Efficient transformation of papaya by coat protein gene of papaya ringspot virus mediated by Agrobacterium following liquid-phase wounding of embryogenic tissues with caborundum

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Summary. Generation of transgenic papaya (Carica papaya L.) has been hampered by the low rates of transformation achieved by conventional Agrobacterium infection or microprojectile bombardment. We describe an efficient Agrobacterium-mediated transformation method based on wounding of cultured embryogenic tissues with carborundum in liquid phase. Embryogenic tissues were obtained from cultured immature zygotic embryos collected 75-90 days after pollination. The expressible coat protein (CP) gene of a Taiwan strain of papaya ringspot virus (PRSV) was constructed in a Ti binary vector pBGCP, which contained the NPT-II gene as a selection marker. The embryogenic tissues were vortexed with 600 mesh carborundum in sterile distilled water for 1 min before treating with the disarmed A. tumefaciens containing the pBGCP. Transformed cells were cultured on kanamycin-free medium containing 2,4-D and carbenicillin for 2-3 weeks and then on the kanamycin medium for 3-4 months. The developed somatic embryos were transferred to the medium containing NAA, BA and kanamycin and subsequently regenerated into normal-appearing plants. Presence of the PRSV CP gene in the putative transgenic lines was detected by PCR and the expression of the CP was verified by Western blotting. The transgene was nuclearly inherited as revealed by segregation analysis in the backcrossed R1 progeny. From five independent experiments, the average successful rate of transformation was 15.9% of the zygotic embryos treated (52 transgenic somatic embryo clusters out of 327 zygotic embryos treated), about 10-100 times higher than the available methods previously reported. Thus, wounding highly regenerable differentiating tissues by carborundum vortexing provides a simple and efficient way for papaya transformation mediated by Agrobacterium.

Key words: Agrobacterium vector, Papaya ringspot virus, Coat protein gene

Introduction

Introduction of foreign genes into plants has been achieved by several DNA delivery methods. Agrobacterium-mediated transformation is the method most extensively employed (Klee et al. 1987). The other transformation approaches, such as electroporation (Formmm et al. 1986) or direct DNA uptake (Krens et al. 1982) performed on protoplasts, and delivery by microprojectile bombardment (Klein et al. 1987), can circumvent the host range limitation of Agrobacterium. However, these methods generally yield low frequencies of transformation.

Regeneration of papaya plants has been reported from protoplast (Chen and Chen 1992), cotyledon (Litz et al. 1983), petiole (DeBryjine et al. 1974), hypocotyl (Yie and Liaw 1977), root (Chen et al. 1987), anther (Tsay and Su 1985), ovule (Litz and Conover 1982) and immature embryo (Fitch and Manshardt 1990) cultures. Pang and Sanford (1988) were able to transform leaf disks, stems and petioles of Carica papaya with oncopositive Agrobacterium, but attempts to regenerate the transformed cells into plantlets were not successful. Transgenic papayas expressing the coat protein (CP) of papaya ringspot virus (PRSV) and the bacterial GUS gene have been obtained via microprojectile bombardment (Fitch et al. 1990) or Agrobacterium-mediated transformation (Fitch et al. 1993). In these two reports, embryogenic tissues were used as explants and transformation was achieved at a low frequency, 0.42% for particle bombardment and 0.6% for Agrobacterium-mediated transformation (Fitch et al. 1990, 1993). Recently, the bacterial GUS gene has been delivered into papaya by Agrobacterium-mediated transformation using papaya petioles as explants (Yang et al. 1996). However, the lengthy regeneration process (10-11 months after transformation) and the high frequency of abnormalities in the regenerated plants limit the application of this method (Yang et al. 1996).
High rates of regeneration were obtained when embryogenic tissues derived from immature zygotic embryos of papaya were used as explants (Fitch and Manshardt 1990). In this investigation embryogenic callus tissues derived from the immature zygotic embryos were treated with Agrobacterium after mechanical wounding by carborundum in liquid phase. After co-cultivation and selection, putative transgenic embryos were regenerated into normal-appearing plants and the expression of the foreign gene in the transgenic papaya was verified by Western blotting and PCR analysis. The transgene was nuclearily inherited as revealed by segregation analysis in backcrossed R1 progeny. Our results indicate that wounding with carborundum prior to Agrobacterium treatment of embryogenic tissues is a reliable and efficient method for papaya transformation.

Materials and Methods

Plant material. Immature zygotic embryos were obtained from papaya (Carica papaya L. var. Tainung No.2) fruits 75-90 days after pollination. Embryos were cultured on the induction medium which consisted of half-strength MS (Murashige and Skoog 1962) salts, 50 mg l-1 myo-inositol, full-strength MS vitamins, 400 mg l-1 glutamine, 6% sucrose, 2 mg l-1 2,4-D and 1% Difco Bactoagar, pH 5.8, as described by Fitch et al. (1990). Apical domes of the excised zygotic embryos became enlarged 2 wk later and 10-20 somatic embryos budded from the enlarged apical domes were obtained 4-5 wk after culture. Embryogenic tissues derived from immature zygotic embryos, 3-4 wk after culture but before the maturation of the developing somatic embryos, were used for subsequent transformation.

Construction of PRSV CP gene. Plasmid pTMD9 containing most of the Nib gene, the complete CP gene and the entire 3' noncoding region of the genome of a severe mosaic-type strain PRSV YK from Taiwan was described previously (Wang et al. 1994). Plasmid pH121 containing the NPT II and the GUS gene was purchased from Clontech (Palo Alto, California). A NcoI site was introduced into pH121 and pTMD9 by in vitro mutagenesis at the site immediately before the GUS and CP reading frames. The NcoISacI fragment of the mutagenized pTMD9 was introduced into the mutagenized pH121, resulting in a substitution of the GUS reading frame by the PRSV CP reading frame and its 3' noncoding region. Because the construction of the NcoI site was in frame with the reading frame of the CP gene, two amino acids of methionine and alanine were added in front of the putative N-terminal end of the CP gene. The plasmid containing the CP gene was designated as pBGCP. A different construction of the CP gene in pBGCP was generated by substituting the GUS leader sequence with the 5' GUS leader (nt 1-347) of PRSV HA strain, which contained the complete viral leader sequence and the 87 amino acids encoding the CP reading frame of the CP gene, two amino acids of methionine and alanine frames. The construction of pBGCP was performed with 1 min melting at 94°C, 2 min annealing at 55°C and 3 min extension at 72°C for 30 cycles. PCR products were analyzed by electrophoresis in 1% agarose gel.

DNA extraction and polymerase chain reaction. Total DNA was extracted from putative transgenic plants or non-transformed papaya following the procedure described by Mettler (1987). One µg of Rnase A-treated DNA was used for PCR as template. The upstream primer MO928, 5'TACCGTTCTGAATGAGAAGC 3', and the downstream primer MO1008, 5'GTGCATGTCTCTGGTAC 3', reflecting nucleotide positions 9277-9296 and 10077-10096 of the PRSV YK RNA sequence (Wang et al. 1994), respectively, were used for amplification. The PCR was performed with 1 min melting at 94°C, 2 min annealing at 55°C and 3 min synthesis at 72°C for 30 cycles. PCR products were analyzed by electrophoresis in 1% agarose gel.

Western blotting analysis. The expression of the CP was analyzed by Western blotting using anti-PRSV serum (Yeh et al. 1984) as the primary antibody and goat anti-rabbit IgG conjugated with alkaline phosphatase as the secondary antibody. Leaves or calli of papaya were homogenized in 4 volumes (w/v) of dissociation buffer (62.5 mM Tris.HCl, pH 6.8, 2% SDS, 3% 2-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue). The extracts were heated at 95°C for 5 min and centrifuged at 8000 g for 5 min for removing plant debris. Total proteins of each sample (15 µl) were loaded on 12% gel for SDS-PAGE (Laemmli 1970), and subsequently transferred to PVDF membranes (Millipore Co.). The immunostaining procedures were performed as described by User's manual of GUS Gene Fusion System (Clontech).

Segregation analysis of the transgene. R0 plants of putative transgenic lines were micropropagated by tissue culture (Yang et al. 1996) and challenge inoculated with PRSV YK. Inoculation was performed by rubbing the two youngest leaves with a 1/20 dilution of leaf extract of PRSV-infected Cucumis metuliferus in 0.01 M potassium phosphate buffer, pH 7.0. These plants were monitored by symptom development and ELISA assays using the polyclonal antibody to PRSV (Yeh et al. 1984). The putative transgenic lines GCP16-0, GCP17-0 and GCP17-1 were highly resistant to PRSV infection. Plants of the line GCP17-1 were grown in green house and R1 plants were obtained by backcrossing with non-transformed plants of the parental cultivar Sunrise. The inheritance of the transgene in the R1 progeny of GCP17-1 was analyzed by PCR detection with primers specific to the CP gene and by the resistance to PRSV infection. Genomic DNA from leaves of R1 plants was used for PCR.