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Comparison of the effects of kanamycin and geneticin on regeneration of papaya from root tissue

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Abstract

Kanamycin and geneticin are commonly used for the selection of neomycin phosphotransferase II (*npt* II) transformed plants. Since papaya tissue is sensitive to both antibiotics, it is difficult to explore their effects on the regeneration process solely based on using non-transformed tissues. Adventitious roots derived from *npt* II-transgenic and non-transgenic papaya shoots *in vitro* were used as explants in this investigation. The effects of kanamycin and geneticin on callus formation, embryogenesis, and conversion of somatic embryos to shoots were compared. Callus growth derived from *npt* II-transformed root explants was apparently enhanced on kanmycin within 50–200 mg l^{-1} or on geneticin within 12.5–50 mg l^{-1} as compared to those on antibiotic-free controls. The percentages of *npt* II-transformed somatic embryo-forming callus were not significantly different (16.3–18.3%) on geneticin less than 6.25 mg l^{-1} and only slightly reduced (11.2–15.7%) on geneticin within 12.5–50 mg l^{-1}, whereas, formation of somatic embryos was strongly suppressed on kanamycin media. Conversion rates of *npt* II-transformed somatic embryos to shoots were not significantly different among all kanamycin or geneticin treatments. Percentages of the callus derived from non-transformed root explants were greatly reduced on the medium containing more than 25 mg l^{-1} kanamycin or geneticin, and no somatic embryos formed from untransformed callus on any kanamycin or geneticin media. Our results indicated that somatic embryogenesis of callus derived from *npt* II-transformed root explants of papaya was strongly inhibited by kanamycin. Thus, to regenerate *npt* II-transformed cells from papaya root tissue, we recommend using the lower concentration geneticin (12.5–25 mg l^{-1}) to avoid the adverse effects of kanamycin on embryogenesis.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid; BA – 6-benzyladenine; IBA – indole-3-butyric acid; *npt* II – neomycin phosphotransferase II; PRSV – papaya ringspot virus

Introduction

A marker gene for selection is often used to recover transformants during a gene transfer process. Plant transformants emerge from the mass of non-transformed tissue because of the advantage provided by the expression of the selection gene (Guerineau, 1995). The most commonly used selectable gene is *npt* II, which codes for the enzyme neomycin phosphotransferase (NPT) that detoxifies several aminoglycoside antibiotics such as kanamycin, geneticin (G418), paromomycin, and neomycin (Galun and Breiman, 1997). The enzyme NPT consists of 264 amino acids and has high substrate specificity. It catalyses the ATP-dependent phosphorylation of the 3′-hydroxy moiety of the aminohexose ring (Nap et al., 1992).

Kanamycin is a trisaccharide composed of one deoxystreptamine and two glucosamine units (2-deoxystreptamine-6-D-glucosamine) (Nap et al., 1992), and is most frequently used for the selection of *npt* II-transformed plants (Norelli and Aldwinckle,

1993). Geneticin is a 3′-OH-containing gentamycin derivative with chemical properties similar to kanamycin (Curtis et al., 1995). Combination of geneticin and *npt* II was found useful for selection of transformants for *Phaseolus acutifolius* (Dillen et al., 1997), *Pinus radiasta* (Walter et al., 1998), *Gossypium hirsutum* (Rajasekaran et al., 2000), and *Allium cepa* (Eady et al., 2000). However, most plant species are extremely sensitive to kanamycin and geneticin (Dandekar, 1992), and it is difficult to investigate their effects on morphogenesis from non-transformed plant tissue. In transformation of papaya, kanamycin is normally used to select transformed cells expressing the *npt* II gene from among non-transformed tissues, and is inhibitory for regeneration from leaves (Pang and Sanford, 1988; Cabrera-Ponce et al., 1996), immature embryos (Fitch et al., 1990, 1993; Cheng et al., 1996; Cai et al., 1999) or petioles (Yang et al., 1996) at the concentrations ranging from 75 to 150 mg l^{-1}. However, detailed information that kanamycin may affect the callus growth and regeneration of *npt* II-transformed papaya tissue is still lacking.

Recently, we established an efficient system for inducing adventitious roots from *in vitro* shoots derived from selected hermaphroditic plants with good horticultural qualities (Yu et al., 2000). The adventitious roots can be cultured to produce somatic embryos within 4 months (Lin and Yang, 2001). To develop an effective method of papaya transformation using root tissue, this study was to investigate and compare the effects of kanamycin and geneticin on regeneration of *npt* II-transformed papaya cells from adventitious root segments. Susceptibility to NPT phosphorylation, induction of callus growth, initiation of somatic embryogenesis, and conversion of somatic embryos into shoots during the plant regeneration process from root tissues of *npt* II-transformed papaya lines were analyzed.

Materials and methods

Plant materials and culture conditions

Source of adventitious roots

Multiple shoots of non-transformed papaya (*Carica papaya* L. cv. Tainung No. 2) or transformed papaya with the coat protein (CP) gene of a Taiwan strain of *Papaya ringspot virus* (PRSV), constructed in Ti binary vector pBGCP including the *npt* II gene as a selection marker, were micropropagated *in vitro* as described previously (Cheng et al., 1996). Individual shoots with 2–3 leaves were first placed in the root induction medium for 1 week in darkness, and then transferred to vermiculite with 1/2 MS solution for 2 weeks for root growth (Yu et al., 2000). The developed adventitious roots were cut into segments of about 5 mm and used as explants. Three PRSV-CP transgenic lines 16-0-1, 17-0-5, and 18-0-9 (Bau et al., 2003) were used as plant materials. Each transgenic line was used as an individual treatment. There were three replicates per treatment, with 30 root segments per replicate.

Culture media

The basic medium consisted of MS salts (Murashige and Skoog, 1962) and B_5 vitamins (Gamborg et al., 1968), 3% (w/v) sucrose, and 0.8% (w/v) agar. The root induction medium contained 2.5 μM indole-3-butyric acid (IBA) in the basic medium. The medium for callus formation and somatic embryogenesis (CEM) included 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.45 μM 6-benzyladenine (BA) to the basic medium. The medium for conversion of somatic embryos into shoots (SM) was supplemented with 0.1 μM α-naphthaleneacetic acid (NAA) and 0.8 μM BA (Yang and Ye, 1992). The pH of all the media was adjusted to 5.7±0.1 with 1 N KOH before autoclaving at 1.1 kg cm^{-2} (121 °C) for 20 min. Concentrated solutions of kanamycin and geneticin (Sigma Co, St. Louis, MO) were added to the autoclaved medium in prescribed volume after sterilization by filtering through a 0.22 μm membrane filter.

Culture conditions

All treated explants were cultured in a growth chamber at 28±1 °C under dark conditions for induction of callus and somatic embryos from individual root segments. For conversion of somatic embryos into shoots, a light intensity of 53 μmol m^{-2} s^{-1} with cool white fluorescent lamps was provided for 14 h daily.

Effects of kanamycin and geneticin on callus formation and somatic embryogenesis

To investigate the effects of kanamycin and geneticin on the formation of callus and somatic embryos from root segments, the adventitious root segments were cultured on CEM medium containing 0, 6.25, 12.5, 25, 50, 100 and 200 mg l^{-1} kanamycin or geneticin.

Four weeks after incubation, percentages of callus formation were calculated from the numbers of root segments with callus out of total root segments treated and the fresh weights of callus-forming explants were also measured. To compare the effects of kanamycin and geneticin on somatic embryogenesis, percentages of somatic embryo-forming callus out of total calluses were determined at 16 weeks after culturing on CEM medium.

Effects of kanamycin and geneticin on conversion of somatic embryos to shoots

To study the effects of kanamycin and geneticin on conversion of somatic embryos to shoots, individual somatic embryos derived from somatic embyro-forming callus were cultured on the SM medium containing 0, 6.25, 12.5, 25, 50, 100 and 200 mg l^{-1} of kanamycin or geneticin. The conversion rates of somatic embryos were determined at 4 weeks after culturing. There were three replicates per treatment, with 30 somatic embryos per replicate.

Results and discussion

Effects on callus formation and growth

The rates of callus formation 4 weeks after culturing root segments on CEM medium containing different concentrations of antibiotics are summarized in Figure 1. On media with various concentrations of kanamycin or geneticin, the percentages of the callus forming explants of *npt* II-transformed papaya tissue of line 16-0-1 approached 100%. For the non-transformed tissue, callus formation was dramatically reduced (20–47%) in the media with kanamycin more than 25 mg l^{-1} (Figure 1), and no callus formed using geneticin in the same concentration range (Figure 1).

Fresh weight of callus-forming explants of the *npt* II-transformed tissue of line 16-0-1 was significantly enhanced (94–103%) at kanamycin more than 50 mg l^{-1}, while an extreme reduction in growth was observed with callus from non-transformed tissue at kanamycin more than 25 mg l^{-1} (Figure 2A). Fresh weight of transformed tissues increased significantly (62–159%) at geneticin within 12.5–50 mg l^{-1}, however, no significant differences were noticed among other concentrations. Callus growth of non-transformed tissues was completely inhibited at geneticin more than 12.5 mg l^{-1} (Figure 2B). Callus formation and growth from the root segments of transgenic lines 17-0-5 and 18-0-9 treated with both antibiotics were similar to those of line 16-0-1 (data not shown).

Pang and Sanford (1988) reported that callus formation from non-transformed papaya leaves was inhibited at 20 mg l^{-1} kanamycin or from stems and petioles at 150 mg l^{-1} kanamycin. Pena et al. (1997) also reported that a low concentration of geneticin (10 mg l^{-1}) effectively inhibited shoot regeneration of non-transformed *Citrus aurantifolia* Swing. In this study, we showed that the development of callus from non-transformed root segments was also more sensitive to geneticin than kanamycin, but callus growth from the root tissue of *npt* II-transformed papaya was not inhibited by both antibiotics.

Effects on somatic embryogenesis

The percentages of somatic embryo-forming callus cultured in CEM media containing different concentrations of kanamycin or geneticin are summarized in Figure 3. There were no significant differences in somatic embryo formation on the antibiotic-free medium between calluses derived from *npt* II-transformed (18.3%) of line 16-0-1 and untransformed tissue (17.9%). For the callus derived from untransformed tissue, no somatic embryos formed in any kanamycin treatments. The highest frequency of somatic embryo-forming callus of the *npt* II-transformed tissue of line 16-0-1 on the antibiotic-free medium was 18.3%, compared with 0.7–3.2% on the media containing various concentrations of kanamycin (Figure 3A). Similar inhibitory effects of kanamycin on somatic embryo formation were also noticed for the callus derived from the root tissues of transgenic lines 17-0-5 and 18-0-9 (data not shown).

At geneticin concentrations ranging from 6.25 to 50 mg l^{-1}, the results showed that the percentages of somatic embryo-forming callus of transformed tissue of line 16-0-1 were gradually declined (16.3–11.7%) as compared to that of the geneticin-free control (18.3%) and no embryos formed over 50 mg l^{-1} geneticin (Figure 3B). A similar trend was also noticed for the calluses formed from the root tissues of transgenic lines 17-0-5 and 18-0-9 (data not shown).

The first somatic embryo derived from callus of transformed explant was noticed 8 weeks after culturing and the number of somatic embryos increased after further culturing at concentrations of geneticin

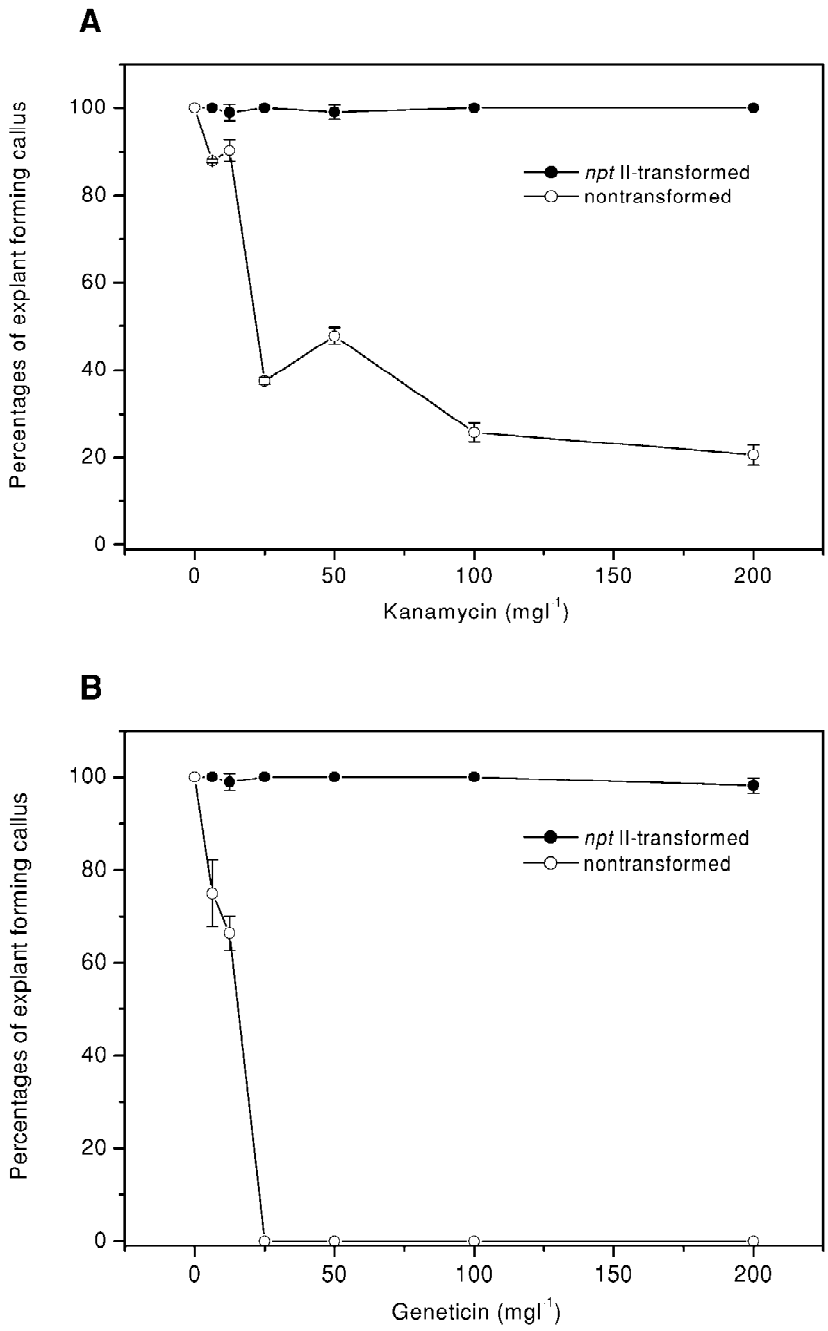

Figure 1. Effects of different concentrations of kanamycin (*A*) and geneticin (*B*) on the percentages of callus forming-explants from root tissues of non-transformed or *npt* II-transformed papaya line 16-0-1. The results were taken 4 weeks after culturing the root segments on CEM medium. Values represent means from three replicates with a total of 90 explants for each treatment, bar=±S.D.

less than 50 mg l^{-1}. More somatic embryos (40–50) from individual callus appeared on media with geneticin ranging from 0 to 25 mg l^{-1} (Figure 4A) than those appeared on the medium with 50 mg l^{-1} (10–20) (Figure 4C), and most embryos were easily separated and detached from callus. No embryos formed from callus at 100 mg l^{-1} geneticin (Figure 4E). However, in kanamycin treatments, somatic embryo formation was delayed at least 4 weeks and somatic embryos from *npt* II-transformed callus were

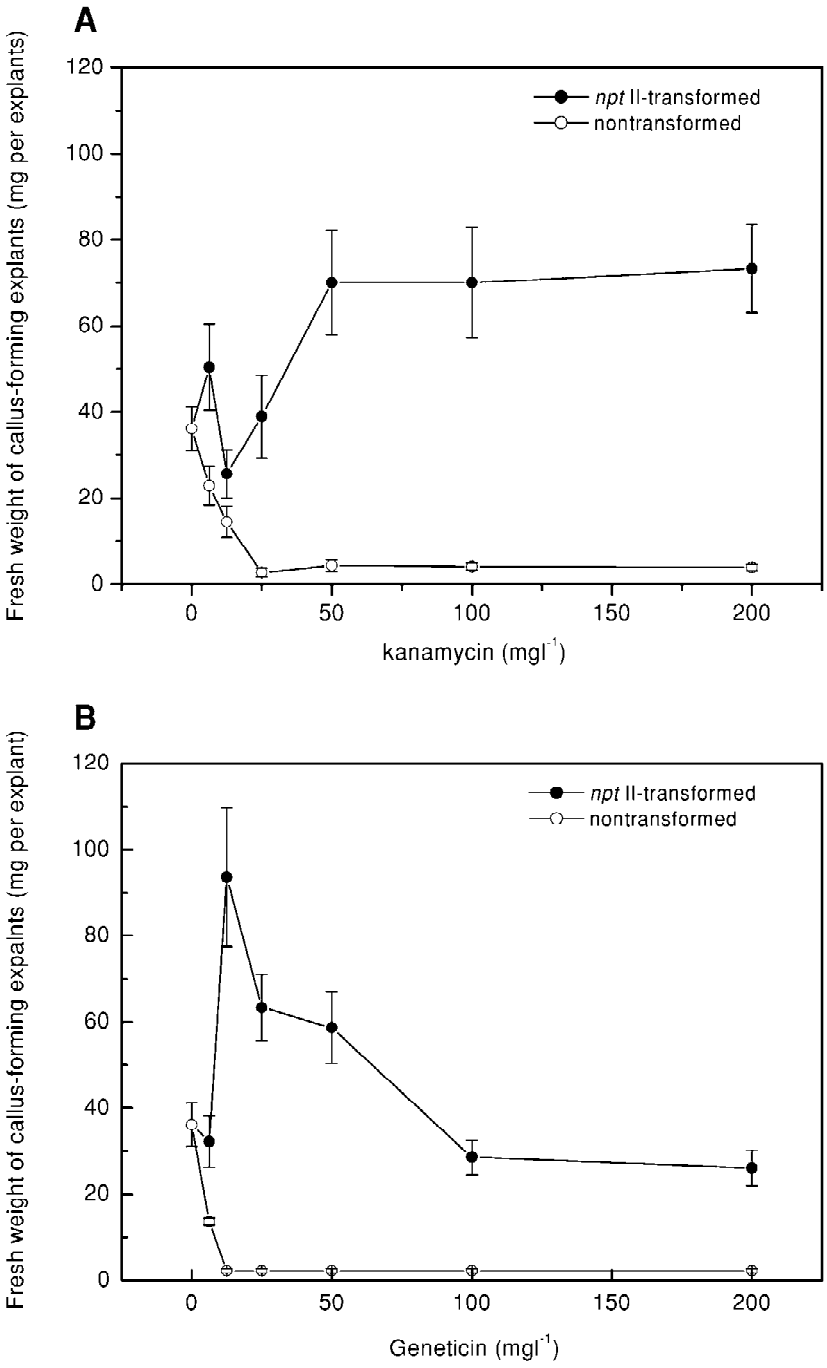

Figure 2. Effects of kanamycin (*A*) and geneticin (*B*) on the fresh weight of callus-forming explants from the root tissues of non-transformed or *npt* II-transformed papaya line 16-0-1. The results were recorded 4 weeks after culturing root segments on CEM medium. Values represent means from three replicates with a total of 90 explants for each treatment, bar=±S.D.

strongly inhibited at higher concentrations of kanamycin. Fewer somatic embryos (2–4) derived from transformed callus were observed in the medium with 25 mg l^{-1} kanamycin (Figure 4B), and there were very few or no embryos formed at kanamycin more than 50 mg l^{-1} (Figure 4D,F).

Kanamycin has been shown to promote morphogenesis in tobacco and carrot (Owens, 1979), and

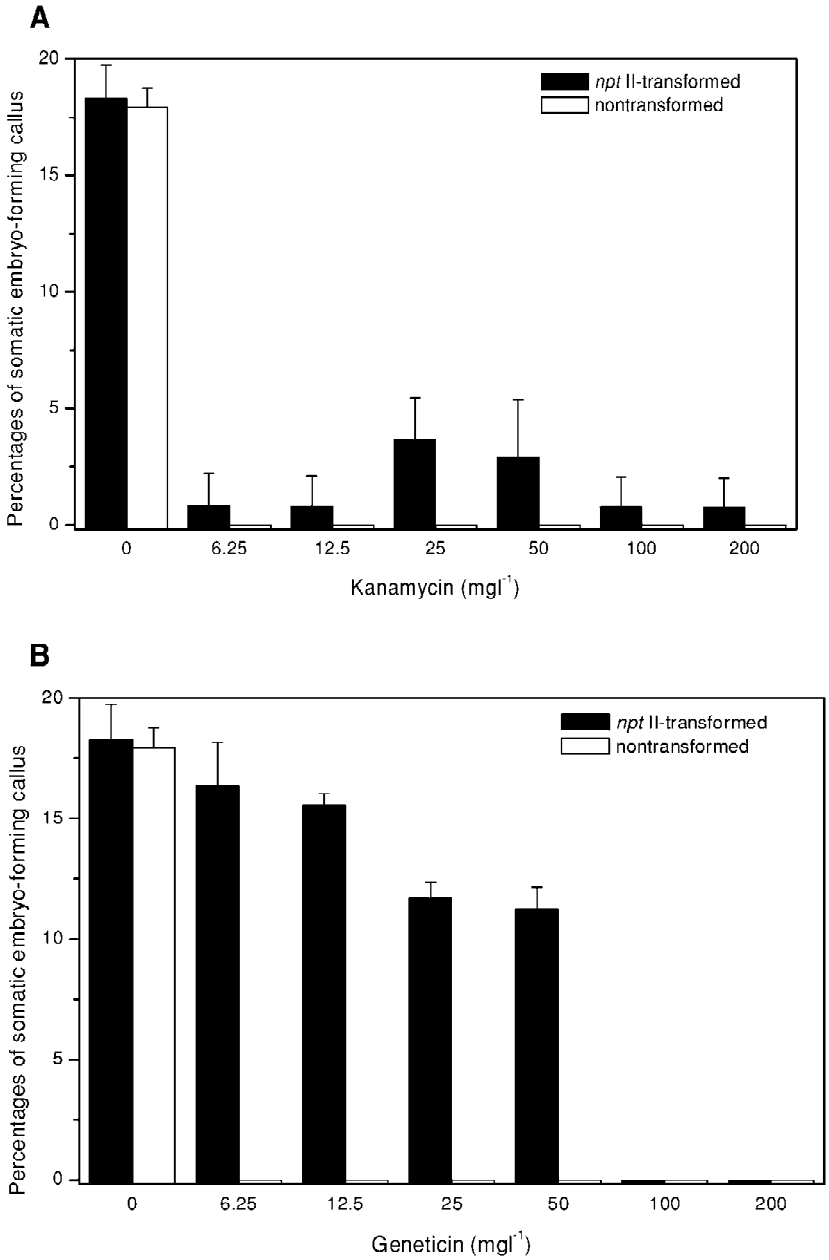

Figure 3. Effects of kanamycin (*A*) and geneticin (*B*) on percentages of somatic embryos-forming callus derived from root tissues of non-transformed or *npt* II-transformed papaya line 16-0-1. The results were taken 16 weeks after culturing root segments on CEM medium. Values represent means from three replicates with a total of 90 explants for each treatment, bar=±S.D.

has been used routinely to select transformants in several *Solanaceous* species (Klee and Rogers, 1989). Although certain species, e.g. walnut, are quite resistant to kanamycin, possibly due to the presence of endogenous non-specific kanamycin phosphotransferase activity (Dandekar, 1992), others are extremely sensitive. Kanamycin has been reported to completely inhibit morphogenesis in several non-transformed fruit species, e.g. in *Vitis* at 7 mg l^{-1} for *V. vinifera* and *V. rupestris* (Gray and Meredith, 1992), and in *Rubus* spp., another member of Rosaceae, at 10 mg l^{-1} (Fiola et al., 1990). In non-transformed *Malus* spp., kanamycin strongly inhibited regeneration at 5–10 mg l^{-1} (Yepes and Aldwinckle, 1994a). In this

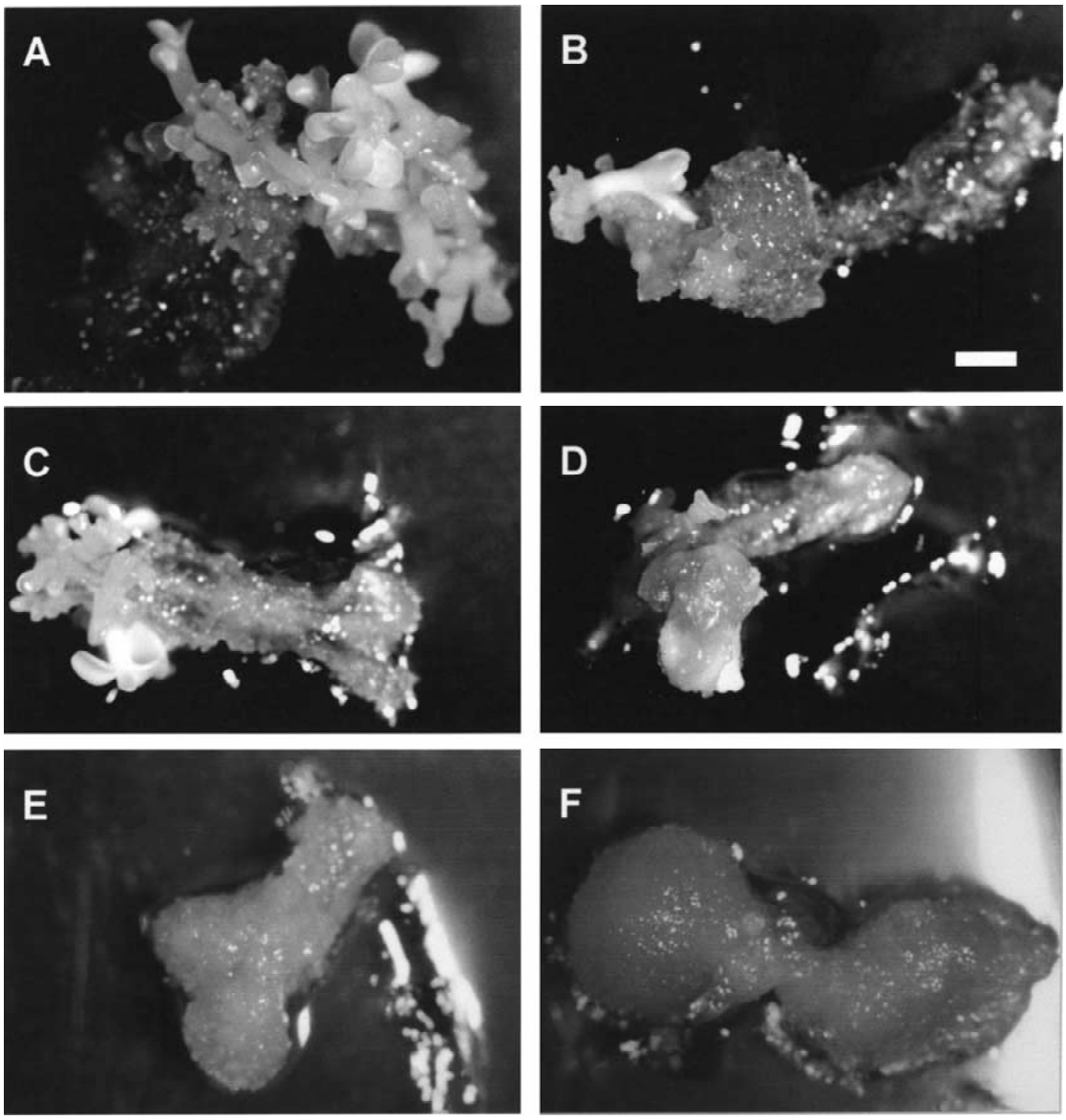

Figure 4. Formation of somatic embryos from callus derived from root tissues of *npt* II-transformed papaya line 16-0-1. The results were recorded 16 weeks after culturing the root segments on CEM media with 25 (*A*), 50 (*C*), and 100 (*E*) mg l^{-1} geneticin or 25 (*B*), 50 (*D*) and 100 (*F*) mg l^{-1} kanamycin, bar=1 mm.

study, although, 60–90% callus derived from non-transformed root explants was still noticed at concentrations of kanamycin or geneticin ranging from 6.25 to 12.5 mg l^{-1}, but these calluses were scant and pale in color, and no somatic embryos formed after further culture. The rate of somatic embryo-forming *npt* II-transformed callus was not significantly different between media with 0 and 6.25 mg l^{-1} geneticin, and slightly decreased on media containing 12.5~50 mg l^{-1}. Furthermore, the numbers and appearance of somatic embryos derived from individual root segments at geneticin concentrations below 25 mg l^{-1} were similar to the control. On the contrary, somatic embryogenesis was strongly inhibited by kanamycin.

Effect on conversion of somatic embryos into shoots

Individual somatic embryos were placed on SM media containing kanamycin or geneticin. During the

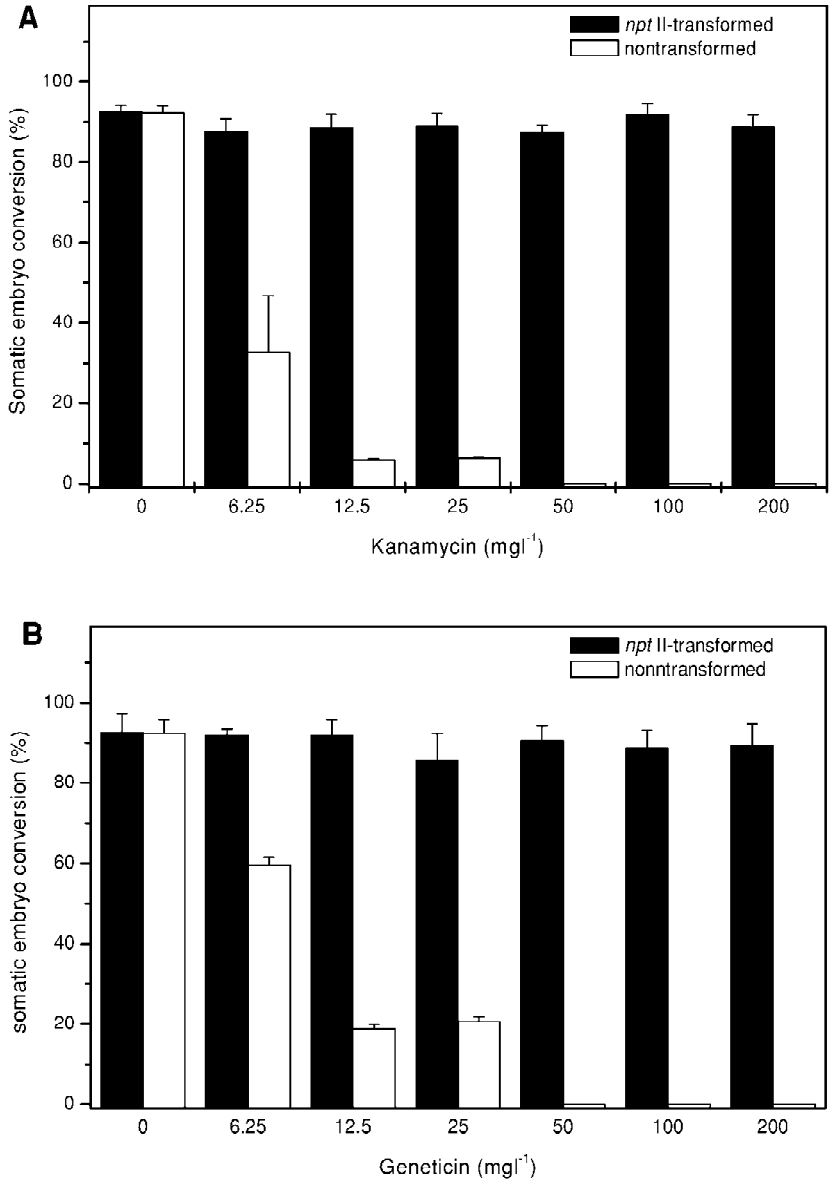

Figure 5. Effects of different concentrations of kanamycin (*A*) and geneticin (*B*) on conversion rates of somatic embryos derived from root tissue of non-transformed or *npt* II-transformed papaya culturing on SM medium for 4 weeks. Values represent means from three replicates with a total of 90 explants for each treatment, bar=±S.D.

conversion of a somatic embryo to a plant, the hypocotyl is elongated and straightened to raise cotyledons and the shoot apex away (Yu et al., 2001). The conversion rates of somatic embryos after culturing for 4 weeks are shown in Figure 5. Germination percentages of non-transformed somatic embryos dramatically decreased (6–32%) in media with kanamycin ranging from 12.5 to 25 mg l^{-1} and no somatic embryos germinated at kanamycin higher than 50 mg l^{-1}. Conversion rate of *npt* II-transformed somatic embryos from line 16-0-1 was not significantly different among all the kanamycin treatments (>87%) (Figure 5A). The conversion rates of the somatic embryos derived from transgenic lines 17-0-5 and 18-0-9 under kanamycin conditions were similar to those of line 16-0-1 (data not shown). In geneticin treatments, the results were also similar to kanamycin treatments (Figure 5B).

Kanamycin acts on the 70 S ribosomal proteins and blocks protein synthesis in organelles and geneticin acts on the 80 S ribosomal protein to block eukaryotic protein synthesis (Eady and Lister, 1998). Both antibiotics are aminoglycoside compounds that are detoxified by the *npt* II-enzyme (Pena et al., 1997). In plant cells, kanamycin exerts its effect on mitochondria and chloroplasts by impairing protein synthesis, resulting in chlorosis (Weide et al., 1989). For several *Malus* cultivars and rootstocks, kanamycin at 50 mg l^{-1} is phytotoxic and causes shoot chlorosis and necrosis (Yepes and Aldwinckle, 1994b). Our investigation showed that at kanamycin or geneticin more than 50 mg l^{-1} the conversion process of a non-transfomed somatic embryo of papaya into a shoot was blocked and the embryos became pale and brown in color. Some non-transformed somatic embryos developed small yellow leaves at kanamcycin or geneticin less than 25 mg l^{-1}, and were able to grow again at antibiotic-free condition. Somatic embryos derived from *npt* II-transformed callsus grew with vigor and converted into normal plants at various concentrations of kanamycin or geneticin.

When transforming papaya with *Agrobacterium,* 75–150 mg l^{-1} kanamycin was used to kill non-transformed cells, low efficiency in transformation and many abnormal transformed somatic embryos are noticed during the selection process (Fitch et al., 1993; Cabrera-Ponce et al., 1996; Yang et al., 1996). In this investigation, we noted that somatic embryos derived from *npt* II-transformed callus were strongly inhibited and very few somatic embryos developed at kanamycin less than 50 mg l^{-1} and no somatic embryos formed at kanamycin more than 50 mg l^{-1}. However, we found that there were fewer adverse effects during regeneration process if lower concentrations of geneticin (12.5–25 mg l^{-1}) were used.

The root segments of PRSV-CP transgenic lines 16-0-1, 17-0-5, and 18-0-9 were used as materials to study the effects of antibiotics on regeneration. The segregation analysis showed that the *npt* II transgene in line 18-0-9 has an inheritance of two dominant loci, whereas both of lines 16-0-1 and 17-0-5 have a single locus (Bau et al., 2003). All transgenic line were also maintained on kanamycin medium (100 mg l^{-1}) and grew vigorously, indicating that all the three lines are not npt II silencers and that kanamycin does not affect micropropagation of the transgenic lines. For most successful papaya transformation, once the somatic embryos are regenerated from transformed cells, it was shown that kanamycin (75–150 mg l^{-1}) has no obvious detrimental effects during conversion of somatic embryos into shoots and consequent micropropagation thereafter (Fitch et al., 1990; Cheng et al., 1996; Cai et al., 1999). Therefore, embryogenesis from callus into somatic embryos is considered the major limiting factor for regeneration of *npt* II-transformed cells. Our results indicated that kanamycin enhances callus growth of the *npt* II-transgenic root tissue and has no adverse effects on the conversion of somatic embryos, but somatic embryogenesis is strongly inhibited by kanamycin, even at very low concentration.

References

Bau HJ, Cheng YH, Yu TA, Yang JS & Yeh SD (2003) Broad-spectrum resistance to different geographic strains of Papaya ringspot virus in CP-gene transgenic papaya. Photopathology 93: 112–120

Cabrera-Ponce JS, Vegas-Garcia A & Herrera-Estrella L (1996) Regeneration of transgenic papaya plants via somatic embryogenesis induced by *Agrobacterium rhizogenes*. In Vitro Cell. Dev. Biol. Plant 32: 86–90

Cai W, Gonalves C, Tennant P, Fermin G, Souza M, Sarinud N, Jan FJ, Zhu HY & Gonsalves D (1999) A protocol for efficient transformation and regeneration of *Carica papaya* L. In Vitro Cell. Dev. Biol. 35: 61–69

Cheng YH, Yang JS & Yeh SD (1996) Efficient transformation of papaya by coat protein gene of papaya ringspot virus mediated by *Agrobacterium* following liquid-phase wounding of embryogenic tissue with caborundum. Plant Cell Rep. 16: 127–132

Curtis IS, Power JB & Davey MR (1995) NPT II assays for measuring gene expression and enzyme activity in transgenic plants. In: Jones H (ed) Methods in Molecular Biology. Plant Gene Transfer and Expression Protocols Vol. 49 (pp. 149–159). Humana Press Inc, Totowa, NJ

Dandekar AM (1992) Transformation. In: Hammerschlag FA & Litz RE (eds) Biotechnology of Perennial Fruit Crops (pp. 141–168). C.A.B. International, Wallingford

Dillen W, De Clercq J, Gossens A, Van Montagu M & Angenon G (1997) *Agrobacterium*-mediated transformation of *Phaseolus acutifolius* A. Gray. Theor. Appl. Genet. 94: 151–158

Eady CC & Lister CE (1998) A comparison of selective agents for use with *Allium cepa* L. immature embryos and immature embryo-derived cultures. Plant Cell Rep. 18: 117–121

Eady CC, Weld RJ & Lister CE (2000) *Agrobacterium tumefaciens*-mediated transformation and transgenic-plant regeneration of onion (*Allium cepa* L.). Plant Cell Rep. 19: 376–381

Fiola JA, Hassan MA, Swartz HJ, Bors RH & McNicols R (1990) Effect of thidiazuron, light influence rates and kanamycin on *in vitro* shoot organogenesis from excised *Rubus* cotyledons and leaves. Plant Cell Tiss. Org. Cult. 20: 223–238

Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL & Sanford JS (1990) Stable transformation of papaya via microprojectile bombardment. Plant Cell Rep. 9: 189–194

Fitch MMM, Manshardt RM, Gonsalves D & Slightom JL (1993)

Transgenic papaya plants from *Agrobacterium*-mediated transformation of somatic embryos. Plant Cell Rep. 12: 245–249

Gamborg OL, Miller RA & Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell. Res. 50: 151–158

Galun E & Breiman A (1997) Transgenic Plants: Tools for Genetic Transformation. In: Transgenic Plants: Tools for Genetic Transformation (pp. 44–76). Imperial College Press, London

Gray DJ & Meredith CP (1992) Grape. In: Hammerschlag FA & Litz RE (eds) Biotechnology of Perennial Fruit Crops (pp. 229–262). C.A.B. International, Wallingford

Guerineau F (1995) Tools for expressing foreign genes in plants. In: Jones H (ed) Methods in Molecular Biology. Plant Gene Transfer and Expression Protocols Vol. 49 (pp. 1–32). Humana Press Inc, Totowa, NJ

Klee HJ & Rogers SG (1989) Plant genetic vectors and transformation: Plant transformation systems based on the use of *Agrobacterium tumefaciens*. In: Schell J & Vasil IK (eds) Cell Culture and Somatic Cell Genetics of Plants. Molecular Biology of Plant Nuclear Genes Vol. 6 (pp. 2–25). Academic Press, San Diego

Lin CM & Yang JS (2001) Papaya somatic embryo induction from fruiting-bearing field plants: Effects of root supporting material and position of the rooting explants. Acta Hort. 560: 489–492

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Nap JP, Bijvoet J & Stiekema WJ (1992) Biosafety of kanamycin-resistant transgenic plants. Trans Res. 1: 239–249

Norelli JN & Aldwinckle HS (1993) The role of aminoglycoside antibiotics in the regeneration and selection of neomycin phosphotransferase-transgenic apple tissue. J. Am. Soc. Hort. Sci. 118: 311–316

Owens LD (1979) Kanamycin promotes morphogenesis of plant tissue. Plant Sci. Lett. 16: 225–230

Pang SZ & Sanford JC (1988) *Agrobacterium*-mediated gene transfer in papaya. J. Am. Soc. Hort. Sci. 113: 287–291

Pena L, Cervera J, Juarez J, Nararro A, Pina JA & Navarro L (1997) Genetic transformation of lime (*Citus aurantifolia* Swing.): factors affecting transformation and regeneration. Plant Cell Rep. 16: 731–737

Rajasekaran K, Hudspeth RL, Cary JW, Anderson DM & Clevel TE (2000) High-frequency stable transformation of cotton (*Gossypium hirsutum* L.) by particle bombardment of embryogenic cell suspension cultures. Plant Cell Rep. 19: 539–545

Walter C, Grace LJ, Wagner A, White DWR, Walden AR, Donaldson SS, Hinton H, Gardner RC & Smith DR (1998) Stable transformation and regeneration of transgenic plants of *Pinus radiata* D. Don. Plant Cell Rep. 17: 460–468

Weide R, Koornneef M & Zabel P (1989) A simple, non-destructive spraying assay for the detection of an active kanamycin resistance gene in transgenic tomato plants. Theor. Appl. Genet. 78: 169–172

Yang JS & Ye CA (1992) Plant regeneration from petioles of *in vitro* regenerated papaya (*Carica papaya* L.) shoots. Bot. Bull Acad. Sin. 33: 375–381

Yang JS, Yu TA, Cheng YH & Yeh SD (1996) Transgenic papaya plants from *Agrobacterium*-mediated transformation of petioles of *in vitro* propagated multishoots. Plant Cell Rep. 15: 459–464

Yepes LM & Aldwinckle S (1994a) Factors that affect leaf regeneration efficiency in apple, and effect of antibiotics in morphogenesis. Plant Cell Tiss. Org. Cult. 37: 257–269

Yepes LM & Aldwinckle S (1994b) Micropropagation of 13 *Malus* cultivars and rootstocks, and effect of antibiotics on proliferation. Plant Growth Reg. 15: 55–67

Yu TA, Yeh SD, Cheng YH & Yang JS (2000) Efficient rooting for establishment of papaya plantlets by micropropagation. Plant Cell Tiss. Org. Cult. 61: 29–35

Yu TA, Yeh SD & Yang JS (2001) Effects of carbenicillin and cefotaxime on callus growth and somatic embryogensis from adventitious roots of papaya. Bot. Bull. Acad. Sin. 42: 281–286