillin or 5 mg L^{-1} cefotaxime in suspension cultures for complete inhibition of *Agrobacterium*. However, higher concentrations of carbenicillin or cefotaxime, 250-500 mg L^{-1}, has widely been used in plant tissue culture, *e.g., Arabidopsis thaliana* (Akama *et al.,* 1992) and *C. papaya* (Fitch *et al.,* 1993, Cabrera- Ponce *et al.,* 1996, Cheng *et al.,* 1996 and Yang *et al.,* 1996). For *Agrobacterium* mediated gene transfer in papaya, carbenicillin (Fitch *et al.,* 1990, Fitch *et al.,* 1993, Yang *et al.,* 1996, Cheng *et al.,* 1996 and Tsong-Ann *et al.,* 2001) and cefotaxime (Fitch *et al.,* 1993 and Cabrera-Ponce *et al.,* 1996) were often added to the medium during plant regeneration to control the growth of *Agrobacterium.*

Molecular characterization of transformants

PCR, dot blot hybridisation and real-time PCR: Genomic DNA isolated from putative transformants were subjected to PCR using gene specific primers which amplified 341 bp amplicon of *cp* gene (**F**: 5'- GGG AAT TCC CGC AGC AAA TTG AC -3' and **R**: 5'- AGG GTA CCC TCT TGA TTC CAT AC -3') (Fig. 6). A total number of 8 plants were positive for *cp* gene after repeated PCR using *cp* specific primers. Transformants showed presence of *cp* gene.

Table 3. Effect of polyamines and their concentrations on different parameters of somatic embryogenesis

Polyamine	Concentration				
	0 µM	50 µM	75 µM	100 µM	Mean
	Explant callused (%)				
Spermidine	72.66	25.70	19.20	11.50	32.27
Putrescine	72.66	16.00	11.60	7.03	26.82
Spermine	72.66	20.00	14.30	10.60	29.39
Mean	72.66	20.57	15.03	9.71	
	Number of somatic embryos explant				
Spermidine	18.60	45.30	53.30	70.60	46.95
Putrescine	18.60	22.00	25.00	26.60	23.05
Spermine	18.60	27.00	35.30	40.60	30.38
Mean	18.60	31.43	37.87	45.93	
	Weight of embryonic clump (mg)				
Spermidine	328.00	463.00	600.00	899.00	572.50
Putrescine	328.00	363.00	380.00	400.00	367.75
Spermine	328.00	376.00	425.00	484.00	403.25
Mean	328.00	400.67	468.33	594.33	

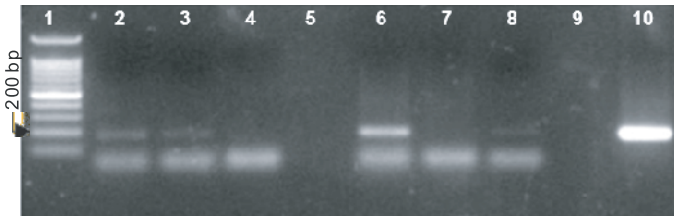

Fig. 6. Molecular confirmation of T1 transgenic lines by PCR using cp-specific primers. Lane 1: Marker, Lane 2: Negative Control, Lane 3, 4, 7, 9, II, 13 & 16 showing positive Tl transgenic lines, Lane 18: Positive Control

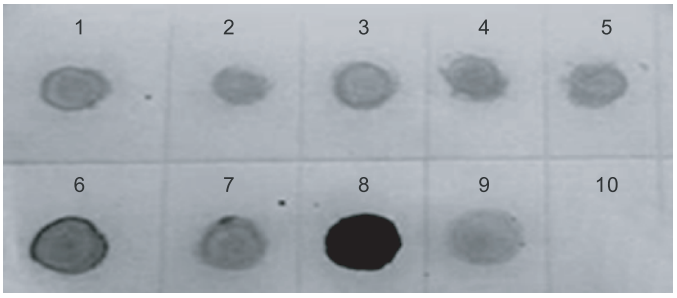

Fig. 7. Molecular confirmation of T1 transgenic lines by dot blot hybridization. Spot 1, 2, 3, 4, 5, 6, 7 & 9 showing positive transformants, Spot 8: Positive Control, Spot 10: Negative Control

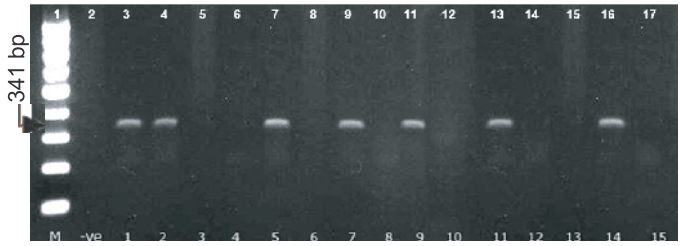

Fig. 8. Molecular confirmation of T1 transgenic lines by RT-PCR: Lane 1: Marker, Lane 2, 3 & 4: Positive T1 transgenic lines, Lane 5: Negative Control, Lane 6: Papaya wild type I, Lane 7: Papaya wild type 2, Lane 8: Pigeon pea as control & Lane 10: Positive Control (Plasmid)

These PCR confirmed plants were screened for *cp* gene integration through dot blot hybridisation (Fig. 7) and were further subjected to real-time PCR using gene specific primers (Fig. 8). Out of 8 plants confirmed through PCR and dot blot hybridisation, only three showed positive results in real-time PCR analysis. These stable transformants were acclimatized for evaluation.

Out of the 3 transgenic papaya lines (RNAi-PRSV-CP), two plant lines (#1, #2 and #3) showed expression of the transgene (RNAi-PRSV-CP). Out of the 2 papaya non-transgenic controls (wild type) leaf tissues, one is mildly infected by PRSV, although it may not be showing symptoms and the other one (#2) is healthy and free of PRSV. Additionally, pigeon pea was used as negative control and there was no amplification of CP (~200 nt). EIF4 primers were used as Internal Control for RT-PCR.

Acknowledgement

We appreciate the Director of ICAR-Central Institute for Subtropical Horticulture, Lucknow, India, for providing laboratory facilities and ICAR for financial support through the project "ICAR-Network Project on Transgenics in Crops".

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Received: December, 2022; Revised: January, 2023; Accepted: March, 2023