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Transgenic papaya plants from *Agrobacterium*-mediated transformation of somatic embryos

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Summary. Transgenic papaya (*Carica papaya* L.) plants were regenerated from embryogenic cultures that were co-cultivated with a disarmed C58 strain of *Agrobacterium tumefaciens* containing one of the following binary cosmid vectors: pGA482GG or pGA482GG/cpPRV-4. The T-DNA region of both binary vectors includes the chimeric genes for neomycin phosphotransferase II (NPTII) and ß-glucuronidase (GUS). In addition, the plant expressible coat protein (*cp*) gene of papaya ringspot virus (PRV) is flanked by the NPTII and GUS genes in pGA482GG/cpPRV-4. Putative transformed embryogenic papaya tissues were obtained by selection on 150 µg·ml^{-1} kanamycin. Four putative transgenic plant lines were obtained from the *cp* gene$^-$ vector and two from the *cp* gene$^+$ vector. GUS and NPTII expression were detected in leaves of all putative transformed plants tested, while PRV coat protein expression was detected in leaves of the PRV *cp* gene$^+$ plant. The transformed status of these papaya plants was analyzed using both polymerase chain reaction amplification and genomic blot hybridization of the NPTII and PRV *cp* genes. Integration of these genes into the papaya genome was demonstrated by genomic blot hybridizations. Thus, like numerous other dicotyledonous plant species, papayas can be transformed with *A. tumefaciens* and regenerated into phenotypically normal-appearing plants that express foreign genes.

Introduction

Papaya is a tropical and subtropical perennial tree crop, inbred in some areas like Hawaii, and subject to a fairly long generation time of ten months from seed to seed (Storey 1953). Papayas are highly susceptible to papaya ringspot virus (PRV), and virtually no resistance has been found in cultivars available for conventional breeding (Cook and Zettler 1970, Conover 1976). Introgression of resistance genes from wild species into commercial cultivars has been attempted (Horovitz and Jimenez 1967, Khuspe et al. 1980, Moore and Litz 1984, Manshardt and Wenslaff 1989a, 1989b, Chen et al. 1991), but PRV resistant cultivars have not yet been produced. Consequently, crop improvement to solve disease problems of a tree species like papaya can be enhanced by gene transfer techniques (Grumet 1990).

We have developed protocols for transforming commercial papaya cultivars with the particle gun (Fitch et al. 1990). Based on the strategy of Powell Abel et al. (1986), who first demonstrated coat protein-mediated virus protection, the coat protein (*cp*) gene of PRV was cloned, sequenced, (Quemada et al. 1990), and engineered into an *Agrobacterium tumefaciens* binary cosmid vector (Ling et al. 1991, Slightom 1991, Slightom et al. 1991). This vector was transferred via microprojectiles into embryogenic papaya tissues, and expression of the reporter gene ß-glucuronidase (GUS) and selection gene neomycin phosphotransferase II (NPTII) was reported in putative transgenic somatic embryos and regenerated plants (Fitch et al. 1990). Furthermore, transgenic papayas containing the PRV *cp* gene ranged from susceptible to moderately resistant to apparently immune to PRV HA, the virulent Hawaiian strain (Fitch et al. 1992).

Binary vectors based on pGA482 (An 1986) had been constructed for papaya transformation via *Agrobacterium tumefaciens*, and with the advent of the particle gun both transformation methods became available. We initiated bombardment experiments at the same time as co-cultivation of papaya somatic embryos with *A. tumefaciens* using the method of McGranahan et al. (1988). Because Pang and Sanford (1988) transformed papaya leaf disks with *A. tumefaciens* but did not regenerate plants, we developed protocols for efficient embryogenesis and plant regeneration from immature zygotic embryos of papaya (Fitch and Manshardt 1990) and from young hypocotyl sections (Fitch 1993) prior to initiating co-cultivation experiments.

The *Agrobacterium*-mediated gene transfer mechanism can be used to transfer T-DNA regions of binary cosmid vectors into papaya tissues. We have now demonstrated that foreign genes can be transferred into papaya tissues by the biological *A. tumefaciens*-mediated T-DNA transfer method and the mechanical microprojectile-mediated gene transfer method (Fitch et al. 1990, 1992).

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Materials and methods

Plant material and culture conditions. Papaya (*Carica papaya* L.) seeds of the cultivar "Kapoho" were germinated as described (Fitch 1993). Hypocotyls from seedlings about 10 d old were sliced into 2- to 3-mm sections and co-cultivated with *A. tumefaciens* immediately after slicing.

Embryogenic calluses and somatic embryos were initiated from "Kapoho" hypocotyl sections as described (Fitch 1993). The hypocotyl sections were cultured in the dark at 27°C on induction medium that consisted of half-strength MS salts medium (Murashige and Skoog 1962), modified according to Fitch and Manshardt (1990) and containing 0.5 to 25 $mg \cdot l^{-1}$ 2,4-D. Cultures were maintained without subculturing for six to eight months prior to co-cultivation with *A. tumefaciens*. About one week prior to co-cultivation, the cultures were transferred to fresh induction medium containing 10 $mg \cdot l^{-1}$ 2,4-D. Somatic embryo cultures that were initiated from 90- to 105-d-old immature zygotic embryos (Fitch and Manshardt 1990) and maintained in the dark with 0, 0.5, or 10 $mg \cdot l^{-1}$ 2,4-D without subculturing for six to eight months were also co-cultivated with *A. tumefaciens*.

Agrobacterium cultures. Disarmed *A. tumefaciens* strain C58-Z707 (Hepburn et al. 1985) was transformed with modified binary cosmid vectors pGA482GG (17.0 kb) or pGA482GG/cpPRV-4 (18.6 kb) which were derived from pGA482 (An 1986). Both plasmids contain a bacterial GUS gene (Jefferson et al. 1986) in addition to a polylinker region and the kanamycin resistance gene, neomycin phosphotransferase II (NPTII) which is driven by the nopaline synthase (Nos) promoter and is designated Nos-NPTII. The plasmid pGA482GG/cpPRV-4 is a further modification of pGA482GG that includes a 35S-driven PRV *cp* gene of the mild mutant strain PRV HA 5-1 (Yeh and Gonsalves 1984) inserted into the *Hin*dIII site of the polylinker region (Ling et al. 1991, Slightom 1991, Slightom et al. 1991).

A. tumefaciens cultures were prepared for co-cultivation by the method of Hookyaas (1988). Overnight cultures of bacteria were induced for 4 h with 50 µM acetosyringone (Aldrich Chemical Co., Milwaukee, WI) prior to co-cultivation.

Agrobacterium co-cultivation and selection for transgenic somatic embryos. Sliced hypocotyl sections were immediately placed into 18 ml of callus induction medium to which 2.0 ml of an induced or uninduced overnight *A. tumefaciens* culture was added. Callus induction medium that is mentioned hereafter contained 10 $mg \cdot l^{-1}$ 2,4-D. Tissues were incubated for 30 min to allow for *A. tumefaciens* attachment, blotted dry, and co-cultivated on induction medium for 2 d at 27°C in the dark.

Approximately 500 mg of embryogenic tissues from zygotic embryos and hypocotyl callus (Fitch 1993) were suspended in 10 ml of induction medium containing 50 µl of induced or uninduced *A. tumefaciens*. Flasks of tissues were co-cultivated for 2 d at 27°C.

Following the co-cultivation period, tissues were rinsed twice with induction medium, transferred to 10 ml of induction medium containing 500 $mg \cdot l^{-1}$ cefotaxime (Calbiochem, San Diego, CA), and shaken overnight at 27°C. The antibiotic-treated tissues were transferred to plates of induction medium containing 200 $mg \cdot l^{-1}$ cefotaxime and incubated in the dark.

In four to six weeks, equal portions of tissues in each treatment were transferred to maturation or induction medium containing 200 $mg \cdot l^{-1}$ cefotaxime. Maturation medium was induction medium without 2,4-D. Half of the media contained 75 $mg \cdot l^{-1}$ kanamycin, the other half did not. Tissues were grown in subdued light (<10 $\mu mol \cdot m^{-2} \cdot sec^{-1}$), monitored for selective growth, and regenerated as described (Fitch et al. 1992).

Bacterial contamination of cultures despite the presence of cefotaxime required a more stringent antibiotic treatment. Carbenicillin (Sigma Chemical Co., St. Louis, MO) was added at a concentration of 500 $mg \cdot l^{-1}$. Some contaminated tissues were suspended for 3 d in induction medium containing 50 $mg \cdot l^{-1}$ rifampicin (Sigma). Tissues contaminated with bacteria having apparent resistance to the three antibiotics were discarded. Once bacterial growth appeared to be controlled, all cultures were grown in the presence of 75 or 150 $mg \cdot l^{-1}$ kanamycin and were not subcultured for five months.

GUS histochemical assay. Leaves were sliced and incubated overnight at 37°C in filter-sterilized 0.5 mM 5-bromo-4-chloro-3-indolyl-ß-glucuronide (X-gluc) in 200 mM sodium phosphate buffer, pH 7.0 (Jefferson 1987). Stained tissues were assayed as described (Fitch et al. 1992).

DNA extraction. DNA was extracted using "CTAB" methods (Dellaporta et al. 1983, Saghai-Maroof et al. 1984) as described (Fitch et al. 1992).

Polymerase chain reaction. Genomic DNA was subjected to amplification by the polymerase chain reaction (PCR) (Saiki et al. 1988) as described (Fitch et al. 1992).

Southern blot hybridization. Probes for NPTII and PRV *cp* were prepared and labeled with (α-^{32}P)dCTP or digoxigenin-11-dUTP by random priming (Feinburg and Vogelstein 1983) and genomic DNAs isolated from putative transgenic leaves were digested with six-fold excess of *Hin*dIII and *Bam*HI or *Hin*dIII, separated, and hybridized as described (Fitch et al. 1992).

ELISA assays for NPTII and PRV CP expression. Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) technique (Clark and Adams 1977), employing polyclonal and monoclonal antibodies, was used to assay for PRV coat protein and NPTII (5 Prime 3 Prime, Inc., West Chester, PA) in putative transgenic leaves. Controls were either PRV-infected leaves or NPTII standards. Absorbance units for PRV coat protein assays were based on 175 µg protein in 500 µl extraction buffer. An absorbance reading of $A_{405} = 1.0$ for NPTII was obtained from 10 ng NPTII/mg protein.

Results and Discussion

Response of freshly explanted hypocotyl cultures

Hypocotyl sections treated with both induced and uninduced *A. tumefaciens* C58-Z707 [pGA482GG] and C58-Z707 [pGA482GG/cpPRV-4] became swollen, and epidermis and cortex layers fell away from the central vascular bundles within 25 d on induction medium containing cefotaxime. Brown spots and streaks on the epidermis were observed on some treated sections but never on untreated control sections. Cell enlargement and loosening of the *A. tumefaciens*-infected sections were delayed by three weeks compared to the controls. When the sections were transferred to selection medium three weeks following co-cultivation, further growth ceased. Residual contamination with *A. tumefaciens* was controlled by growing cultures on medium containing 500 $mg \cdot l^{-1}$ carbenicillin and 200 $mg \cdot l^{-1}$ cefotaxime, but about half of the samples were lost. After about a year of maintenance, freshly explanted hypocotyl sections did not produce transgenic cell lines. Embryogenesis in the hypocotyl sections may have been inhibited by infection with *A. tumefaciens* strain C58-Z707. The presence of carbenicillin, cefotaxime, and kanamycin in the induction medium did not appear to have an inhibitory effect on control sections since they became embryogenic in the presence of the three antibiotics (data not shown).

Somatic embryos and embryogenic calluses

Yellow to ivory-colored embryogenic tissues turned light brown after co-cultivation with *A. tumefaciens* C58-Z707 [pGA482GG] or [pGA492GG/cpPRV-4]. Some somatic embryos developed brown spots that resembled hypersensitivity reactions on papaya cotyledons, hypocotyls,

and radicles. Growth of embryogenic tissues was inhibited for a month by 75 mg·l^{-1} kanamycin. Thereafter, the concentration of the selective agent was doubled because all tissues appeared to resume growth. Nearly 75% of the embryogenic callus cultures also became contaminated with *A. tumefaciens* three weeks after co-cultivation. Carbenicillin and cefotaxime treatment inhibited the regrowth of bacteria; however, about half of the samples were lost due to *A. tumefaciens* contamination.

Six months after co-cultivation, light-colored sectors of embryogenic tissues were observed on cultures treated with induced *A. tumefaciens*, and nine months after co-cultivation, selectively growing, light green somatic embryo clusters were evident among the brown tissues (Fig. 1). Portions of the selectively growing clusters were subjected to the GUS histochemical assay and many turned blue within 1 h. The positive response was not due to contamination by *A. tumefaciens*, because no bacterial growth was detected when embryos were placed on antibiotic-free potato dextrose agar.

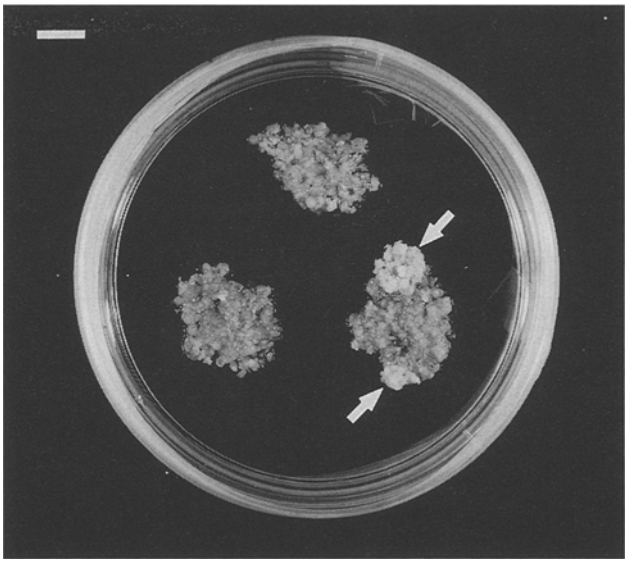

Fig. 1. Clusters of papaya somatic embryos (arrows) growing selectively on medium containing 150 mg·l^{-1} kanamycin after co-cultivation with *A. tumefaciens* C58-Z707 [pGA482GG]. Scale = 1.0 cm.

Identification of transgenic papaya tissues

Three putative transgenic plant lines were readily regenerated from a total of 8.0 g fresh weight (FW) of embryogenic hypocotyl tissues co-cultivated with *A. tumefaciens* C58-Z707 [pGA482GG] that contains the Nos-NPTII and GUS genes in its T-DNA (Table 1). Eight other isolate lines in this group were either lost or were found to be difficult to regenerate into normal-looking plants. The extended period of culture of the embryogenic hypocotyl tissues in the presence of 2,4-D (six to eight months) prior to co-cultivation may have adversely affected the regeneration ability of many of these selectively growing somatic embryo lines. Leaves from two plant lines were GUS$^+$ and NPTII$^+$ in the histochemical assay and ELISA test, respectively (Table 1). NPTII absorbance values were high (A_{405} = 0.9) for both AI-1D-1 and AI-1D-4 from [pGA482GG] and were equivalent to the highest positive control NPTII concentration, 1 pg/µl (A_{405} = 1.0). GUS expression was sometimes negative in older leaves, while young fully expanded leaves were found to be GUS$^+$. Since hundreds of somatic embryos were produced from the original selectively growing clusters (Fig. 1), plants that consistently expressed GUS were selected to represent the isolate.

Only two putative transgenic plant lines were recovered from 13 g FW of embryogenic tissues (the combined FW of the hypocotyl- and zygotic embryo-derived samples) co-cultivated with *A. tumefaciens* C58-Z707 [pGA482GG/cpPRV-4] (Table 1). The two plant lines, obtained from hypocotyl-derived embryogenic tissues, were unusual in that they both produced chlorotic leaves. Once rooted, the plants produced green leaves and appeared normal *in vitro*, but under greenhouse conditions they again produced chlorotic yellow leaves and survived as long as green leaves remained intact. The green leaves of PI-2B-1 were positive for PRV coat protein in the ELISA test (A_{405} = 0.030), while the pale leaves were equal to the negative controls (A_{405} = 0.015). The chlorotic leaves showed lower NPTII expression than green leaves, A_{405} = 0.334 (chlorotic) vs. 0.805 (green). The low positive values in both assays may have been due to sampling of senescent chlorotic leaves since it was difficult to distinguish healthy chlorotic leaves from senescent ones. Chlorotic leaves were not totally devoid of chlorophyll; chloroform extracts of leaves were as dark green in color as those from green leaves.

Molecular analysis of T-DNAs inserted into the transgenic papayas

The presence of transferred genes in four different regenerated papaya plant lines was first tested by PCR amplification using their genomic DNAs as substrate. Two plant lines transformed with *A. tumefaciens* C58-Z707 [pGA482GG] exhibited two DNA fragment sizes, one, a band of the expected 1.0-kb size, and an unexpected band of about 1.3 kb, following PCR amplification for Nos-NPTII (Fig. 2). The smaller fragment was identical in size to that amplified in the pGA482GG positive control DNA and in particle gun-transformed plant lines. Both PCR-generated fragments hybridized to the Nos-NPTII gene probe (Fig. 2B). The larger PCR-generated fragment cannot be explained at this time, but it could be indicative of an abnormal T-DNA transfer that may involve tandem or multiple insertions. Because these tissues are capable of growth in the presence of 150 µg/ml kanamycin, they apparently contain at least one functional Nos-NPTII gene.

The Nos-NPTII gene probe hybridized with a characteristic internal 2.0-kb *Bam*HI/*Hin*dIII fragment (Chee et al. 1989, Chee 1990, Chee and Slightom 1991, 1992) in Southern blots of digested genomic DNAs (Fig. 3). Larger fragments at 3.3, 4.4, 7.0, and 19 kb were observed in two plant lines from *A. tumefaciens* C58-Z707 [pGA482GG] but

Table 1. Putative transgenic isolates from co-cultivation of papaya hypocotyl sections and embryogenic tissues with disarmed *Agrobacterium tumefaciens* strain C58Z707 to determine which tissues are most efficiently transformed

Plasmid construct	Tissue type	Initial amount treated	Total # of "transgenics"	Assay results: total positive/total # assayed		
				GUS	NPTII	PCR CP
Control	HYP	~180 sections	0	0	0	0
pGA482GG	HYP	~180 sections	0	0	0	0
pGA482GG/PRV4	HYP	~180 sections	0	0	0	0
Control	HYP SE	8.5 g	0	0	0	0
pGA482GG	HYP SE	8.0 g	11[a]	8/8	2/2	0/2
pGA482GG/PRV4	HYP SE	7.5 g	2	2/2	2/2	2/2
pGA482GG/PRV4	ZE SE	5.5 g	0	0	0	0

Note: pGA482GG = binary plasmid containing genes for NPTII and GUS, pGA482GG/PRV4 = same as pGA482GG but containing PRV *cp* gene, HYP = hypocotyl sections, HYP SE = somatic embryos derived from hypocotyl calli, ZE SE = somatic embryos derived from zygotic embryos, GUS = histochemical assay, NPTII = Both ELISA test and PCR amplification with Nos-NPTII gene primers, and PCR CP = PCR amplification with PRV *cp* gene primers.

[a]3 isolates lost before GUS assay.

not in one plant line from C58-Z707 [pGA482GG/cpPRV-4] (Fig. 3). Digests with *Hin*dIII contained up to five larger fragments ranging from 3.3 to 19 kb that result from cleavage at the *Hin*dIII site internal to the Nos-NPTII gene and a second adjacent *Hin*dIII site located in the plant genome. Hybridization of the *Hin*dIII digests shows that the gene has been integrated into the genome of these papaya plant lines (Fig. 3).

PCR analysis for the presence of the PRV *cp* gene in both plant lines derived from pGA482GG/cpPRV-4 is shown in Fig. 4 and in Southern blot hybridizations. Genomic DNA from plant line PI-2B-1, digested with *Hin*dIII and *Bam*HI showed the predicted 1.7-kb *Hin*dIII PRV *cp* gene fragment (Slightom 1991, Slightom et al. 1991) that is not present in plants AI-1D-1 and AI-1D-4 derived from pGA482GG (data not shown).

Comparison of papaya transformation methods

Gene transfer into papaya somatic embryos and embryogenic calluses has now been demonstrated with two transformation systems, the particle gun (Fitch et al. 1990) and here with *A. tumefaciens*. Freshly explanted hypocotyl sections were not suitable for *Agrobacterium*-mediated transformation. The time required for the regeneration of putative transgenic papaya tissues differed between the two transformation systems. Putative transgenic embryos appeared six to nine months after co-cultivation of embryogenic calluses and somatic embryos with *A. tumefaciens* compared to four months after similar tissues were transformed via microprojectile bombardment (Fitch et al. 1990, 1992). The delay in the *Agrobacterium* system may have been due to difficulties encountered in decontaminating tissues. The largest number of transgenic plants in the microprojectile-mediated system was regenerated from 2,4-D-treated zygotic embryos that were exposed to the growth regulator for less than one month prior to bombardment (Fitch et al. 1992). The *A. tumefaciens*-mediated system may have been hindered by the use of cultures from both hypocotyls and zygotic embryos that had been grown for six to eight months in the presence of 2,4-D prior to co-cultivation.

Differences between putative transgenic plant lines from the two transformation systems have been observed. Although the number of transgenic lines recovered following co-cultivation was small, GUS was expressed in all 13 somatic embryo lines as well as in the five plant lines regenerated from them. In contrast, only about one third of the isolates from particle bombardment expressed GUS (Fitch et al. 1990, 1992), reflecting vector genes that apparently became unlinked, a common problem associated with the

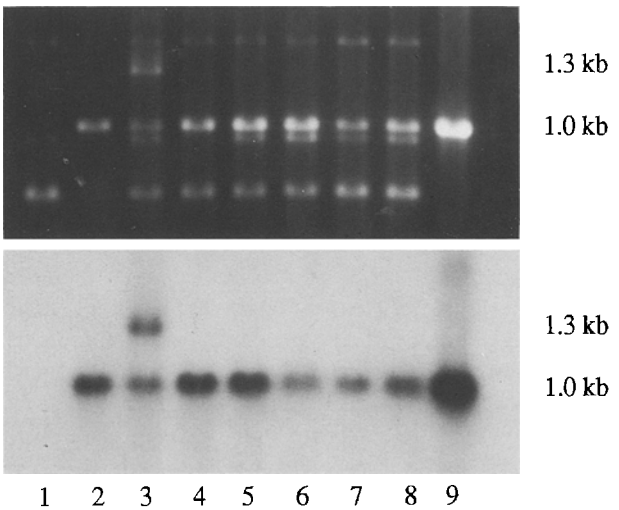

Fig. 2. PCR analysis of DNAs isolated from putative transgenic papaya plants. PCR amplifications were accomplished using the oligonucleotide primers for Nos-NPTII (Chee et al. 1989). Frame A) Ethidium bromide-stained gel showing fragments amplified in papaya DNAs after *A. tumefaciens* co-cultivation and particle gun-mediated transgenic plants (Fitch et al. 1990). Frame B) Southern blot hybridization of PCR products in Frame A. Lane 3 contains the DNA from a plant transformed with *A. tumefaciens* C58-Z707 [pGA482GG]. The 1.0-kb PCR product is common to all transgenic plants, but the *A. tumefaciens*-mediated transgenic plant contains, in addition, the 1.3-kb band. Lane 1) untransformed papaya, lanes 2, and 4 to 8) particle gun-mediated transgenic plants S55-1, S49-2, S59-2, K37-1, S63-1, and S60-4, lane 3) AI-1D-4 transformed with *A. tumefaciens* C58-Z707 [pGA482GG], lane 9) pGA482GG positive control DNA.

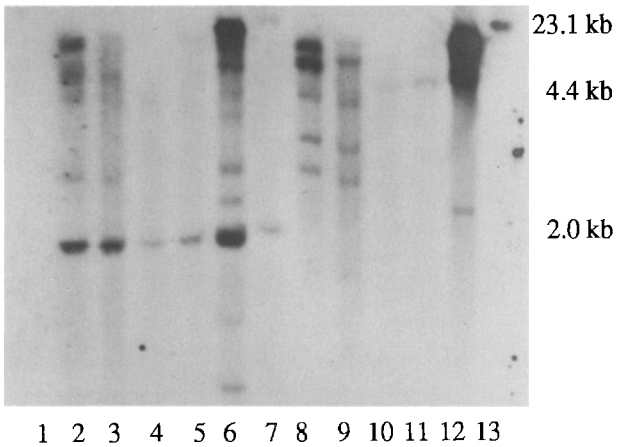

Fig. 3. Genomic Southern blot hybridization of DNAs of plants derived from either *A. tumefaciens*- or particle gun-mediated transformation. A Nos-NPTII gene probe hybridized with several bands in *Hin*dIII/*Bam*HI (lanes 1 to 7) or *Hin*dIII (lanes 8 to 13) digests. Lane 1) untransformed papaya, lanes 2 and 8) AI-1D-1 transformed with *A. tumefaciens* C58-Z707 [pGA482GG], lanes 3 and 9) AI-1D-4 transformed with *A. tumefaciens* C58-Z707 [pGA482GG], lanes 4 and 10) PI-2B-1 transformed with *A. tumefaciens* C58-Z707 [pGA482GG/cpPRV-4], lanes 5 and 11) S55-1 transformed with the particle gun [pGA482GG/cpPRV-4], lanes 6 and 12) K39-1 transformed with the particle gun [pGA482GG/cpPRV-4], lanes 7 and 13) pGA482GG. The 2.0-kb band characteristic of the Nos-NPTII gene digested with *Hin*dIII/*Bam*HI was present in all five transformants. About 10 µg of digest was loaded in each lane. A digoxigenin-labeled 600-bp Nos-NPTII gene fragment was used as the probe.

microprojectile bombardment transformation system (McCabe et al. 1988, Christou et al. 1989, Gordon-Kamm et al. 1990). Two plant lines recovered from *A. tumefaciens* transformation with C58-Z707 [pGA482GG] showed evidence for normal and rearranged Nos-NPTII fragments after PCR analysis and in Southern blot hybridizations. These results indicate that multiple gene transfer may have occurred. The transgenic *Agrobacterium*-derived R_0 plants from the *cp* gene$^-$ vector have just recently begun flowering, and further characterization of transgenic plants and progeny from both transformation systems may reveal other differences between the transformants.

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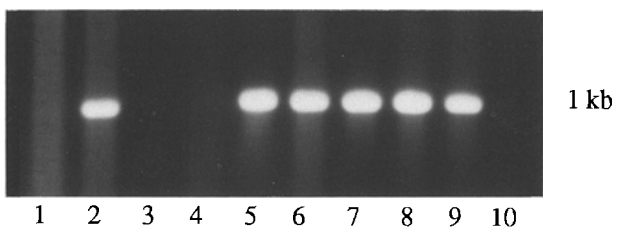

Fig. 4. PCR analysis of DNAs isolated from putative transgenic papaya plants using PRV *cp* gene primers. Lane 1) untransformed papaya, lanes 2, 8, 9, and 10) S55-1, K39-1, S55-1, and S54-1, transformed with the particle gun [pGA482GG/cpPRV-4], lanes 3 and 4) AI-1D-1 transformed with *A. tumefaciens* C58-Z707 [pGA482GG], lane 5) PI-2B-2 transformed with *A. tumefaciens* C58-Z707 [pGA482GG/cpPRV-4], lanes 6 and 7) PI-2B-1 transformed with *A. tumefaciens* C58-Z707 [pGA482GG/cpPRV-4].

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