Plant Cell Reports (1996) 16:127–132

Plant Cell Reports

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

Ying-Huey Cheng [1], Jiu-Sherng Yang [2], and Shyi-Dong Yeh [1]

[1] Department of Plant Pathology, National Chung-Hsing University, Taichung City, Taiwan, R. O. C.
[2] Department of Botany, National Chung-Hsing University, Taichung City, Taiwan, R. O. C.

Received 7 February 1996/Revised version received 11 April 1996 – Communicated by I. K. Vasil

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 R_1 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

Correspondence to: S.-D. Yeh

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 (Formm 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 (DeBryijne 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 nuclearly inherited as revealed by segregation analysis in backcrossed R_1 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 embyos 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 pBI121 containing the NPT II and the GUS gene was purchased from Clontech (Palo Alto, California). A *Nco*I site was introduced into pBI121 and pTMD9 by *in vitro* mutagenesis at the site immediately before the GUS and CP reading frames. The *Nco*I/*Sac*I fragment of the mutagenized pTMD9 was introduced into the mutagenized pBI121, 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 *Nco*I 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 pB5'CP was generated by substituting the GUS leader sequence with the 5' cDNA (nt 1-347) of PRSV HA strain, which contained the complete viral leader sequence and the 87 amino acids of the N-terminal part of the P1 protein of PRSV (Yeh et al. 1992). The third plasmid pBIN was generated from deletion of the GUS reading frame from pBI121. The omission of the CP reading frame in pBIN was used as a control for pBGCP.

The three constructed plasmids (pBGCP, pB5'CP and pBIN) were separately mobilized to *Agrobacterium tumefaciens* LBA4404 by the triparental mating method (Rogers et al. 1986). *A. tumefaciens* cultured in the LB medium containing 50 ppm kanamycin and 100 ppm streptomycin at 28℃ for 36 hr were used for plant transformation.

Plant transformation and regeneration. Centrifuge tubes (Corning) containing 30 ml of distilled water and 0.5 g of carborundum (600 mesh) were sterilized by autoclaving. About 40 pieces of embryogenic calli (3-4 g) derived from immature zygotic embryos were transferred to a sterilized centrifuged tube and vortexed with Vortex Genie-2 (Scientific Industries, Inc., Bohemia, NY) at speed 7 for 1 min. The carborundum-wounded calli were submerged in the culture of *A. tumefaciens* for 5 min. After blotting away the excess bacterial culture, the calli were transferred to the induction medium (Fitch et al. 1990) and co-cultivated for two days. After co-cultivation, they were cultured on the induction medium containing 500 ppm carbenicillin to inhibit the growth of *A. tumefaciens*. Three weeks later, the calli were transferred to the same medium containing 100 ppm kanamycin and 500 ppm carbenicillin for selection of transformed tissues.

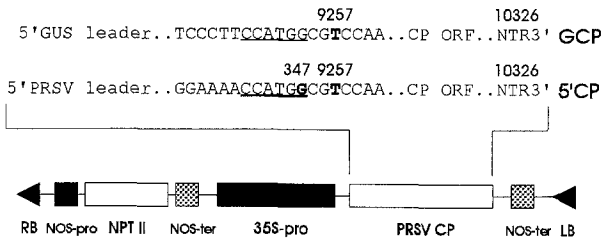

Fig.1. Construction of the PRSV CP gene in a Ti-binary vector. Numbers superscribed above the bold letters indicate the nucleotide positions of PRSV genome. The CP gene was constructed with a GUS leader or a homologous virus leader and transcribed by a CaMV 35S promoter and a NOS terminator. Selection marker, neomycin phosphotransferase gene (NPT II) was included in the vector and transcribed by the NOS promoter and terminator.

Somatic embryos derived from the selected transformed cells were transferred to medium containing 0.2 ppm BA and 0.02 ppm NAA for germination (Yang and Ye 1992). After germination, shoots were excised and transferred to MS medium containing 0.5 ppm IBA for one week and subsequently transferred to vermiculite supplemented with 1/2 volume of MS basal medium for rooting.

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, $^{5'}$TACCGGTCTGAATGAGAAGC$^{3'}$, and the downstream primer MO1008, $^{5'}$GTGCATGTCTCTGTTGACAT$^{3'}$, 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℃, 2 min annealing at 55℃ and 3 min synthesis at 72 ℃ 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 3 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. R_0 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 R_1 plants were obtained by backcrossing with non-transformed plants of the parental cultivar Sunrise. The inheritance of the transgene in the R_1 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 R_1 plants was used for PCR

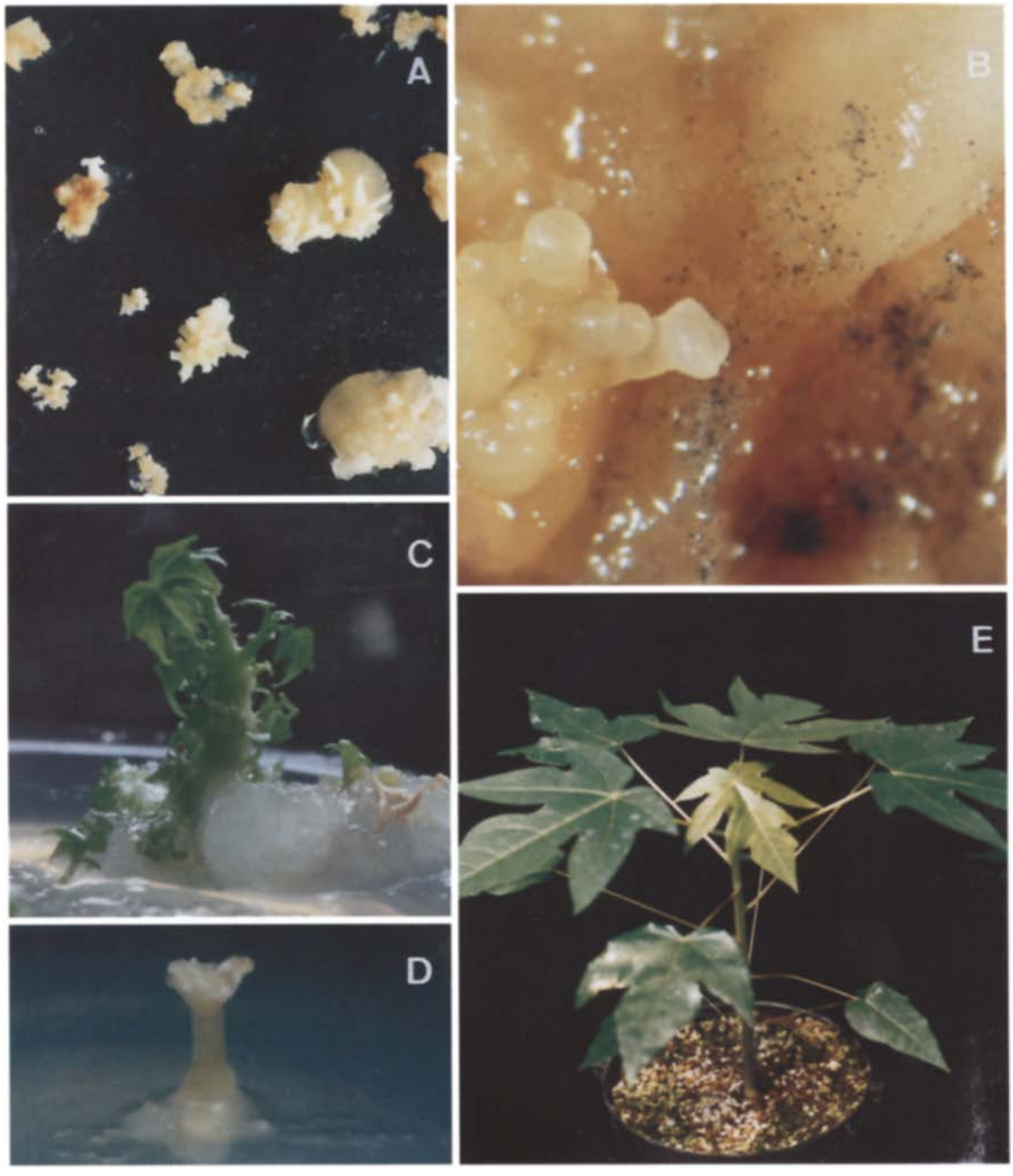

Fig. 2. Regeneration of the putative transgenic papaya by *Agrobacterium*-mediated transformation following vortexing of embryogenic tissues derived from immature zygotic embryos with carborundum in sterile water. Panel A & B, the putative transformed regions of the callus grew gradually to form somatic embryos, but the non-transformed regions turned brown, ceased growing, and resulted in distinct appearance of carborundum. Panel C, putative transgenic embryos germinated on medium containing kanamycin. Panel D, germinated non-transformed shoot turned pale and deteriorated on the selective medium. Panel E, a putative transgenic plant after rooting in vermiculite medium.

detection and evaluation for virus resistance was performed by mechanical inoculation with PRSV on seedlings with 3-5 leaves.

Results

Development of somatic embryos on the kanamycin medium.

The PRSV CP gene and the selective marker NPT II were constructed in pBGCP (Fig. 1) and were delivered into papaya cells by *Agrobacterium*-mediated transformation following wounding by carborundum-vortexing. In each experiment, about 75-107 clumps of embryogenic calli were wounded with carborundum prior to co-cultivation with *Agrobacterium*. Numerous somatic embryos started to develop from embryogenic calli 3-4 wk after co-cultivation on the induction medium containing carbenicillin. However, after they were transferred to the

Table 1. Frequency of *Agrobacterium*-mediated transformation of embryogenic tissues derived from immature zygotic embryos of papaya following vortexing with carborundum

Experiment	Gene construct	Numbers of embryogenic tissue clumps treated[a]	Numbers of transformed clusters[b]	Percentage (%)
1	GCP	35	4	11.4
	5'CP	40	1	2.5
2	GCP	29	21	72.4
	5'CP	49	0	0
3	AL4404[c]	19	0	0
	5'CP	38	6	15.8
4	AL4404	22	0	0
	5'CP	46	3	6.5
	121	39	4	10.3
5	AL4404	32	0	0
	5'CP	24	6	25.0
	BIN	9	1	11.1
	121	18	6	33.3
Total	AL4404	73	0	0
	Foreign genes	327	52	15.9

[a] Embryogenic tissues of one clump were derived from an immature zygotic embryo.

[b] The callus clusters survived and developed 2-3 months after culture on kamamycin selection medium were considered to have originated from transformed cells.

[c] *Agrobacterium* containing plasmid pAL4404 without foreign genes was used as negative control.

kanamycin selection medium, only putative transgenic embryogenic tissues continued to grow (Fig. 2A). Non-transformed embryogenic calli ceased growing on the selective medium and gradually turned brown or pale, resulting in the distinct appearance of the carborundum on the callus (Fig. 2B).

After culturing on the kanamycin medium for 2 to 3 months, embryogenic calli regenerated multiple somatic embryos (Table 1). Somatic embryos derived from the same embryogenic clump were considered to be from the same immature zygotic embryo. Transformation frequencies ranged from 0 to 72.4%, with an average of 15.9% as calculated from a total of 52 putative transgenic embryo clusters obtained from a total of 327 embryogenic calli treated.

Establishment of the putative transgenic lines

Putative transgenic embryos were germinated on the medium containing NAA, BA and kanamycin for 2-4 wk. After germination, the putative transformants grew rapidly and formed multiple shoots (Fig. 2C). Non-transformed embryos were not able to germinate and gradually deteriorated (Fig. 2D). Few pieces of embryogenic tissue turned green but mingled with white pale domains, apparently resulting from chimeric tissue which contained both transformed and non-transformed cells. These tissues required 2-4 wk additional time to develop and germinate. Shoots developed from an individual somatic embryo were sliced and subcultured on the same medium for proliferation. Multiple shoots of 1.0-1.5 cm were transferred to the IBA medium for rooting. The putative transgenic plants established in vermiculite medium were normal-appearing (Fig. 2E). After 2-3 wk hardening in the growth chambers, they were cultured in a temperature-controlled (23-28 ℃) green house for further investigation. A total of 63 plant lines were established from the putative transgenic embryos germinated. Among them, 15 lines were micropropagated by tissue culture and grown in greenhouse for further evaluation.

PCR detection

Total DNA isolated from putative transformants growing on the kanamycin medium were analyzed by PCR, using primers specific for the PRSV CP gene, to verify the presence of the gene in the papaya plants. A DNA fragment of 0.82 kb, corresponding to the expected span between the two primers, was amplified in all five putative transgenic lines GCP16-0, GCP16-1, GCP17-0, GCP17-6 and GCP18-1 (Fig. 3, lanes 4-8). This fragment was not present in non-transformants (Fig. 3, lanes 2, 3). The 0.82 kb DNA fragment was also amplified from all other 10 selected lines when they were detected by PCR.

Western blotting

Total proteins extracted from the selected transformants

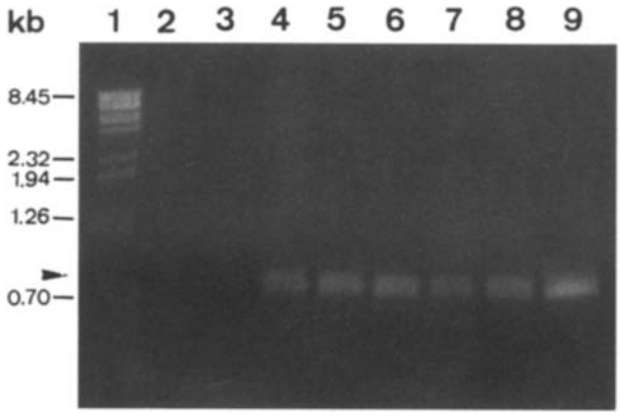

Fig.3. PCR detection of the PRSV CP gene in the putative transgenic papaya lines. The DNAs in lanes 4 to 8 were from lines GCP16-0, GCP16-1, GCP17-0, GCP17-6 and GCP18-1, respectively. The expected DNA fragment of 0.82 kb, indicated by an arrowhead, was amplified by the primers specific to the PRSV CP gene. Plasmid pBGCP containing PRSV CP gene was used as a positive control (lane 9). DNA from non-transformants did not show the specific band (lanes 2 & 3). DNA markers were in lane 1, their sizes are indicated on the left.

were analyzed by Western blotting to detect the expression of the PRSV CP. Protein extracts from calli and shoots of putative transformants or non-transformants were separated by SDS-PAGE and immunobloted with anti-PRSV serum. PRSV CP was detected in shoots of lines GCP16-1, GCP17-1, GCP17-6 and GCP18-1 (Fig. 4, lanes 3-6). In line GCP16-0, intact CP was detected in calli from substratum of multiple shoots (Fig. 4, lane 8) and in degraded form in shoots (Fig. 4, lane 7). In line 17-1, CP was detected in shoots as well as calli (Fig. 4, lanes 2 and 1). Protein corresponding to PRSV CP was not detected in the non-transformed controls (Fig. 4, lanes 9, 10 and 11). CP in calli of transgenic lines GCP17-2, GCP17-3, 5'CP18-2 and 5'CP18-4 was also detected (data not shown).

Segregation of the transgene in R_1 progeny

Among the 15 putative transgenic lines inoculated with PRSV YK, lines GCP 16-0, GCP 17-0 and GCP 17-1 were found highly resistant to PRSV infection. They showed no visible symptoms up to two months after inoculation, whereas the non-transformed controls showed severe mosaic and wilt symptoms within 12 days. The negative reactions in ELISA tests in these three lines indicated that the resistance was due to apparent inhibition of the virus replication. The other lines only showed slight to moderate resistance, as reflected in various degrees of symptom attenuation and in delay of symptom development. Line GCP 17-1 was selected for genetic analysis for the inheritance of the transgene.

A transgene is inheritable if it is integrated into the chromosome of a transgenic plant. The R_1 progeny of GCP17-1 backcrossed with cultivar Sunrise were analyzed by PCR detection and mechanical inoculation with PRSV for the segregation of the CP gene. From a total of 374 plants tested, severe mosaic and wilting symptoms were observed in 186 plants and the other 188 plants remained symptomless 3 wk after inoculation. By the PCR detection in 40 plants, a 0.82 kb DNA fragment of the CP gene was amplified in 21 plants which also conferred resistance to PRSV after mechanical inoculation (data not shown). The 1:1 segregation ratio in the R_1 progeny determined by PCR detection and virus resistance indicated that the CP gene was inserted in the chromosome of the transgenic papaya line 17-1 at a single locus.

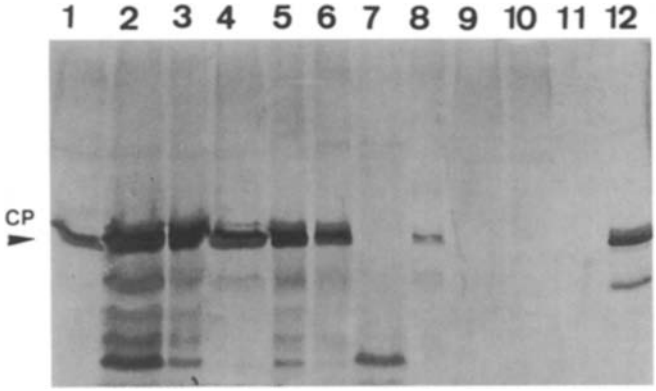

Fig.4. Western blot analysis for the expression of the PRSV CP gene in the putative transgenic papaya lines. Protein extracts from calli or shoots were separated by SDS-PAGE and immunoblotted with anti-PRSV serum. The extract from PRSV-infected *Cucumis metuliferus* was used as a positive control (lane 12). PRSV CP was detected in shoots of lines GCP16-1, GCP17-1, GCP17-6 and GCP18-1 (lanes 3 to 6, respectively). In line GCP17-0, CP was detected in shoot (lane 2) and callus (lane 1). In line GCP16-0, intact CP was detected in callus (lane 8) while degraded in shoot (lane 7). Non-transformant controls are shown in lane 9, 10 and 11.

Discussion

Based on the resistance to kanamycin, PCR detection, Western blotting, and segregation analysis, the PRSV CP gene was successfully integrated into transgenic papaya plants by carborundum wounding in liquid phase prior to *Agrobacterium*-mediated transformation. When transgenic papaya line GCP17-1 was backcrossed with cultivar Sunrise, the segregation ratio of the CP gene was 1:1, indicating that the transgene is inherited like a single dominant gene. The expression of the viral protein in the transgenic papaya lines was easily detected by Western blotting. This is contrary to transgenic papayas reported by Fitch et al. (1990, 1993) and Yang et al. (1996), in which the expression of PRSV CP was not detectable or difficult to detect by Western blotting. In transgenic plants, the effective protection from virus infection provided by expression of the viral CP gene has been demonstrated in several virus groups (Fitchen and Beachy

1993). Resistance to PRSV infection in some of our transgenic papaya lines has been demonstrated in this investigation, but whether higher expressors provide a higher degree of virus resistance needs to be further studied.

Embryogenic tissues of papaya have a higher potential for regeneration than other explants such as shoot tips, stems, petioles, or root tips (Fitch et al. 1990). In this investigation, transgenic papayas were successfully produced when embryogenic tissues derived from immature zygotic embryos were used for transformation. Since the explants used were very small and fragile, the traditional wounding methods such as cutting by scalpel or puncture by needle were very difficult and tedious. The wounding generated by vortexing with carborundum in this investigation provided a very fast and efficient way to generate tiny wounds suitable for infection by *Agrobacterium* of the critical cells which have a high potential to regenerate. During the development of this process, we found that the size of carborundum particles and the speed of vortexing are critical factors affecting transformation efficiency. For example, when 400 or 350 mesh carborundum was used or when the vortexing was set at full speed, severe damage to the tissues was observed and putative transgenic plants were not obtained. A longer period of vortexing up to 2 or 4 min also drastically reduced the rates of regeneration to 1.2 and 0%, respectively.

According to reports by Fitch et al. (1990, 1993), foreign genes were delivered into papaya at a frequency of 0.42% of bombarded embryos (10 lines out of 2300 bombarded zygotic embryos) or 5 transgenic lines out of 19 grams embryogenic callus by microprojectile bombardment, and at 0.15% (0.24 transgenic line per gram) embryogenic callus by *Agrobacterium*-mediated transformation (2 lines out of 13 grams). The average transformation frequency from this investigation, by wounding with carborundum prior to *Agrobacterium*-mediated transformation, was 15.9% or about 1.6 transgenic lines per gram embryogenic tissues (52 transgenic somatic embryo clusters out of 327 clumps of zygotic embryogenic calli treated), approximately 6-100 times higher than the reported methods of conventional *Agrobacterium*-mediated transformation or particle bombardment.

When petioles of papaya were used as explant, it needed a long time for regenerating somatic embryos (Yang and Ye 1992). By *Agrobacterium*-mediated transformation of petioles, it required 10-11 months for the transformed cells to regenerate into a plantlet (Yang et al. 1996). When embryogenic tissues derived from hypocotyl or immature zygotic embryos were used as explant for transformation, the regeneration process took about 13 months (Fitch et al. 1993). Regeneration of somatic embryos of papaya is usually achieved by culturing the explants on medium containing 2,4-D. Because of possible long-term effect of 2,4-D, abnormal shoots, leaves or plants occurred at high frequencies (Fitch et al. 1993; Yang et al. 1996). Our method of wounding embryogenic tissues by carborundum prior to *Agrobacterium*-mediated transformation not only increased the transformation frequency but also greatly shortened the regeneration time (about 9 months). Moreover, this method produced mostly normal-looking transgenic papaya plants which were fertile and produced transgenic progeny.

Acknowledgment. This study was supported by the Council of Agriculture of the Republic of China on Taiwan.

References

Chen MH, Chen CC (1992) Plant Cell Rep 11:404-407
Chen MH, Wang PJ, Maeda E (1987) Plant Cell Rep 6:348-351
DeBruijne E, DeLanghe E, van Rijck R (1974) Int Symp Fytofarm Fytiata 26:637-645
Fitch MMM, Manshardt RM (1990) Plant Cell Rep 9:320-324
Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL (1993) Plant Cell Rep 12:245-249
Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1990) Plant Cell Rep 9:189-194
Fromm ME, Taylor LP, Walbot V (1986) Nature 319:791-793
Fitchen JH, Beachy R (1993) Annu Rev Microbial 47:739-763
Klee H, Horsch R, Rogers S (1987) Annu Rev Plant Physiol 38:467-486
Klein TM, Wolf ED, Wu R, Sanford JC (1987) Nature 327:70-73
Krens FA, Molendijk L, Wullems GJ, Schilperoort RA (1982) Nature 296:72-74
Laemmli UK (1970) Nature 227:680-685
Litz RE, Conover RA (1982) Plant Sci Lett 26:153-158
Litz RE, O'Hair SK, Conover RA (1983) Scientia Hort 19:287-
Mettler IJ (1987) Plant Mol Biol Rep 5:346-349
Murashige T, Skoog F (1962) Physiol Plant 15:472-497
Pang SZ, Sanford JC (1988) J Amer Soc Hort Sci 113:287-291
Rogers SG, Horsch RB, Fraley RT (1986) Methods Enzymol 118:627-640
Tsay HS, Su CY (1985) Plant Cell Rep 4:28-30
Wang CH, Bau HJ, Yeh SD (1994) Phytopathology 84, 1205-1210
Yang JS, Ye CA (1992) Bot Bull Acad Sin 33:377-382
Yang JS, Yu TA, Cheng YH, Yeh SD (1996) Plant Cell Rep (in press)
Yeh SD, Gonsalves D, Provvidenti R (1984) Phytopathology 74, 1081-1085
Yeh SD, Jan FJ, Chiang CH, Doong TJ , Chen MC, Chung PH, Bau HJ (1992) J Gen Virol: 2531-2541
Yie ST, Liaw SI (1977) In Vitro 13:564-567