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Agrobacterium-mediated transformation of *Citrus* stem segments and regeneration of transgenic plants

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Summary. A method for *Agrobacterium*-mediated transformation of Citrus and organogenic regeneration of transgenic plants is reported. Internodal stem segments were co-cultured with *Agrobacterium* harboring binary vectors that contained the genes for the scorable marker ß-glucuronidase (GUS) and the selectable marker NPT-II. A low but significant percentage (≤ 5%) of the shoots regenerated in the presence of 100 µg/ml kanamycin were GUS^+. Polymerase chain reaction (PCR) analysis confirmed that GUS^+ shoots contained T-DNA. Two plants established in soil were shown to be transgenic by Southern analysis.

Key words: Carrizo citrange - Key lime (*C. aurantifolia*) - Genetic transformation

Introduction

A reliable genetic transformation system is important for the introduction of foreign genes into plants. The two most widely used methods of plant transformation are introduction of naked DNA into protoplasts and *Agrobacterium*-mediated transformation of various explants. Kobayashi and Uchimiya (1989) transformed *Citrus sinensis* (L.) Osb. protoplasts via polyethylene glycol-mediated DNA uptake and regenerated transformed embryogenic callus. The transformation efficiency was estimated to be approximately 1.0×10^{-6}. More recently, Vardi et al. (1990), using essentially the same procedure with rough lemon (*C. jambhiri* Lush.) protoplasts, obtained nine stably transformed embryogenic clones, two of which regenerated an unspecified number of transformed plants. Frequency of transformation was not estimated. Finally, Hidaka et al. (1990) transformed embryogenic nucellar *C. sinensis* callus with *Agrobacterium* and regenerated at least one transgenic plantlet. The best transformation frequency was estimated to be 7.0×10^{-3}.

One limitation of these methods is the requirement for embryogenic citrus callus. Callus production is not possible for some citrus types (Gmitter and Moore 1986) and is frequently very difficult even with amenable genotypes. In this report, we present an alternative, and potentially more direct, method for citrus transformation that involves *Agrobacterium*-mediated transformation of internodal stem segments followed by regeneration of shoots. Factors that affect frequency of transformation in this system were extensively analyzed.

Materials and methods

Plant Materials. Citrus seeds were purchased from Willits & Newcomb (PO Box 428, Arvin CA 93203). Seeds were disinfected for 10 min in 70% ethanol, then for 20 min in 20% Clorox + 2 drops of Tween-20 per 100 ml, and were then rinsed 3 times with sterile distilled water. Seeds were placed individually in 150 x 25 mm tubes containing half-strength Murashige and Tucker (1969) medium (MT), 2.5% sucrose, and 0.6% Difco Bacto-agar, pH 5.7. The cultures were maintained at 27°C with 16 h of cool-white fluorescent light (76 $\mu mol.s^{-1}.m^{-2}$).

Bacterial Strains and Plasmids. A variety of wild type *Agrobacterium tumefaciens* strains, kindly provided by Dr. Bill Gurley (University of Florida), were used to test the susceptibility of several *Citrus* genotypes to *Agrobacterium* infection. Seedling hypocotyls were injected with overnight bacterial cultures. The plants were then kept in an illuminated humid chamber for 21 days and scored for the presence of tumors. The engineered *Agrobacterium* strains used for transformation experiments were based on the host strain EHA101 (Hood et al. 1986). The binary vector plasmids pGA472 (An et al. 1985) and pMON9793 were introduced individually into EHA101 by direct DNA uptake (An 1987). Plasmid pMON9793 is a derivative of pMON505 in which a chimeric gene containing a mannopine synthase promotor, the coding region for ß-glucuronidase (GUS) (Jefferson 1987), and the nopaline synthase (NOS) 3'

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polyadenylation signal were cloned into the multilinker (Rogers et al. 1987; C. Gasser, personal communication). The efficacy of the *Agrobacterium*-vector plasmid combinations used in these experiments was verified by transformation of tobacco prior to experiments with *Citrus*.

Cultures of wild type and engineered *Agrobacterium* strains to be used for transformation experiments were initiated from glycerol stocks and grown overnight at 28°C in YEP medium (An 1987) containing the appropriate antibiotics to late or post log phase. Bacteria were then collected by centifugation for 10 min at 2000 x g and resuspended in one-half volume of antibiotic-free YEP.

Transformation/Selection/Regeneration. Explants were cut from 2 to 4 month old citrus seedlings grown aseptically *in vitro*. Leaf disks were cut aseptically with a hole punch and placed on regeneration medium [MB, MT with 5% sucrose, 5 mg/l 6-benzyladenine (BA), and 0.8% agar, pH 5.7]. Internodal stem pieces (1 cm) were inserted vertically into MB medium with either the basal (B↑) or apical (A↑) end of the segment protruding. The protruding ends were inoculated with *Agrobacterium* by placing a small drop of the culture on the end of the segment with a syringe. After a 2 to 3 d coculture period, the stem pieces were transferred to 20 x 100 mm petri plates containing MB medium supplemented with 200 µg/ml mefoxin (MB+M) (mefoxin = cefoxitin sodium, Merck, Sharpe & Dohme) to inhibit further growth of the vector bacteria. For selection, the medium also contained 100 µg/ml kanamycin (MB+MK). Cultures were maintained under the conditions described above for germinating seeds. At 4 weeks, shoots from the stem segments were harvested for testing and the segments were transferred to fresh medium. At 8 weeks, shoots were again harvested and the segments themselves analyzed or discarded. Putatively transformed shoots were rooted in culture cups containing 50 ml of sterile potting soil moistened with 25 ml half-strength MT. In preliminary experiments, rooting was also evaluated on MT/agar medium containing various levels of NAA (0, 0.01, or 0.1, mg/l).

Analysis of Putatively Transformed Tissue. Regenerated shoots were assayed for GUS activity immediately upon excision from stem explants. Freehand sections, cut from the basal ends of the shoots, were placed in round bottom microtiter plates containing 23 µl of stain (1 mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide in 0.1 M $NaPO_4$ buffer, pH 7.0 with 10 mM Na_2EDTA (Jefferson 1987) per well. Tissue was stained for no longer than 4 or 5 h at 37°C because extended periods of staining resulted in many false positives; frequently the entire surface of the section would stain intensely blue. Transformed tobacco and nontransformed citrus tissue were included as positive and negative controls, respectively, with each set of assays. After clearing and fixing the tissue with 100 µl of 95% ethanol:glacial acetic acid (3:1 v/v), it was possible to unambiguously detect even small GUS^+ sectors.

PCR was used to detect specific DNA sequences from small amounts of tissue. DNA was extracted from stained, fixed material using the CTAB (cetyl trimethyl ammonium bromide) method of Rogers and Bendich (1985). DNA from fresh shoot or plant tissue was extracted by this method or by one slightly modified from Dellaporta et al. (1983) (Durham 1990). PCR amplification from fixed tissue was performed on an unquantified amount of DNA; when larger amounts of tissue were available for extraction, approximately 0.2 µg of DNA was used. Reaction mixtures (100 µl) contained 200 µM dNTPs, 0.1 µM of each primer, 2.0 units of Taq polymerase (Promega), and Taq polymerase buffer (Promega). Samples were heated to 94° for 4 min and then subjected to 30 cycles of 2 min at 96°, 2 min at 50°, and 3 min at 72°. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide.

The primers described by Lassner et al. (1989) were used to PCR-amplify a 336 bp T-DNA specific fragment from the NOS gene. Primers 5'TGGAGTACCTGTCCCGTAAGG3' and 5'AGCTGGCGCTAGTAGGTCTAA3' were used to PCR-amplify a 321 bp fragment of the EHA101 *virD* gene, which is highly homologous at the DNA level to that of pTiA6 (Komari et al. 1986, Jayaswal et al. 1987). We verified with PCR of a dilution series of vector bacteria that the NOS product and the *virD* product from the bacteria amplify to comparable amounts under our conditions.

For Southern analysis, DNA was extracted from approximately 100-200 mg of fresh leaf tissue using the modified Dellaporta et al. (1983) procedure. Southern analysis on DNA (approximately 1 µg) was conducted on nylon membranes (Amersham) (Maniatis et al. 1982) according to Church and Gilbert (1984) with the exception that following hybridization, the membranes were subjected to two 1-h washes at 65°C with 0.5 x SSPE, 1% SDS (M. Roose, personal communication). The probe was a 1.1 kb *Bam*HI→*Sst*II fragment containing the NPTII gene isolated from the pMON505 derivative pMON507 and labeled with ^{32}P using a random primer labeling kit (Boehringer Mannheim Biochemicals).

Results and Discussion

Our objective was to develop an *Agrobacterium*-based transformation system similar to the leaf disk procedure of Horsch et al. (1985). Accordingly, we screened several citrus genotypes for their ability to regenerate shoots from various explants. Our results showed that Carrizo citrange [*C. sinensis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.], Swingle citrumelo (*C. paradisi* Macf. x *P. trifoliata*), and Key lime [*C. aurantifolia* (Christm.) Swing.] were sufficiently regenerable for transformation experiments; Hamlin sweet orange [*C. sinensis* (L.) Osb.] regenerated poorly and has not been examined further. Experiments with leaf disks and stem segments of different lengths showed that stem segments ~1 cm in length were the most effective in shoot production. Shorter segments (0.2 - 0.5 cm) produced mostly callus and buds; 0.7 cm leaf disks produced very few shoots. The experiments reported below used 1 cm segments. Shoots arose from the cut ends with little or no callus production.

Citrus genotypes were also tested for susceptibility to various *A. tumefaciens* strains by injecting seedling hypcotyls with bacterial cultures. All tested citrus types were susceptible to *Agrobacterium* as evidenced by the production of tumors at the wound site following inoculation with most wild-type strains (data not shown). *A. tumefaciens* strain A281, which contains the oncogenic Ti plasmid pTiBo542, gave rise to very large, rapidly growing tumors. Because of the apparent hypervirulence of pTiBo542, EHA101, a non-oncogenic derivative of this strain (Hood et al. 1986), was used in combination with the binary plasmids pGA472 and pMON9793 in the transformation experiments below.

Production of transformed shoots. Transformation experiments involved inoculating cut ends of excised stem segments with vector bacteria and then culturing inoculated segments on shoot regeneration medium in the presence of 100 µg/ml kanamycin. Because both of the above binary plasmids contained the NPTII

Table 1. Numbers and percentages of green shoots and GUS$^+$ shoots produced when Carrizo citrange, Swingle citrumelo, and Key lime stem segments oriented with their apical (A↑) or basal (B↑) ends protruding from the medium were inoculated with EHA101(pMON9793) and cultured with and without selection with kanamycin.

Genotype	Explant orientation	Number segments evaluated	No. (%) segments with green shoots	Total no. shoots produced	No. (%) GUS$^+$ shoots	Number (%) segments w/ GUS$^+$ shoots
With selection (100 µg/ml kanamycin) - MB+MK medium						
Carrizo[a]	B↑	490	87 (18)	154	8 (5.2)	6 (1.2)
Carrizo[b]	A↑	1002	437 (44)	1026	18 (1.8)	17 (1.7)
Swingle[c]	B↑	960	162 (17)	382	5 (1.3)	5 (0.5)
Swingle	A↑	84	36 (43)	124	0 (0.0)	0 (0.0)
Key	A↑	750	68 (9)	115	2 (1.7)	2 (0.3)
Without selection - MB+M medium						
Carrizo	B↑	111	32 (29)	58	0 (0.0)	0 (0.0)
Carrizo[a]	A↑	288	150 (52)	753	3 (0.4)	2 (0.7)
Swingle[c]	B↑	224	71 (32)	180	1 (0.6)	1 (0.5)
Swingle	A↑	13	11 (85)	54	0 (0.0)	0 (0.0)
Key	A↑	64	8 (12)	18	0 (0.0)	0 (0.0)

[a]These figures are a summary of three experiments.
[b]These figures are a summary of four experiments.
[c]These figures are a summary of two experiments.

gene, which confers resistance to kanamycin, we anticipated that virtually all of the regenerating shoots would be transformed. In treatments where uninoculated stem segments were cultured on 100 µg/ml kanamycin, the number of shoots produced was reduced by greater than 95% (data not shown). Segments inoculated with vector bacteria and placed on kanamycin-containing medium produced many shoots that quickly bleached while others remained green. Unfortunately, most of the green shoots were apparently not transformed and had "escaped" selection. This was shown most clearly in the experiments with the GUS-containing pMON9793 vector plasmid, in which transformed shoots were identified early in the protocol by GUS staining. As can be seen in Table 1, 95% or more of the shoots regenerated were GUS$^-$; thus regeneration in the presence of kanamycin is not a reliable indicator of transformation in this system.

Evidence that GUS$^+$ staining is a reliable indicator of transformation includes: 1) the *Agrobacterium* strain/vector plasmid combination EHA101(pMON9793) does not stain positive for GUS; 2) staining incubation periods restricted to 4 to 5 h eliminated false positives (see Methods); 3) PCR analysis confirmed that the GUS$^+$ shoots contained T-DNA. DNA was extracted from either very small (<20 mg fresh weight) leaf pieces from regenerated shoots or from fixed GUS$^+$-stained basal sections of regenerated shoots and subjected to PCR analysis (Fig. 1). Amplification was with primers for a segment of the NOS gene (Lassner et al. 1989), which is present in the pMON9793 T-DNA. In addition, we frequently conducted parallel PCR analyses with primers specific for a region of the *virD* locus (not shown). Because these sequences are present on the resident Ti plasmid of the *Agrobacterium* vector, but are not transferred to the plant, they provide a control for the presence of contaminating vector bacteria that could conceivably give rise to the NOS PCR product. DNA from putatively transformed plant tissue amplified with the *vir* region primers never gave rise to a product.

The regeneration of escape shoots under kanamycin selection is not unique to citrus. It has been suggested that such escapes arise from nontransformed cells that are protected from the selective agent by transformed cells in the explant (Jordan and McHughen 1988). However, this may not be the cause of shoot production in citrus. At the end of several experiments the inoculated ends of the stem segments were assayed for GUS$^+$ sectors large enough to be viewed under a dissecting microscope (Table 2). Only 4 to 8 % of the segments contained GUS$^+$ sectors, whereas shoots regenerated from a much higher percentage of segments. It may be possible that a small number of surviving bacteria inactivate the kanamycin in the medium, thereby protecting the plant cells. Experiments are in progress to test this possibility.

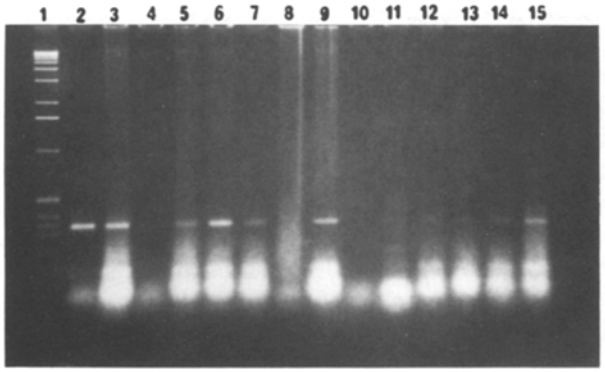

Fig. 1. PCR analysis of putatively transformed, GUS$^+$ tissue from Carrizo citrange shoots regenerated from internodal stem segments inoculated with EHA101(pMON9793). DNA isolated from green shoot leaf tissue or from GUS stained, fixed stem sections was amplified with primers specific for a region of the NOS gene contained in the T-DNA. The 336 bp PCR products (indicated with an arrow) were visualized on an ethidium bromide-stained 2% agarose gel. Lane 1, 1 kb ladder molecular weight markers; lane 2, plasmid DNA from EHA101(9793); lanes 3 through 9, DNA from leaf tissue from seven putatively transformed shoots; lanes 10 through 15, DNA from stained, fixed sections of six putatively transformed shoots. PCR products were detected from all samples except those in lanes 4, 8, 10 and 11. Lack of visualization of product in these lanes may be due to insufficient template DNA. Nontransformed citrus controls and controls lacking template DNA are not shown on this gel but were present when amplifications were done; none of the negative controls yielded a product.

The GUS gene has become indispensable in our experiments because of the ease with which it can be used to identify transformed tissue at an early stage. This reliable scorable marker has allowed us to examine several factors that influence the production of transformed shoots: plant genotype, presence of the selective antibiotic, and stem segment orientation. Production of transgenic shoots was not genotype-dependent; GUS$^+$ shoots were produced from all three citrus genotypes tested (Table 1). Selection with kanamycin was not very effective, but it did appear to increase the number of GUS$^+$ shoots obtained (Table 1). Finally, the orientation of segments in the medium affected GUS$^+$ shoot production (Table 1). When segments were oriented in a vertical position, shoots were only produced from the portion of the segment that protruded from the medium. Carrizo and Swingle segments oriented with their apical ends protruding from the medium (A↑) produced more shoots than when they were placed with their basal end upright (B↑). However, a higher frequency of GUS$^+$ shoots was obtained from B↑ segments. Since Carrizo and Swingle are highly regenerable genotypes, it appears best to culture their segments B↑. In the case of less regenerable genotypes like Key lime, it may be preferable to culture the segments A↑ to maximize shoot production.

Our experiments identified two limiting steps in the production of GUS$^+$ shoots. First, as mentioned above, the transformation rate was low; only 4 to 8% of the inoculated segments evaluated contained GUS$^+$ sectors (Table 2). Second, generally only about 10% containing GUS$^+$ sectors gave rise to GUS$^+$ shoots (Table 2).

Regeneration of Transgenic Plants. A continuing difficulty has been rooting of regenerated shoots, especially since many of the putatively transformed shoots are small and weak. Rooting efficiency was evaluated on medium containing various levels of NAA, on half-strength medium with no growth regulators, and on sterilized soil moistened with half-strength medium. The highest percentage of rooting was obtained in soil. For example, in one large experiment with shoots from uninoculated stem segments, rooting percentages were 9% (19/202) on medium containing 1.0 mg/l NAA, 42% (90/215) on half-strength medium with no growth regulators, and 58% (65/112) in soil. Therefore rooting of shoots obtained from inoculated segments was conducted in sterile soil.

To date, using the described protocol, we have produced two citrus plants in soil that we have shown to be transgenic by Southern analysis. One was identified by a Southern analysis screen of 35 plants; this plant regenerated from a segment inoculated with EHA101(pGA472). This plant appears to contain multiple copies of T-DNA with at least some T-DNA rearrangement. As shown in Fig. 2, digestion of plant

Table 2. Numbers and percentages of stem segments inoculated with EHA101(pMON9793) that contained GUS$^+$ tissue versus those that produced GUS$^+$ shoots.

Genotype	Medium	Number segments evaluated	No. (%) segments producing green shoots	No. (%) segments producing GUS$^+$ tissue	No. (%) segments producing GUS$^+$ shoots
Swingle	MB+MK	795	116 (15)	39 (4.9)	3 (0.4)
Swingle	MB+M	187	61 (33)	10 (5.3)	1 (0.5)
Key	MB+MK	750	68 (9)	29 (3.9)	2 (0.3)
Carrizo	MC+MK[a]	456	312 (68)	38 (8.3)	16 (2.1)

[a]The medium used in this experiment was MT with 1500 mg/l malt extract, 0.01 mg/l 2,4-dichlorophenoxyacetic acid, and 2 mg/l BA.

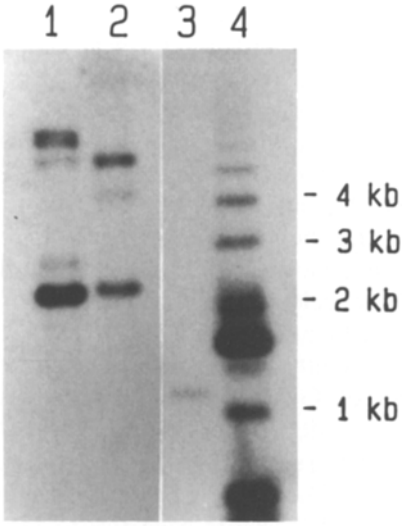

Fig. 2. Southern analysis of a Carrizo citrange plant regenerated from a stem segment inoculated with EHA101(pGA472). The probe was a 1.1 kb restriction fragment from the NPT-II region of the T-DNA. Lane 1, Carrizo DNA cut with *Bam* HI to generate an internal T-DNA fragment; lane 2, Carrizo DNA cut with *Hind* III to generate a right border fragment; lane 3, single copy reconstruction with the NPT-II fragment; lane 4, molecular weight markers. No bands were obtained when nontransformed Carrizo DNA was hybridized with the probe (not shown).

DNA with *Bam* HI yielded the expected internal T-DNA fragment, an intense band at ~2.2 kb, but it also produced additional larger bands of lesser intensity (lane 1). Comparison of the intensity of these bands with a single copy reconstruction (lane 3) indicated that multiple copies of the T-DNA are present per genome. Plant DNA restricted with *Hind* III to yield a T-DNA right border fragment produced several hybridizing bands all of which were more intense than the single copy reconstruction (Fig. 2, compare lanes 2 and 3). None of these bands were the same size as that produced by *Hind* III restriction of the pGA472 plasmid (not shown), i.e. they derived from a T-DNA/plant DNA border. However, the smallest band was too small to represent an intact border fragment even in the unlikely case that the T-DNA inserted immediately adjacent to a *Hind* III site in the plant DNA. This suggests that some of the T-DNA has undergone internal deletion. Additional Southern analyses in which *Eco* RI was used to produce a border fragment confirm this interpretation because they yielded bands much smaller that the theoretical minimum border (data not shown).

One GUS^+ Carrizo plant regenerated from a segment inoculated with EHA101(pMON9793) has also grown sufficiently for leaf tissue to be removed for Southern analysis. This plant, although producing a different pattern of bands, was similar to the first plant in that it was multiply transformed and that the T-DNA appeared to have undergone rearrangement (data not shown).

Future prospects. We have developed a protocol to produce transgenic citrus plants from cultured stem segments. Although transformation frequencies are low, this protocol should allow transfer of single genes into any citrus scion or rootstock cultivar that regenerates via organogenesis with reasonable efficiency. Such experiments are in progress. Further experiments are also being done to identify ways to increase transformation frequencies and shoot survival, to reduce the number of escapes during regeneration under selection, and to increase rooting frequencies.

However, this method of citrus transformation, along with other currently published protocols, produces transgenic plants that are juvenile. These plants will have to be grown in the field for a number of years before the horticultural traits of the mature plants can be evaluated. A method of transforming mature tissue of tree crops would be a great asset for transformation studies in these species.

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