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REGENERATION OF TRANSGENIC PAPAYA (Carica papaya) PLANTS

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ABSTRACT

Stable transformation of papaya (Carica papaya L.) has been achieved following DNA delivery via high velocity micro-projectiles. Three types of embryogenic tissues, including immature zygotic embryos, hypocotyl sections, and somatic embryos derived from both, were bombarded with tungsten particles carrying chimeric genes coding for NPT II, GUS, and the coat protein of a mild mutant strain HA 5-1 of papaya ringspot virus. All tissue types were cultured prior to and following particle bombardment on half-strength MS medium supplemented with 10 mg/l^{-1} 2,4-D, 400 mg/l^{-1} glutamine, and 6% sucrose. Upon transfer to 2,4-D-free medium containing 150 mg/l^{-1} kanamycin sulfate, 12 isolates regenerated somatic embryos, and five of these produced leafy shoots six to nine months following bombardment. Tissues from 11 isolates were assayed for NPTII activity, and nine were positive. Five out of 12 isolates assayed for GUS expression were positive. Three isolates were positive for both NPTII and GUS.

INTRODUCTION

In most papaya-growing regions of the world, papaya ringspot virus (PRV) is a major limiting factor in production. The disease is caused by an aphid-transmitted potyvirus, and symptoms on papaya (Carica papaya L.) include chlorotic mottling and distortion of leaves, watersoaked lesions on stems and petioles, and ringspotting and distortion of fruits (Conover, 1964). Infected plants decline rapidly, become unproductive and eventually die.

Application of insecticides to eliminate aphid vectors is expensive and generally ineffective as a PRV control measure (Conover, 1964; Namba and Kawanishi, 1966). Isolated regions can be maintained free of the virus through a combination of quarantine and roguing, as in the major production areas of Hawaii (Namba and Higa, 1977), but this approach has no merit in areas already infected with PRV.

Papaya germplasm has been screened previously for genetic resistance to PRV, but these efforts revealed only susceptible plants (Cook and Zettler, 1970) or modest levels of polygenic resistance (Conover and Litz, 1978). The latter materials were used in Florida to develop the dioecious cultivar 'Cariflora' (Conover et al., 1986), which may be considered a compromise between useful PRV resistance and acceptable fruit quality.

Other researchers have shown that high levels of resistance to PRV are present in several wild Carica species, and at least one report has suggested that resistance is controlled by a single dominant gene (Horovitz & Jimenez, 1967). In Hawaii, interspecific hybrids between commercial papayas and wild Carica species have been produced through embryo culture (Manshardt and Wenslaff, 1989a, 1989b). Several of these are vigorous and show excellent field resistance to PRV (unpublished results). However, the F1's are quite sterile, and backcrossing to recover fertility may require considerable time and effort.

The failure of other approaches to provide disease control in the short term, and the restricted host range of PRV (Yeh et al., 1984) have made the use of cross-protecting viral strains an attractive option for limiting PRV damage. Cross-protection is the deliberate systemic infection of plants with a nonvirulent or mild form of a virus to preclude subsequent infection by a more virulent strain of the same or closely related virus. Mild strains of PRV have been created by nitrous acid mutation of a virulent Hawaiian isolate (Yeh and Gonsalves, 1984). Greenhouse tests (Yeh and Gonsalves, 1984), small field tests in Hawaii (Yeh et al., 1988) and Taiwan (Wang et al., 1987), and large scale application of cross-protective PRV strains in Taiwan (Yeh et al., 1988) have demonstrated that cross-protection is useful in limiting economic damage from PRV. However, conventional cross-protection has several drawbacks: 1) each papaya generation requires inoculation, 2) the cross-protecting viral strain causes mild disease symptoms and a reduction in yield, 3) breakdown of cross-protection does occur, particularly when the virulent strain is introduced into young, unexpanded leaves around the apex, where the titer of the mild strain may be low, and 4) escape of the mild strain into other susceptible crops is possible.

Through use of new and powerful genetic engineering technology, it has been demonstrated that expression of a viral coat protein gene alone is sufficient in some cases to produce resistance in genetically transformed plants (Abel et al., 1986; Hoekema et al., 1989), and the term "genetically engineered viral resistance" has been suggested to describe this strategy Plants thus engineered may overcome most of the limitations of conventionally cross-protected plants. Stable incorporation of the coat protein gene in a chromosome of the transgenic plant will result in Mendelian inheritance of virus resistance in the seedling progeny, eliminating the need to inoculate each

generation. Expression of the coat protein gene in every cell of the transformed plant should reduce the frequency of breakdown, such as may occur in conventionally cross-protected plants due to poor systemic spread of the mild strain. The absence of a replicating, albeit mild, virus in the genetically engineered plants means 1) that there should be no disease symptoms or yield reductions, and 2) that there will be no possibility of a mild virus escaping to infect other crops. Finally, it seems likely that the time needed to introduce virus resistance into papaya by genetic engineering will be far less than the time required to produce resistant cultivars by traditional breeding methods.

In this paper, we report a major step toward the goal of creating papaya cultivars with genetically engineered virus resistance, namely, the regeneration of transgenic papaya plants from embryogenic tissue cultures that have been successfully transformed with marker genes by microprojectile bombardment (Sanford, 1988).

MATERIALS AND METHODS

Tissue cultures

Embryogenic papaya cultures were initiated from zygotic embryos and seedling hypocotyl segments. Immature zygotic embryos were excised from the cultivars 'Sunset' and 'Kapoho' at 90 to 105 days after pollination. Hypocotyls from 14-day-old 'Kapoho' seedlings, grown on water agar (1%), were sliced into 2-mm segments. Both tissue types were plated on 'induction' medium containing half-strength MS salts (Murashige and Skoog, 1962), 400 mg 1 glutamine, 100 mg/l^{-1} myo-inositol, full strength MS vitamins, 6% sucrose, 10 mg/l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and 1% Sigma A agar at pH 5.8 (this medium, excluding 2,4-D, will henceforth be called 'maturation' medium). Cultures were incubated at 27°C in darkness, with transfers to fresh medium at irregular intervals occasionally exceeding one month.

At intervals following initiation, cultures were subjected to bombardment with DNA-coated microprojectiles. Explanted hypocotyl segments were bombarded one to two days after initiation on induction medium solidified with 1.5% agar. Zygotic embryos were cultured from four to 23 days and transferred, with meristems exposed, into induction medium solidified with 1.5% agar. Bombardment of zygotic embryos occurred one week after transfer.

Three weeks after bombardment, most cultures were transferred to maturation medium supplemented with 75 mg/l^{-1} kanamycin sulfate. Following four more weeks of culture, the kanamycin concentration was increased to 150 mg/l^{-1} in both maturation and induction media. The cultures were not

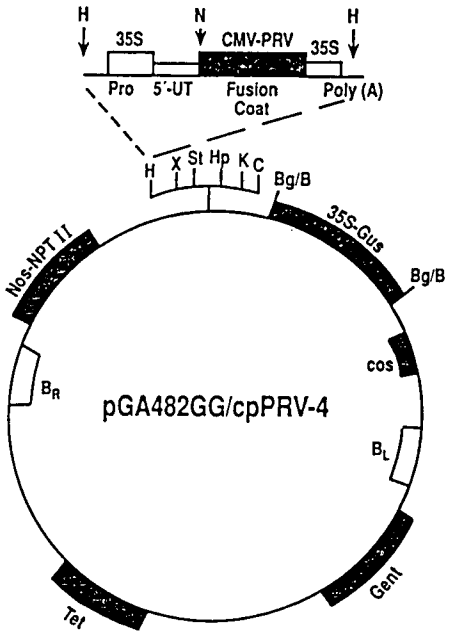

Fig. 1. Plasmid vectors used for coating the microprojectiles were all derived from the *Agrobacterium* binary vector pGA482 (An 1986) which contains the T-DNA-derived border fragments B_R and B_L, the *cos* site from the bacterial phage λ, a restriction enzyme polylinker, and the NOS-NPTII fusion gene. Addition of the bacterial gentamicin gene (Gent) outside of the T-DNA region has been described by Chee et al. (1989). The presence of this gentamicin has no function when used with microprojectile bombardment; it is useful for plasmid transfers into *Agrobacterium*. The bacterial GUS gene, constructed by Jefferson (1987), was cloned into the vector by removing the GUS gene via a partial *Bam*HI digestion and cloning this 3.0 kb fragment into the *Bgl*II site of the polylinker of pGA482 (Quemada, Slightom, unpublished data). Expression cassettes PRV-4 and PRV-19 (Quemada, Slightom, unpublished data) were designed for cloning into the *Hin*dIII site of the polylinker; only the vector pGA482GG/cpPRV-4 is shown since pGA482GG/cpPRV-19-5 differs only in the linkage of the 5'-untranslated region (5'-UT) with the PRV coat protein gene (Quemada, Slightom, unpublished data). Restriction enzyme sites are: Bg/B, non-digestable fusion of *Bgl*II and *Bam*HI; C, *Cla*I; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; N, *Nco*I; St, *Stu*I; and X, *Xho*L

transferred again for eight weeks. The hypocotyl segment cultures, after 5 months on the initial selective medium, were maintained on induction medium containing 150 mg/l^{-1} kanamycin. Thereafter, transfers of all cultures were accomplished monthly to the same respective media.

Plasmids and DNA delivery

The plasmids pGA482GG/cpPRV-4 (Fig. 1) and pGA482GG/-cpPRV-19-5 are derivatives of the micro-T-DNA vector pGA482 (An et al., 1985). Both plasmids have been modified to contain chimeric genes for the coat protein of the mild mutant strain HA 5-1 of papaya ringspot virus (Yeh and Gonsalves, 1984) and bacterial beta-glucuronidase (GUS) (Jefferson, 1987), in addition to the neomycin phosphotransferase II (NPTII) gene of pGA482. Coding sequences for the PRV coat protein gene (Quemada et al., 1990) and the GUS reporter gene were inserted between cauliflower mosaic virus 35s promoter and nopaline synthase (NOS) 3' termination sequences. The plasmids pGA482GG/cpPRV-4 and pGA482GG/cpPRV-19-5 differ in arrangement of leader sequences in the chimeric PRV coat protein gene. The NPTII gene is flanked by NOS promoter and termination sequences.

Plasmid DNA was precipitated from ethanol, centrifuged at 15,000 rpm for 15 minutes and vacuum dried for ten minutes. The DNA was suspended in deionized water at a concentration of 1 ug/ul. Particle coating and bombardment were essentially as reported by Klein et al. (1988). Immediately prior to loading the coated particles onto macroprojectiles, the microfuge tubes were sonicated briefly or finger vortexed to suspend the particles. Two ul of suspension were delivered per bombardment, and each tissue culture plate was bombarded three or four times.

GUS histochemical assay

Three weeks after particle bombardment, and at irregular intervals thereafter, papaya tissue cultures were examined for GUS expression using the 5-Br-4-Cl-3 indolyl-beta-D-glucuronic acid (X-gluc) histochemical assay (Jefferson, 1987). Samples of zygotic embryos, hypocotyl segments, or somatic embryos and calli having fresh weights of about 100 mg each were transferred to 1.5-ml microfuge tubes and treated with 400 ul of 0.96 mM X-gluc in 200 mM sodium phosphate buffer, pH 7. Color development was recorded after one to 12 hours of incubation at 37C.

NPTII assay

Six months following particle bombardment, somatic embryos that grew on kanamycin were assayed for NPTII expression following the protocol of McDonnell et al. (1988). Fresh weights of tissue samples ranged from less than 10 mg to 220 mg. Scintillation counts were recorded, and auto- radiographs were developed 48 hours to ten days after the assay.

RESULTS AND DISCUSSION

Tissue cultures

Seventy-seven plates of embryogenic zygotic embryos, embryogenic calli derived from hypocotyls and from zygotic embryos, and explanted hypocotyl segments were bombarded with DNA-coated microprojectiles. Seven of the plates were badly contaminated and had to be discarded. Of the remaining 70 plates, ten gave rise to a total of 15 isolates that were putatively transgenic, based on expression of scorable and/or selectable marker genes.

Growth on kanamycin

Cultures were allowed to recover from bombardment for a total of three to five weeks before transfer to selective media containing 75 mg/l^{-1} kanamycin. This level of kanamycin, while adequate to suppress induction of embryogenesis in freshly explanted tissues, was not sufficient to completely inhibit growth of somatic embryos that had developed prior to being plated on selective medium. Consequently, after four to six weeks at 75 mg/l^{-1} kanamycin, the concentration was doubled to 150 mg/l^{-1} for most of the cultures, and the higher level effectively stopped growth of untransformed papaya tissues in the bombarded cultures.

Four months after bombardment, some calli and somatic embryos with apparent resistance to kanamycin were observed growing on selective media. In some cases, growth of the putative transformants was rapid. One month after such isolates were observed, parts of plants or somatic embryo clusters could be assayed to confirm that transformation had occurred. In one case, the resistant embryo was clearly chimeric, having a green, developing sector set off sharply from the pale ivory-colored embryonic tissues.

GUS assay

Starting three weeks after bombardment, we began to assay the cultures for GUS expression using X-gluc substrate. We tested 240 zygotic embryos, or about 10% of the total number bombarded, and observed 47 dark blue, GUS-positive spots on 23 zygotic embryos. In 29 of these cases, GUS expressing cells were located in the cotyledons and therefore lacked the potential for plant regeneration in our tissue culture system. The remaining 18 GUS spots were observed in the swollen, embryogenic apical domes of the zygotic embryos, and these had the potential to give rise to transformed plants. In apical domes assayed one month after bombardment, blue spots became visible after ten hours of incubation. However, when embryogenic tissues derived from apical domes were assayed five months after bombardment, GUS expression was evident after only 50 minutes of incubation,

presumably due to the larger number of transformed cells in each blue spot at the later date.

Bombardment of somatic embryo preparations and hypocotyl segments was not as productive of GUS-positive spots as zygotic embryos. Compared with zygotic embryos, assays of about the same amount of bombarded surface area of somatic embryos/calli yielded only eight, dark blue spots. These were observed on hypocotyls and cotyledons of somatic embryos and on masses of globular embryos or calli. Hypocotyl segments were not assayed extensively, because they showed only one GUS-positive cell in initial tests conducted soon after bombardment. However, six months following bombardment, one embryogenic callus derived from a hypocotyl segment produced a strong GUS-positive response, demonstrating that hypocotyl tissue also possesses potential for transformation with the particle gun.

GUS-expressing tissues detected within about four months of bombardment were lost due to the small amounts of transformed tissue involved and the destructive nature of the assay. However, after we observed rapid growth in a few somatic embryos and calli on kanamycin, we were able to isolate these and assay portions of the tissues without sacrificing the entire isolate.

The GUS response varied considerably in different transformation events. Some somatic embryos appeared uniformly dark blue, while others were paler, as if only the epidermis had been transformed. In 41-1, globular somatic embryos showed an intense blue GUS response, while cotyledons of more mature somatic embryos from this isolate showed only pale blue GUS expression.

NPTII assay

A total of 12 somatic embryo or plant isolates from nine bombarded plates were tentatively identified as transgenic, based on growth on selective medium four to eight months after particle bombardment. The results of NPTII assays confirmed that nine papaya somatic embryos showing uninhibited growth on selective medium were indeed transformed. Table 1 shows the correspondence between growth on kanamycin and NPTII and GUS expression in putative transformed cultures. Three of the nine isolates expressing NPTII also showed GUS activity.

All of the isolates listed in Table 1 are presently being grown and propagated for further testing. Some or most of them may be chimeras. Consequently, subcultures of each have been transferred to micropropagation medium containing 150 mg/l^{-1} kanamycin to promote axillary growth of transgenic sectors, so that wholly transformed individuals may be obtained.

Table 1. Expression of scorable (GUS) and selectable (NPTII) marker genes in putative transgenic papaya isolates growing on selection medium (150 mg/l^{-1} kanamycin).

Isolate No.	Tissue bombarded	GUS	NPTII	Growth on kan.
Control	SE	-	-	-
9-1	H	+++	ns	++
25-1	SE		+	+++
27-1	SE	+++	++	+++
29-1	SE	+++	+	+++
41-1	ZE	+++	-	+++
47-1	ZE	-	++	++
50-1	ZE	-	+	+++
60-1	ZE	+++	+	++
60-2	ZE	-	-	++
62-1	ZE	-	+++	+++
62-2	ZE	-	++	+++
62-3	ZE	-	+++	+

Control culture was not bombarded with microprojectiles.

H = hypocotyl segments, SE = somatic embryos, ZE = zygotic embryos
- = no expression, + = slight, ++ = moderate, +++ = strong, ns = not assayed

NPTII data based on scintillation cpm ug^{-1} protein

CONCLUSIONS

An efficient tissue culture system for regenerating papaya via somatic embryogenesis from immature zygotes and seedling hypocotyl sections has been developed and employed to regenerate plants genetically transformed by micro- projectile bombardment. Evidence of successful transformation includes expression of GUS and NPTII marker genes and growth on selective medium. Further characterization of transgenic plants for expression of the coat protein gene of papaya ringspot virus is in progress. Our objective is to employ the latter trait to produce papaya cultivars with genetically engineered resistance to papaya ringspot virus.

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