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Shoot tip transformation in papaya

Rajesh Pati
Shoot Tip Transformation in Papaya (Carica papaya L.)

R. Chandra1, M. Mishra1, R. Pati1, S. Agarwal1 and R.K. Jain2
1Biotechnology Laboratory, Central Institute for Subtropical Horticulture, Lucknow- 227 107, India
2Indian Agricultural Research Institute, New Delhi, India

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Abstract
In vitro regeneration and transformation protocol has been developed in papaya across the globe using somatic embryo as explant. Transgenic papaya has been developed from hypocotyl or immature zygote. The transformation efficiency remains very low through embryo mediated transformation due to lack of synchronized maturity and subsequently poor germination. We report here for the first time transformation of young, in vitro grown shoot tips of papaya with dual gene (cp+rep) construct through Agrobacterium.

INTRODUCTION
India is one of the leading papaya producing countries of the world contributing 25% of world's total production. Papaya cultivation is threatened world over by a deadly viral disease, Papaya ringspot virus (PRSV). This disease is transmitted by aphid with characteristics ring spot lesions on fruit and leaves which affects productivity and marketability. Papaya leaf curl virus (PaLCuV) is another viral disease that affects papaya productivity but it is mostly confined to North India. The details of this geminivirus were studied by Saxena et al. (1998). This disease was discovered in the papaya orchards of southern Taiwan in 2002. Infected papaya developed symptoms such as downward curling of leaves, twisted petioles, veinulation, and stunting. Diseased plants produced small and distorted fruits that tend to fall prematurely.

Efforts have been made to combat these diseases. Cultural practices cross protection and resistant breeding has not yielded promising results for PRSV. r-DNA technology has successfully produced papaya conferring resistance against PRSV in Hawaii (Fitch et al., 1993; Cai et al., 1999; Gonsalves, 1998). However, similar efforts have not been made in India. In order to deliver gene of interest in the plant genome, somatic embryos developed from immature zygotic embryo or hypocotyl segment (Fitch et al., 1993; Cai et al., 1999) has been the preferred explant world over. However, high recovery of transformants through embryogenesis mediated pathway remains low due to lack of synchronized maturity and poor germination of somatic embryos and finally poor rooting in embryos derived plantlets. We report here a method of Agrobacterium mediated shoot tip transformation of papaya cv. Pusa Delicious using dual gene (cp+rep genes) in pBINAR binary vector.

MATERIALS AND METHODS
Papaya seedlings were germinated in vitro from immature seeds of cultivar Pusa Delicious grown in the germplasm block of CISH, Lucknow on half strength Murashige and Skoog medium.

Dual genes construct (cp+rep) in pBINAR binary vector mobilized in Agrobacterium tumifaciens strain LBA 4404 developed at Indian Agricultural Research Institute, New Delhi, was utilized for transformation. It has npt II selection marker and CMV 3SS promoter. For activation of bacterial culture, single colony of Agrobacterium containing dual gene (cp and rep) was inoculated in 50 ml of LB liquid medium and left overnight at 28°C (OD 0.8 at 600 nm) in incubator shaker at 100 rpm. Overnight grown Agrobacterium culture was centrifuged (10,000 rpm) and pellets were dissolved in liquid MS medium supplemented with different concentration of spermidine (1, 2 and 3 μM), acetylosiringone (50, 100 and 150 μM) and kept for 3 h prior to infection. 0.5 cm long
Shoot tips were taken from 1 week old seedlings, meristems were excised and wounded with different methods, such as vortexing the shoot tip with carbaboranum, bombardment of tungsten from gene gun (Gene Pro-HE 2000) and a u n g h i n g the meristems tip with fine needle. The wounded tissues were inoculated on MS liquid medium fortified with acetosyringone for 30 min under agitated condition for infection. After infection, the explants were blotted dry on sterile filter paper and further inoculated on agarified MS medium (without hormone) containing acetosyringone (50, 100 and 150 μM) for co-cultivation under dark for different periods (24, 48 and 72 h).

After co-cultivation the shoots were washed with different antibiotics (cefotaxime 500 mg/L, carbenicillin 500 mg/L and ampicillin 500 mg/L) and transferred to regeneration medium (1/2 MS + BAP 0.2 mg/L + NAA 0.1 mg/L + glutamin 400 mg/L) containing cefotaxime 500 mg/L, carbenicillin 500 mg/L and ampicillin 500 mg/L for controlling growth of Agrobacterium. After one week shoot tips were transferred to selection medium having antibiotics (1/2 MS + 1.5 mg/L BAP, 0.2 mg/L NAA and 75 mg/L kanamycin). The shoots were selected up to 12 weeks in kanamycin. Initially they were exposed to 75 mg/L kanamycin for 4 weeks than survived shoots were further transferred to 100 mg/L for 4 weeks and finally the surviving shoot tips were shifted to 150 mg/L Kanamycin another for 4 weeks. Kanamycin selected shoot tips were elongated in regeneration medium for another 4 weeks. Finally elongated shoot tips (1-2 cm) were further inoculated on kanamycin free MS medium supplemented with different concentrations of IBA (1, 2 and 3 mg/L) and activated charcoal (500 mg/L) for roots induction.

Molecular Analysis

Total genomic DNA kanamycin resistant plantlets were isolated (Qiagen, Plant genomic DNA Isolation kit). Approximately 250 ng of genomic DNA was used as template for PCR. In order to show the integration of T-DNA and Ti-DNA of Ti plasmid in the transformed plantlets, one region (npt II) was amplified with a pair of gene specific primers. npt II specific gene was detected by PCR with primers specific to npt II (~800 kb), such as forward primer (5'-TCTCCACTCTGCTCTCGCC-3') and reverse primer (5'-AGGCATGGGCGATG-3'). The amplification conditions for npt II, initial duration was at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 54°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. PCR products were analyzed by electrophoretic separation on 1% agarose gel (w/v) in 1X TBE buffer and stained with ethidium bromide.

RESULTS AND DISCUSSION

One week old wounded seedlings explant is prerequisite for Agrobacterium mediated transformation. The data (Fig. 1) clearly indicated that shoot tips vortexed with carbaborandum (for 1 min) gave higher transformation efficiency (11.20%) compared to other winding methods. Zhu et al. (2006) described an Agrobacterium-mediated plant transformation by carborandum wounding in papaya embryogenic calli. While in another experiment in C. papaya, Agrobacterium tumefaciens has been used in conjunction with carborandum in a liquid phase which improved transformation efficiency (Yeh et al., 1998). This method gave the higher transformation efficiency which on average produced at least one positive plant during PCR analysis and southern blot analysis from 10-20% of callus cluster co-cultivated with Agrobacterium tumefaciens. The density of bacteria used for infection is adjusted either by monitoring the time of overnight cultures during incubation or by diluting the overnight bacterial cultures (Yeh et al., 1998). The bacterial density and inoculation time is directly correlated to each other. The optimum bacterial culture densities varied in different fruit crops. The explant after infection (30 min) with Agrobacterium is blotted dry on sterile filter papers and then transferred to agarified regeneration medium for co-cultivation at different hours (24, 48 and 72 h). The data (Fig. 2) clearly revealed that tissues co-cultivated for 72 h in dark with Agrobacterium, gave higher putative transformati ons up to 8.80%. In citrus, explants after agro infection were
co-cultivated for 3 days (Cervera et al., 1998; Perez et al., 1998; Han et al., 1999a). C. sinuosus and C. reticulata (3 days) and sweet orange (2 days). In grapes, co-cultivation period of 1-5 days have been reported. In V. vinifera, co-cultivation periods of 1 day (Barbaut et al., 1990), 2 days (Harst et al., 2000), 3 days (Nakano et al., 1994) and 5 days (Hoshino et al., 2000) have been reported. The data (Fig. 3) clearly shows that cefotaxime 500 mg/L is very effective in control of Agrobacterium from tissue surface and gave higher putative transformants (8.80%) compared to other antibiotics (carbenicillin and augmentin). Lin et al. (1995) used 10 mg/L carbenicillin or 5 mg/L cefotaxime in suspension cultures for complete inhibition of Agrobacterium. However, higher concentrations of carbenicillin or cefotaxime, 250-500 mg/mL, have been widely 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; Yang et al., 1996). For Agrobacterium mediated gene transfer in papaya, carbenicillin (Fitch et al., 1990, 1993; Yang et al., 1996; Cheng et al., 1996; Tsong-Ann et al., 2001) and cefotaxime (Fitch et al., 1993) were often added to the medium during plant regeneration to control the growth of Agrobacterium. The usage of phenolics (acetosyringone) may raise 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. It is evident from our results that (Fig. 4) acetosyringone (100 μM) added during co-cultivation period enhanced putative transformants (11.20%). Fitch et al. (1993) also suggested that overnight grown bacterial cultures induced for 4 h with 50 μM acetosyringone prior to co-cultivation improved the transformation efficiency. In tamarillo, addition of acetosyringone to bacterial culture and co-cultivation medium increased transformation efficiency (35%) significantly (Atkinson and Gardner, 1993). Our results showed (Fig. 5) that polyamine such as spermidine at 1.0 mM concentration increased the transformation efficiency when used during co-cultivation process. The spermidine (1 mM) enhances the vir gene induction when Agrobacterium cells were treated prior to acetosyringone addition (Kumar, 2003; Kumar and Rajam, 2006). It has been suggested that the optimum cellular polyamine level in the host plant through the modulation of polyamine metabolism either 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 by Agrobacterium (Khanna and Daggard, 2005). Transformed shoots (1-2 cm long) subjected to IBA (2.0 mg/L) in the absence of kanamycin produced more roots (3 roots/culture). Such plants were easily acclimatized on sterilized coconut husk supplemented with MS salt mixture.

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Literature Cited


Cui, W.Q., Gonsalves, C., Tenent, P., Fermin, G., Jr. Souza, M., Sarindu, N., Jam, F.J.,


Saxena, S., Hallan, V., Singh, B.P. and Sane, P.V. 1998. Leaf curl disease of Carica papaya from India may be caused by a bipartite geminivirus. Plant Dis. 82(1):126.


**Figures**

![Diagram](image)

*Fig. 1. Effect of wounding method on transformation efficiency.*
Fig. 2. Effect of co-cultivation periods on transformation efficiency.

Fig. 3. Effect of antibiotics on control of growth of *Agrobacterium*. 
Fig. 4. Effect of acetosyringone on transformation efficiency.

Fig. 5. Effect of spermidine on transformation efficiency.