Regeneration of Plants from Peach Embryo Cells Infected with a Shooty Mutant Strain of *Agrobacterium*

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Abstract. Immature 'Redhaven' peach [*Prunus persica* (L.) Batsch] embryos were infected with a shooty mutant strain of *Agrobacterium tumefaciens*, tms328::Tn5, which carries an octopine-type Ti plasmid with a functional cytokinin gene and a mutated auxin gene. Shoots were regenerated from embryo-derived callus that was initiated on MS medium lacking phytohormones. Shoots exhibited increased frequency of branching and were more difficult to root than the noninfected. Transcripts of the tms328::Tn5-cytokinin gene were detected using northern analyses of total plant RNA. Polymerase chain reaction of genomic DNA and cDNA resulted in amplification of DNA fragments specific for the cytokinin gene, as determined by restriction enzyme and Southern analyses. The concentrations of the cytokinins zeatin and zeatin riboside in the leaves of regenerated plants were on the average 51-fold higher than in leaves taken from nontransformed plants. None of the shoots or callus tissues were positive for octopine. The expression of the T-DNA encoded cytokinin gene promotes growth of peach cells in the absence of phytohormones, thus serving as a marker for transformation. In addition, this gene appears to promote morphogenesis without an auxin inductive step.

Asexual gene transfer techniques hold much promise for genetic improvement of peach and other crop species. The *Agrobacterium*-mediated gene transfer technique has proven to be the simplest and most successful method for introducing novel genes into a wide variety of plants (Schell, 1987). Transfer of agronomically important genes into peach cells derived from mature plants would facilitate rapid improvement of commercially important cultivars. Transfer of genes into juvenile tissues could lead to either the release of novel peach cultivars and rootstocks or to the production of novel germplasm useful for breeding purposes.

Reports of *Agrobacterium*-mediated transformation of mature and immature peach tissues have been published recently (Hammerschlag et al., 1989; Martin et al., 1990; Scorza et al., 1990). Martin et al. (1990) and Scorza et al. (1990) used armed *Agrobacterium* strains carrying either the Ri or Ti plasmid plus a binary plasmid with either the *uidA* gene encoding the enzyme β -glucuronidase or the gene for neomycin phosphotransferase as a marker for selection of transformed cells. In these studies, no transgenic plants were recovered. In addition, in one case, the evidence presented for transformation does not rule out possible contamination of the tissues with *Agrobacterium*, as stated by the authors (Martin et al., 1990), and in another case, limited data are presented as evidence of transformation and integration of foreign DNA into the peach genome (Scorza et al., 1990). In an attempt to obtain transgenic peach plants, Hammerschlag et al. (1989) infected peach stem explants (derived from mature plants) with a shooty mutant strain of *A. tumefaciens* (tms328::Tn5) that is known to induce shoots from tobacco tumors (Van Slogteren et al., 1983). Cytokinin-independent growth

and the production of octopine was observed, but shoots could not be regenerated from the transformed cells.

It is well established that it is often impossible to regenerate plants from tissues derived from mature woody plants and, therefore, most of the studies on transformation of such species have used explants derived from juvenile tissues (Fillati et al., 1987; McGranahan et al., 1988; Sederoff et al., 1986). Peach plants can be regenerated from immature embryos (Hammerschlag et al., 1985). The study reported here was undertaken to investigate the possibility of obtaining transgenic plants from peach embryos. This study also explores the effects of expression of a cytokinin gene on peach morphogenesis by examining plant regeneration in the presence and absence of cytokinin following *Agrobacterium* infection.

Materials and Methods

Plant tissue culture and infections. Immature 'Redhaven' peach embryos were isolated from fruit at 63 and 65 days after flowering as described by Hammerschlag et al. (1985). Embryos were wounded with forceps and transferred to liquid MS salts medium, pH 5.6 (Murashige and Skoog, 1962). *Agrobacterium* were grown at 30°C to a density of 1×10^9 cells/ml in LB medium (pH 5.6) supplemented with 20 μ M acetosyringone (Hammerschlag et al., 1989; Sheikholeslam and Weeks, 1987). Embryos were cocultivated overnight with either *A. tumefaciens* strain tms328::Tn5 or strain Al36, each at a final concentration of 5×10^8 cells/ml of MS medium, or in MS salts as a control. Strain tms328::Tn5 has a Tn5 transposon inserted into auxin gene 1 (*iaaM*) in the T-DNA of the Ti plasmid (Fig. 1) (Garfinkel et al., 1981; Schell, 1987) and strain Al36 is a noninfectious derivative that does not carry a Ti plasmid (Watson et al., 1975). Infected and control embryos were washed in liquid MS medium, blotted, and transferred to fresh MS medium either without hormones or with (per liter) 0.1, 1.0, or 5.0 mg 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg 1-naphthaleneacetic acid (NAA), or 0.1 mg *N*-(phenylmethyl)-1H-purin-6-

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Abbreviation: PCR, polymerase chain reaction.

Fig. 1. Map of T-left (TL) DNA of an octopine-type Ti plasmid with size and direction of possible mRNA transcripts indicated by arrows. The *tms328::Tn5 A. tumefaciens* strain has a Tn5 transposon inserted in the *iaaM* gene. The enlarged area is of the cytokinin gene (*ipt*) in which the oligomer primers used in the PCR reaction are indicated by the small arrows.

amine (BA) and 1.0 mg 2,4-D in addition to 30 g of sucrose, 6 g of Phytagar (GIBCO, Grand Island, N.Y.), and 100 mg of cefotaxime (Sigma, St. Louis). Ten embryos were plated per treatment. Callus produced after 6 weeks was transferred to a highly regenerative callus medium and then, after another 6 weeks, to shoot induction medium (Hammerschlag et al., 1985). Each of the above media was supplemented with 100 mg cefotaxime/liter. Callus was also cultured in the absence of BA in the above media. Four calli were plated per treatment and incubated at 26°C in either darkness or light (16-h photoperiod of 40 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by cool-white fluorescent lamps). Shoots were transferred to shoot-elongation medium (Hammerschlag, 1982) and were either maintained on this medium or rooted in half-strength MS medium supplemented with 0.53 mg NAA/liter, 3.0% sucrose, and 0.6% Phytagar and then transferred to soil (Hammerschlag et al., 1987). Cocultivation and regeneration experiments were repeated twice.

Nucleic acid preparations and blot hybridizations. Genomic DNA was extracted from plants by a rapid miniprep method (Dellaporta et al., 1984). Tissues analyzed included in vitro-grown shoots, leaves from greenhouse plants, and callus established from leaves. DNAs were treated with RNase A (100 $\mu\text{g}\cdot\text{ml}^{-1}$) for 30 min at 37°C followed by a 1 phenol : 1 chloroform (v/v) extraction. Ten to 20 μg of the DNA was denatured in 0.4 M NaOH for 15 min and blotted onto a sheet of Zeta-Probe nylon membrane (Bio-Rad, Richmond, Calif.) in a slot-blot apparatus as recommended by the manufacturer. For Southern analysis, 10 to 20 μg of DNA was digested with restriction enzymes *EcoRI* or *BamHI* (New England Biolabs, Beverly, Mass.), separated on a 0.7% agarose gel, and transferred to Zeta-Probe nylon membrane in 0.4 NaOH. Blots were hybridized with a radioactive probe in 1 mM EDTA, 0.5 M NaH_2PO_4 , pH 7.2, and 7% sodium dodecyl sulfate (SDS) at 65°C for 24 to 36 h. The probe, a 7.3-kb pair *EcoRI* fragment from the T-DNA region of pTiB6S3 (Van Larebeke et al., 1974), was labeled to a specific activity of 2 to 4 $\times 10^8$ counts per minute/ μg using a random primer kit (Boehringer Mannheim, Indianapolis) and added in excess to the hybridization solution. Membranes were washed twice in 1 mM EDTA, 40 mM NaHPO_4 , pH 7.2, and 5% SDS at 65°C for 30 to 60 min and exposed to X-AR5 film at -70°C with two intensifying screens (Cronex Lightning Plus, DuPont, Wilmington, Del.). Single and five-copy reconstructions consisting of genomic DNA from untransformed plants and the Ti plasmid from strain *tms328::Tn5* were included as positive controls on the blots.

The method of Chirgwin et al. (1979) was used to isolate total RNA. RNA (40 μg) was separated on agarose/formaldehyde gels and transferred to nitrocellulose membranes (Maniatis et al., 1982). Prehybridizations and hybridizations were done

in 6 $\times$ SSC (1 $\times$ SSC, 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), 0.25% (w/v) dry milk, 0.1 mg of salmon sperm DNA/ml, and 50% (v/v) formamide at 42°C. Blots were hybridized with the probe described above. Following hybridization, filters were washed twice in 2 $\times$ SSC and 0.1% SDS at room temperature for 30 min and exposed to film.

Polymerase chain reaction. One microgram of genomic DNA was subjected to polymerase chain reaction (PCR) (Saiki et al., 1985, 1988) (Perkin Elmer Cetus, Norwalk, Conn.). Commercially synthesized primers (Synthecell, Rockville, Md.) specific for the isopentenyl transferase (*ipt*) gene (Barker et al., 1983) were used in PCR (Fig. 1). The primers were 18 and 20 nucleotides long and, with respect to the ATG, commenced at nucleotide 28 and 511. The PCR reactions were done in an automated thermal cycler (Perkin Elmer Cetus). For the initial three cycles, DNA was denatured at 94°C for 1 min, primers were annealed at 42°C for 2 min, and the enzyme allowed to extend the DNA at 52°C for 6 min. Subsequent 45 cycles of denaturation, annealing, and extension were done at 94, 45, and 72°C, respectively, all for 1 min. In addition, 1 μg of total RNA was pretreated with six units of RNase-free DNase in the presence of 28 units of RNasin (Stratagene, La Jolla, Calif.) (Grillo and Margolis, 1990) and reverse transcribed into cDNA by addition of 100 pmole random hexamers and 200 units of MuLV reverse transcriptase (Hawkins, 1988; Rappolee et al., 1988) (Boehringer Mannheim; BRL, Gaithersburg, Md.) in a final volume of 20 μl 1 $\times$ PCR buffer and 1 mM deoxynucleotides. The reaction was allowed to proceed for 1 h at 42°C and was terminated by heating at 90°C for 10 min. The cDNA was then amplified by PCR. Ten to 30 μl of PCR products from each reaction mixture were electrophoresed on 1.7% agarose gels and transferred to Zeta-Probe membranes in 0.4 M NaOH. Prehybridization and hybridizations were done as described above. A 570 base pair *AvaII/HincII* DNA fragment homologous to the amplified 5' region of the *ipt* gene was used as probe.

Zeatin/zeatin riboside assays. Plant leaf extracts were prepared in 80% methanol and purified on immunoaffinity columns packed with monoclonal antizeatin riboside antibody (De Danske Sukkerfabrikker, Copenhagen). The immunoaffinity column material has a highly selective adsorption of zeatin and related cytokinins combined with very low nonspecific binding. Bound zeatin and zeatin riboside were eluted and quantified by enzyme-linked immunosorbent assay as previously reported (Smigocki, 1991; Smigocki and Owens, 1988, 1989).

Octopine assay. Shoot and callus tissues were analyzed for the production of octopine (Aerts et al., 1979; Hammerschlag et al., 1989). About 50 mg (fresh weight) of tissues was cultured for 4 to 6 days on MS medium containing 5 mM arginine. Plant extracts (5 μl) were analyzed by electrophoresis on Whatman 3MM paper, stained in phenanthrenequinone, and visualized by ultraviolet illumination.

Results

Regeneration of shoots. The percentage of embryos callusing (averaged for all media) after infection with strain *tms328::Tn5* or Al36 was 49% and 36%, respectively, in comparison to 30% for noninfected embryos. Only embryos infected with strain *tms328::Tn5* produced callus in the absence of any phytohormones in the medium (Fig. 2). Callus was induced from control embryos and from embryos exposed to strain Al36 only after exposure to either 0.1 mg NAA/liter or 0.1 mg 2,4-D/liter. Shoot induction from either infected or noninfected embryos occurred best when callus was cultured either in light on MS

Fig. 2. Callus production from embryos cocultivated with *A. tumefaciens* strain *tms328::Tn5* and cultured for 6 weeks on hormone-free medium.

Fig. 3. Axillary shoot production from peach shoots regenerated from embryos infected with the shooty mutant strain *tms328::Tn5* and cultured on shoot elongation medium.

medium with 0.5 mg BA/liter and 0.05 mg NAA/liter followed by MS medium with 2.5 mg BA/liter and 0.01 mg NAA/liter or in darkness on MS medium with 0.05 mg NAA/liter followed by MS medium with 0.01 mg NAA/liter.

Two separate experiments, performed in Summer 1987, yielded 23 shoots from 48 calli. Of these, nine were from the *tms328::Tn5* infections and seven each from Al36 infections and control tissues. Only four shoots survived, two from the *tms328::Tn5* infection (99-1 and 94-1) and two from the controls (22-1). The experiment was repeated in Summer 1988, and similar results were obtained. Shoots resulting from *tms328::Tn5* infections (99-1, 94-1, and RG40) exhibited increased frequency of branching on shoot elongation medium (Fig. 3) and were more difficult to root on rooting medium than controls (RG3). Frequencies of rooting of axillary shoots derived from the original 94-1 and 99-1 shoots were 50% (12/22) and 60% (9/15), respectively, as compared to 81% (17/21) for the control (RG3). The young plants of 94-1 and 99-1 (Fig. 4A) tended to be smaller and shorter (left two shoots) than the controls (RG3,

Fig. 4. Plants grown from axillary shoots of 94-1 and 99-1 (derived from *A. tumefaciens* strain *tms328::Tn5*-infected embryos) exhibiting slower initial growth in soil when compared to the control, RG3. (A) Plants derived from 94-1 (left two) and RG3 (right two) after 2 weeks in soil. (B) Plants from 99-1 (left two) and RG3 (right two) after 5 weeks in soil.

right two shoots). After 1 month in soil, the difference in height was not as apparent as the increased frequency of branching (Fig. 4B, left two shoots).

Nucleic acids analyses. RNA extracted from callus and leaves of regenerated plants was analyzed for T-DNA transcript levels. Using the 7.3 kb *EcoRI* fragment from the T-DNA region as probe, one would expect to detect only the *ipt* gene transcripts since the *iaaM* gene is inactivated in strain *tms328::Tn5*. Transcripts *6a* and *6b* would not be detected since these are only predicted from the T-DNA region sequence analysis (Fig. 1). Low levels of the *ipt* gene message were detected by northern analysis under low stringency conditions used in washing the blots (Fig. 5). Hybridizations were most likely not complicated by contaminating *Agrobacterium* in plant material since, after the initial passages, the shoots were propagated for more than 2 years on media lacking cefotaxime without showing bacterial outgrowth. In addition, no outgrowth was observed after culturing of shoot explants in bacterial media. When genomic DNAs extracted from these tissues were slot-blotted on a filter membrane and hybridized with the same probe, weak signals were detected (data not shown). However, Southern blot analysis of the DNAs digested with *EcoRI* or *BamHI* restriction enzymes, which would confirm integration of the foreign DNA into the peach genome, did not reveal any hybridization with this probe except with the single and five-copy reconstructions used on the blot as positive controls (data not shown).

PCR analyses of nucleic acids. PCR amplification of cDNAs derived from RNA target sequences was used to detect specific RNA messages present at a very low copy number (Hawkins, 1988; Rappolee et al., 1988). Using this technique, the detection and analysis of gene expression can be accomplished at the RNA level. The RNA was first pretreated with DNase under

Fig. 5. Northern blot of peach RNA from transformed (+) and control (-) shoots probed with the *EcoRI* fragment from the T-DNA region (Fig. 1). The expected size of the cytokinin gene transcript is ≈ 800 bp and is indicated by the arrow. Tobacco tissues transformed by the cytokinin gene were used as positive control.

Fig. 6. Southern blot of PCR products of cDNA from RNA of transformed (99-1, RG-40, 94-1) and control (22-1) shoots probed with a 570-bp fragment specific for the *ipt* gene. The expected PCR product for the undigested (U) DNA is 503 bp (lanes 1, 5, 9, 13, and 17). For DNA digested with the restriction enzymes *HpaII* (H), *PstI* (P), and *BamHI* (B), the corresponding pairs of fragments are 402 and 101 (lanes 2, 6, and 10), 414 and 89 (lanes 3, 7, 11, and 18), and 265 and 238 (lanes 4, 8, 12, and 19), respectively. The amount of DNA in lanes U, 18, and 19 is one-third to one-fifth of that in the other lanes.

specified conditions to ensure that the product resulting from the RNA-PCR is due to amplification of cDNA that has been synthesized during the reverse transcriptase reaction. RNA from leaves and callus was subjected to RNA-PCR using primers specific for the cytokinin gene. When used together, these primers specifically amplify a 503-bp region of the *ipt* gene. Figure 6 shows a Southern blot of PCR products of four peach cDNAs that were hybridized with a 570-bp probe specific for the amplified *ipt* gene region. Three of the RNAs from shoots regenerated from *tms328::Tn5*-infected embryos (99-1, RG-40, and 94-1) show the expected 503-bp DNA fragment in the uncut (U) lane. In addition, when these PCR products were digested with restriction enzymes known to specifically cut the *ipt* gene within this 503-bp fragment, DNA bands of expected sizes were detected. Pairs of fragments obtained were 402 and 101, 414 and 89, and 265 and 238, for the *HpaII* (H), *PstI* (P), and *BamHI* (B) digestions, respectively (Fig. 6). DNA in lane 11 appears to be only partially digested with the *PstI* enzyme. The other bands that hybridized with this probe appear not to be specific to the *ipt* gene. To eliminate the possibility that samples were contaminated and not transformed with the cytokinin gene,

three or four preparations of RNAs from each sample were made at different times from different sources using different PCR reagents. Consistently, each RNA sample showed a similar pattern of PCR products. No significant hybridization between RNA from a control shoot, 22-1, and the *ipt* probe was detected. In addition, numerous genomic DNAs prepared from the same samples were also amplified using PCR. In all cases, similar results were obtained (data not shown).

Cytokinin levels. Leaf tissues were analyzed for zeatin and zeatin riboside cytokinins since these are the major cytokinins in *ipt*-transformed shoots and crown galls. Regenerated shoots 99-1 and 94-1 averaged 113 and 91 pmoles zeatin plus zeatin riboside per gram of leaf tissue, respectively, as compared to 2 pmoles $\cdot g^{-1}$ in the control (22-1) (Table 1). This represents a greater than 56-fold increase in these cytokinins in 99-1 and a 46-fold increase in 94-1. Callus tissues and in vitro-propagated shoots were not used in these analyses since they are maintained on media that include low levels of cytokinin.

Octopine synthase assay. All callus and leaf tissues were screened for octopine production encoded for in the T-DNA region (Garfinkel et al., 1981). All tissues tested were negative for octopine (data not shown).

Discussion

The *Agrobacterium*-mediated gene transfer method has been used successfully to transform and regenerate woody plants such as walnut (*Juglans regia* L.) (McGranahan et al., 1988), apple (*Malus pumila* Mill.) (James et al., 1989), and poplar (*Populus alba* $\times$ *P. grandidentata*) (Fillati et al., 1987). Our work demonstrates regeneration of peach plants from embryo-derived callus infected with the shooty mutant strain of *A. tumefaciens*. We chose to work with the embryo callus because, currently, only roots have been induced on callus derived from explants of mature plants (Hammerschlag, 1988). The shooty mutant strain of *A. tumefaciens* has been shown to induce regeneration of shoots from tumors in tobacco (*Nicotiana tabacum* L.) (Van Slogteren et al., 1983), *Physalis minima* (Steffen et al., 1986), and *Kalanchoe daigremontiana* (Memelink et al., 1987). It appears, from our results, that the introduction of the functional cytokinin gene into peach cells may have altered the cytokinin : auxin ratio in planta so as to favor callus formation on phytohormone-free medium followed by regeneration of shoots from embryo-derived peach callus. Regeneration of shoots or roots from many tissue-cultured cells can be induced by increasing or decreasing, respectively, the cytokinin : auxin ratio of the growth medium (Skoog and Miller, 1957) or by introducing genes that are involved in phytohormone biosynthesis directly into plant cells (Klee et al., 1987; Smigocki and Owens, 1988). The transformants were distinct from normal untransformed shoots in that they had increased frequency of branching and were more difficult to root, as expected of tissues enriched in cytokinins. Low levels of the cytokinin gene transcripts were detected by

Table 1. Concentration of zeatin (Z) and zeatin riboside (ZR) in leaves from peach plants regenerated after infection with *A. tumefaciens* strain *tms328::Tn5*.

Regenerated shoots	Z + ZR (pmoles $\cdot g^{-1}$)	
	Rep 1	Rep 2
99-1	125	100
94-1	105	77
22-1 (control)	3	1

northern analysis and further confirmed by the reverse transcriptase PCR that amplifies low-copy-number mRNA. Similarly, PCR of genomic DNA resulted in amplification of DNA fragments specific for the cytokinin gene. A corresponding increase in zeatin and zeatin riboside levels was also observed in leaves from these plants. However, despite positive signals on slot blots, no hybridization was observed on Southern blots of genomic DNA with a T-DNA-specific probe to indicate integration of the foreign DNA into the peach cell genomes. All shoots were negative for octopine production, which is encoded for within the T-DNA region. Others have reported similar findings, and, most recently, James et al. (1989) reported a very low frequency of opine production after assaying hundreds of putative transgenic apple shoots.

The regenerated plants may be chimeras composed of transformed and untransformed cells. This possibility is plausible because crossfeeding of hormones does occur in plant cells, and the shooty mutant strain of *Agrobacterium* carries no marker for selection of transformed cells such as the neomycin phosphotransferase gene regulated by a eukaryotic promoter. Such chimeras would most likely yield weak signals on northern blots, but would be amenable to PCR technology for amplification. It is unlikely that the source of the positive signals is from residual *Agrobacteria*, since for years no bacterial outgrowth has been observed from regenerated plant tissues being cultured on media free of antibiotics or from leaf pieces cultured in bacterial media. In addition, if plant genomic DNA preparations from transformed tissues were in fact contaminated with *A. tumefaciens* DNA, positive signals should have been observed on the Southern blots. In the past, chimeric tissues have been subjected to single-cell cloning from protoplasts to select out the transformed cells. Such an approach is presently not an option for peach since techniques do not exist for regeneration of plants from protoplasts.

In conclusion, we have demonstrated regeneration of peach plants in which some of the cells are expressing a foreign gene introduced by an *Agrobacterium* strain. Since integration of the foreign DNA into the peach genome appears to have occurred at a low frequency, experiments with vectors carrying selectable markers in addition to the cytokinin gene may enhance regeneration of transgenic plants with fewer untransformed cells. Such a system potentiates the transfer of other agriculturally important genes, such as those for disease resistance, into peach. In addition, cytokinin effects on peach morphogenesis and growth habit should be further investigated as a continuing effort to improve morphogenesis from cell cultures and to explore the possibility of introducing a compact growth habit into peach cultivars.

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