Towards Development of Transgenic Papaya (*Carica papaya* L.)

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Abstract

Papaya suffers huge losses due to prevalence of *Papaya ring spot virus* and *Papaya ringspot virus* diseases. Efforts have been made to transfer papaya with Cp gene across the globe. However, transgenic research in India has just begun. Present paper describes the genetic transformation work being carried out in India using microprojectile and *Agrobacterium* mediated.

INTRODUCTION

Papaya (*Carica papaya* L.) is a major tropical fruit grown commercially in India, Brazil, Mexico, Australia, Hawaii, Thailand, South Africa, Philippines, Indonesia and Taiwan. India is the largest producer of papaya contributing 25% of total world production. Papaya cultivation is severely hampered by prevalence of papaya ring spot virus and papaya leaf curl virus. Intergeneric tolerance breeding for virus resistance has proved to be futile in papaya. PRSV is wide spread across the country and is a major limiting factor in papaya production. *Papaya leaf curl virus* (PaLCuV) disease causes severe economic losses in North India. The disease is transmitted by vector whitefly (*Bemisia tabaci*) and characterized by severe curling, downward cupping and crinkling of leaves. Early infection leads to severe reduction in yield and management of disease is urgently required.

Recently DNA recombinant technology has opened up new vistas for development of virus resistant papaya. Genetic engineering for virus resistance has been found effective whereby transgenic plants expressing virus genome sequence resist attack by corresponding viruses. Coat protein mediated resistance (CPMR) has shown promise to develop PRSV resistant papaya in Hawaii (Fitch et al., 1992, 1993; Cai et al., 1999; Gonsalves, 1998). However, similar success story is still awaited in India. The Hawai transgenic papaya variety does not show resistance against PRSV in India because of difference in PRSV strains. Many groups are engaged in transgenic research in papaya viz., Central Institute of Subtropical Horticulture, Lucknow in collaboration with IARI, New Delhi is engaged in development of transgenic papaya conferring resistance to papaya ring spot virus and papaya leaf curl virus under networking mode. Indian Institute of Horticultural Research, Bangalore and Tamil Nadu Agriculture University, Coimbatore are also pursuing research on transgenic papaya. This paper deals with success made so far at this institute. Somatic embryos of papaya cv. Pusa Delicious were transformed using both the methods viz.; micro projectile and *Agrobacterium*. The paper describes initial success in genetic transformation of Indian papaya variety with dual gene construct.

MATERIALS AND METHODS

Under ICAR Networking Project on Transgenics in Crops, a collaborative project between IARI, New Delhi and CISH, Lucknow, efforts are being made to develop transgenic papaya conferring resistance against both *Papaya ring spot virus* and *Papaya leaf curl virus*. Gene construct (dual gene construct having *cp* gene for PRSV resistance and *rep* gene for PaLCuV resistance) has been developed indigenously by Dr. R.K. Jain, IARI, New Delhi and transformation is being carried out at CISH, Lucknow. Somatic embryogenesis and regeneration from immature zygotic embryo of papaya has been developed by Fitch and Manshardt (1990) and papaya cv. Pusa Delicious by Mishra et al.

(2007). The present paper describes transformation protocols followed in our lab using *Agrobacterium* and microprojectile transformation. Somatic embryos of papaya cv. Pusa Delicious were developed as per the protocol described by Mishra et al. (2007). Young (12 weeks old) embryogenic tissues, including a slimy yellow brown substance squashed or spread over a filter paper (Whattman) placed on the induction medium (½ MS + 10 mg/L 2,4-D + 400 mg/L L-glutamine + 8 gm/L agar) for three weeks. Numerous somatic embryos regenerated from this tissues forming loose layer of somatic embryos. Hundred somatic embryos of similar size and age were used for microprojectile and *Agrobacterium* mediated transformation. *Cp* gene in antisense orientation mobilized in pBI121 binary vector driven by 35S promoter and *nos* terminator having *uidA* as reporter gene and *npt-II* as selection gene was utilized for the purpose of microprojectile transformation. Dual gene construct having *rep* + *cp* gene in pBINAR binary vector was utilized for *Agrobacterium* mediated transformation. For microprojectile transformation, *E. coli* containing gene construct was grown overnight in LB (10 g/L trypton, 5 g/L yeast extract and 10 g/L NaCl) medium containing 50 mg/L kanamycin. Plasmid was isolated using alakaline lysis method (Maniatis et al., 1982). Microprojectile suspension containing 50 µl tungsten in an eppendorf tube, having 50 µl plasmid DNA (1 µg/µl), 50 µl 2.5 M $CaCl_2$ and 20 µl filter sterilized 0.1 M spermidine was prepared. Gene gun (Gene Pro He-2000) kept in laminar airflow was sterilized under UV overnight. The helium gas pressure was kept at 12 kg/cm^2 and tissue was kept at 9 cm distance from the micro holder on sliding tray. Four µl plasmid suspension was loaded to the micro-holder. The microprojectile suspension was shot at 600 Hg initial pressure. Each plate bombarded at least twice. Bombarded cultures were transferred on the MS medium devoid of any hormone and kept at 27°C in the dark for a week. In order to bring maturity in transformed somatic embryos, all the embryos were transferred to MS medium containing osmoticum such as polyethylene glycol (15,30,45 mg/L), Mannitol (1,2,3%) and Sorbitol (1,2,3%) along with 75 mg/L kanamycin, 0.5 mg/L BAP, 20% sucrose and 0.8% agar. Media was autoclaved at 121°C on 20 psi for 20 min. pH of the media was kept at 5.8. The cultures were incubated under 16/8 hour light (4000 lux) and dark cycle at 25±2ºC with 50% RH. The data pertaining to maturation, conversion and germination was recorded periodically.

Dual genes construct (*cp+rep*) in pBINAR binary vector mobiliszed in *Agrobacterium tumifaciens* strain LBA 4404 was utilized for transformation. It has npt II selection marker and CMV 35S 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), acetosyringone (50, 100 and 150 µM) and kept for three hours prior to infection. 0.5 cm long shoot tips from 1, 2, 3 and 4 weeks old seedlings were excised and meristem was wounded with different methods such as vortexing the shoot tip with carborandum, bombardment of tungsten from gene gun (Gene Pro-HE 2000) and notching the meristems tip with fine needle. The wounded tissues were inoculated on MS liquid medium fortified with acetosyringone for different duration (15, 30 and 45 min) under agitated condition for infection. After infection , the explants were blotted dry on sterile filter paper and further inoculated on agrified MS medium (without hormone) acetosyringone (50, 100 and 150 µM) containing for co-cultivation under dark for different periods (24, 48 and 72 h).

After co-cultivation the shoot tips were washed with different antibiotics (cefotaxime 500 mg/L, carbenicillin 500 mg/L and augmentin 500 mg/L) and transferred to regeneration medium (½ 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 augmantin 500 mg/L for controlling growth of *Agrobacterium*. After one week shoot tips were shifted to selection medium having antibiotics (½ 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 four weeks than survived shoots were further

transferred to 100 mg/L for 4 weeks and finally they were shifted to 150 mg/L kanamycin. Kanamycin selected 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) + activated charcoal (500 mg/L) for roots induction.

Molecular Analysis

Total genomic DNA form 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_L-DNA and T_R-DNA of Ti plasmid in the transformed plantlets, one regions (*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'-TCTCACCTTGCTCCTGCC-3') and reverse primer (5'-AGGCGATAGAAGGCGATG-3'). The amplification conditions for *npt-II* was initial duration 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 analysed by electrophoretic separation on 1% agarose gel (w/v) in 1X TBE buffer and staining with ethidium bromide.

RESULTS AND DISCUSSION

Microprojectile Transformation

Gene construct- the *GUS* gene fused with CP in antisence orientation under the influence of 35S promoter and NOS terminator was used for bombardment. Plasmid DNA was isolated from *E. coli* cells as per the protocol of Maniatis et al. (1982) followed by coating it on tungsten particles of size 0.6-1.0 µ after washing the tungsten with ethanol and double distilled water at a concentration of 1 µg/µl with the help of 2.5 M $CaCl_2$ and 0.1 M spermidine. 4 µl of DNA microprojectile suspension was delivered per bombardment (Genepro-2000He) at 12 kg/cm^2 pressure and the explants were kept at 9 cm distance from tray holder. Micro-holder containing microprojectile suspension was screwed at the upper portion of the gene gun; the sliding tray was adjusted at 9 cm distance from the microholder in the slot of target-shelf, where the targeted tissues were placed. Most of the plates were bombarded twice. Cultures were kept in the dark for 10-12 days and transferred to fresh induction medium for three weeks. Sequentially, cultures were transferred to regeneration medium (½ MS with 0.5 mg/L BAP and 0.1 mg/L NAA + 400 mg/L glutamin) supplemented with 75 mg/L kanamycin for four weeks. The kanamycin was elevated for 100 mg/L for 4 weeks and 150 mg/L kanamycin for another 4 weeks. Explants were selected for a total of 12 weeks to obtain putative transformants. Transformed embryos were easily matured and germinated with the help of polyethylene glycol at 45 mg/L (Fig. 1). Other osmoticum such as mannitol and sorbitol did not influenced maturation significantly. Over exposure of kanamycin leads to slow in vitro growth of plant and it also hampers root formation, therefore the transformed plants were pulsed with 500 ppm IBA for 10 minutes in dark and shifted to ½ MS medium fortified with IBA (4 mg/L) prior to shifting in autoclaved.

Transient GUS assay (Jefferson, 1987) was performed on bombarded tissue after 10 days of bombardment. Color development was recorded after 12 h of incubation at 37°C. Twelve week following particle bombardment, segments of putative transforments that survived on selective media were assayed for stable GUS expression. Bombarded embryos were picked randomly, the blue foci were recorded under a dissecting microscope. PCR was performed on genomic DNA extracted from putative transformants, which survived kanamycin selection for three months, for *npt-II*, *GUS* and *CP* sequences. The amplicon size for *npt-II* was of 464 bp, *GUS* was of 611 bp and *CP* was of 861 bp.

***Agrobacterium* Mediated Transformation**

Age of explant plays an important role in transformation efficiency. Our results

clearly indicated that shoot tips excised from one week old seedling gave higher (8.8%) transformation efficiency compared to older explants. The results are in accordance with reports of other crops such as grape where higher transformation efficiency was observed was observed in young shoot apex (Baribault et al., 1990). In papayas, explants such as leaf discs, stem segments (Pang and Sandford, 1988), petioles (Pang and Sandford, 1988; Yang et al., 1996) cell suspensions (Ying et al., 2000) and embryonic tissue from immature zygotic embryos (Yeh et al., 1998; Chen et al., 2001) have been reported for transformation studies. However, none of the report suggests shoot tip transformation in papaya. Wounding is prerequisite for *Agrobacterium* mediated transformation. Shoot tips vortexed with carborandum (for 1 min) gave higher transformation efficiency (11.20%) compared to other wounding 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 carborundum 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 bacterium used for infection is adjusted either by monitoring the time of overnight cultures during incubation or by diluting the over night bacterial cultures. The bacterial density and inoculation time is directly correlated to each other. The optimum bacterial culture densities varied in different fruit crops. Higher transformation efficiency (8.4%) was observed in papaya by us, when the shoot tips were infected for 30 min with *Agrobacterium*. In strawberry, overnight culture (OD_{600} nm at 0.8) was found optimum (James et al., 1990) to infect explants for 20-30 min. while in case of leaf disks (Mansouri et al., 1996) 20 min infection of the explants was found optimum. In apple, cultivar Royal Gala, 4-6 min infection time was required. In almond, Archilletti et al. (1995) reported that shoot tips infected for 30 min in (0.6 OD_{600}) with *Agrobacterium* culture gave higher transformation efficiency. The co-cultivation of explants with *Agrobacterium* is necessary to allow the bacterial cell to infect explant cells. 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 for different hours (24, 48 and 72 h). The tissues co-cultivated for 72 h in dark with *Agrobacterium*, gave higher transformation efficiency 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., 1999), *C. sinensis* and *C. reticulate* (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 (Baribault et al., 1990), 2 days (Harst et al., 2000), 3 days (Nakano et al., 1994) and 5 days (Hoshino et al., 2000) have been found optimum. cefotaxime 500 mg/L is very effective in control of *Agrobacterium* from tissue surface and gave higher transformation efficiency (8.80%) compared to other antibiotics. 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/L, 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; Cabrera-Ponce et al., 1996) are 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 that acetosyringone (100 µM) added during co-cultivation period enhanced transformation efficiency (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). 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 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, 2003). Transformed shoots (1-2 cm long) subjected to IBA (2.0 mg/L) in the absence of kanamycin produced more roots (3.0 roots/culture). Such plants were easily acclimatized on sterilized coconut husk supplemented with MS Salt mixture. Presence of kanamycin in the medium prohibits in vitro root formation and subsequent development.

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