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Producing Transgenic 'Thompson Seedless' Grape (*Vitis vinifera* L.) Plants

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Abstract. **Transgenic grape plants were regenerated from somatic embryos derived from leaves of in vitro-grown plants of 'Thompson Seedless' grape (*Vitis vinifera* L.) plants. Somatic embryos were either exposed directly to engineered *Agrobacterium tumefaciens* or they were bombarded twice with 1-μm gold particles and then exposed to *A. tumefaciens.* Somatic embryos were transformed with either the lytic peptide Shiva-1 gene or the tomato ringspot virus (TomRSV) coat protein (CP) gene. After cocultivation, secondary embryos proliferated on Emershad/Ramming proliferation (ERP) medium for 6 weeks before selection on ERP medium containing 40 $\mu g \cdot mL^{-1}$ kanamycin (kan). Transgenic embryos were identified after 3 to 5 months under selection and allowed to germinate and develop into rooted plants on woody plant medium containing 1 μM 6-benzylaminopurine, 1.5% sucrose, 0.3% activated charcoal, and 0.75% agar. Integration of the foreign genes into these grapevines was verified by growth in the presence of kanamycin (kan), positive β-glucuronidase (GUS) and polymerase chain-reaction (PCR) assays, and Southern analysis.**

In the United States, seedless grapes represent about 80% and 98% of the total table and raisin grape production, respectively (California Table Grape Commission, 1995). Only a few seedless cultivars make up this production, of which 'Thompson Seedless' is the most important. This cultivar accounts for the most production of any single grape variety in the United States. In 1992, 'Thompson Seedless' was grown on 263,621 acres in California (California Agricultural Statistics Service, 1993). Thirty-five percent of the table grape production in California in 1994 was 'Thompson Seedless' (23,244,683 boxes, 10 kg/box). In 1993, 97% of the grapes grown for raisin production was 'Thompson Seedless' (Raisin Administrative Committee, 1994).

While improving grape is possible by conventional breeding, it is difficult and time consuming due to the 2- to 3-year generation cycle, the long period of time required for reliable progeny testing and selection, and inbreeding depression that prohibits selfing (Gray and Meredith, 1992). These characteristics make introgression of desirable traits into existing grape cultivars difficult if not impossible to achieve in an individual breeder's lifetime. Thus, the alternative, and potentially less time-consuming, approach of using gene transfer to insert desirable genes is particularly promising for grape, even considering the time necessary for field testing transgenic lines. The ability to improve the disease and/or pest resistance of a major grape cultivar such as 'Thompson Seedless' offers the possibility of improving a large portion of the grape production in a relatively short time, assuming that cultivar integrity would not be compromised by the transgene or the insertion event. Such a change could also reduce pesticide use for a significant portion of grape production.

Incorporating beneficial genes into established grape cultivars is a goal of many research programs (Baribault et al., 1990; Clog et al., 1990; Hébert et al., 1993; Mullins et al., 1990; Stamp et al., 1990). While several recent reports demonstrated successful regeneration of transgenic grapevines (Kikkert et al., 1995; Krastanova et al., 1995) there are no documented reports of successful transformation of a major *Vitis vinifera* scion cultivar. We recently reported the transformation of grape somatic embryos derived from zygotic embryos (Scorza et al., 1995). In the current report we describe the use of this method for transforming somatic embryos derived from 'Thompson Seedless' in vitro-grown leaves.

Materials and Methods

Plant materials and culture. Leaves from 'Thompson Seedless' in vitro cultures were used to produce somatic embryos following the method of Stamp et al. (1990). Briefly, expanding leaves 0.5 cm long excised from in vitro-grown shoots were cultured on a modified Nitsch and Nitsch (1969) (NN) medium with 5 μM 2,4-D, 1 μM BA, 60 $g \cdot L^{-1}$ sucrose, 2 $g \cdot L^{-1}$ activated charcoal, and 7 $g \cdot L^{-1}$ agar (ultrapure, USB, Cleveland), pH 5.7. After a 3- to 12-week culture period, somatic embryos formed. These were transferred to a modified Murashige and Skoog (1962) (MS) medium with 120 $g \cdot L^{-1}$ sucrose, 2 $g \cdot L^{-1}$ activated charcoal, and 7 $g \cdot L^{-1}$ agar, pH 5.7. After 3 years of continual culture on the modified MS medium with transfers each 4 to 6 weeks, somatic embryos were transferred to Emershad and Ramming proliferation (ERP) medium (Emershad and Ramming, 1994) for several transfers and then exposed to

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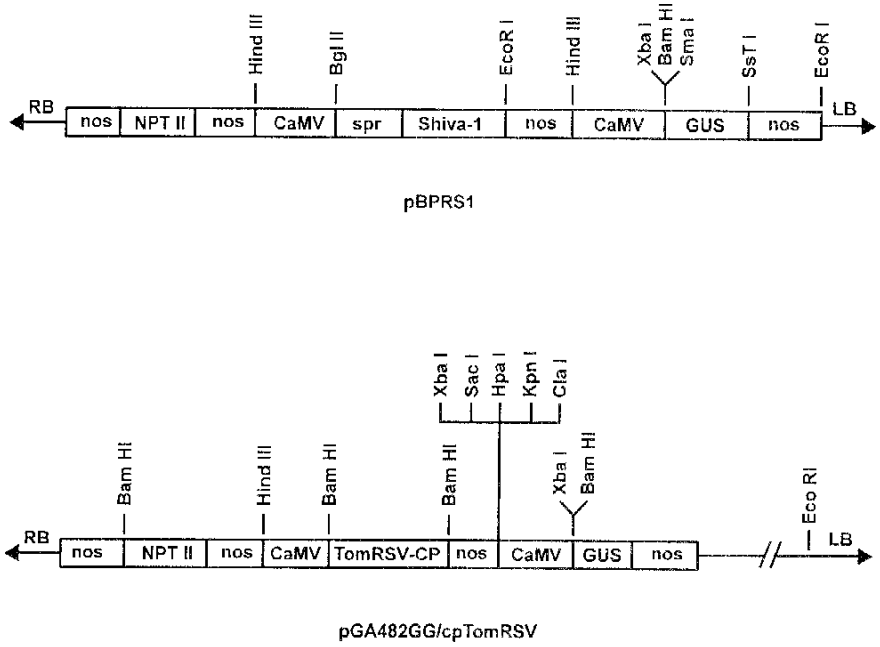

Fig. 1. Partial maps of the T-DNA regions of pGA482GG/cpTomRSV and pBPRS1.

transformation treatments. After cocultivation and selection on ERP medium, putatively transformed embryos were induced to germinate and root on woody plant medium (Lloyd and McCown, 1981) with 15 g·L^{-1} sucrose, 1 μM BA, 3 g·L^{-1} activated charcoal, and 7.5 g·L^{-1} agar, pH 6.0, following the protocol of Emershad and Ramming (1994).

Agrobacterium strain and plasmid descriptions. The *Agrobacterium tumefaciens* strains used were EHA101 and EHA105 (supplied by E. Hood) (Hood et al., 1986) containing plasmid pGA482GGcpTomRSV (Slightom, 1991; Slightom et al., 1991) or pBPRS1 (supplied by J. Norelli and H. Aldwinkle, Cornell Univ.), respectively. Both plasmids contained chimeric gusA [β-glucuronidase (GUS)] and kanamycin (kan) [neomycin phosphotransferase II (NPTII)] genes. Plasmid pGA482GG/cpTomRSV contained the tomato ringspot virus coat protein (TomRSV-CP) gene and pBPRS1 contained the Shiva-1 lytic peptide gene (Destefano-Beltran et al., 1990; Jaynes, 1993) (supplied by Helix Phytonetix, Inc., Baton Rouge, La.) (Fig. 1).

Transformation. Somatic embryos were either bombarded with gold microprojectiles and then exposed to *A. tumefaciens* as described previously (Scorza et al., 1995) or they were exposed to *A. tumefaciens* without prior bombardment. Microprojectile bombardment used the Biolistic PDS-1000/He device (Bio-Rad Laboratories). A total of 700 somatic embryos was separated into groups of 100. Each group was placed onto a 25-mm polycarbonate membrane in the center of a 100-mm petri plate containing ERP medium 24 h before bombardment. Somatic embryos were shot with 1.0-μm-diameter gold particles following the general procedures of Sanford et al. (1991) with parameters as previously described (Scorza et al., 1995). All plates were bombarded twice. Within 2 h of bombardment, embryos were cocultivated with *A. tumefaciens*. An additional 700 somatic embryos were exposed to *A. tumefaciens* without prior bombardment.

Cocultivation and selection. Putative transformants were cocultivated and selected as previously described (Scorza et al., 1995). Briefly, *A. tumefaciens* cultures were grown overnight at 28 °C in LB medium containing selective antibiotics for each plasmid. These cultures were centrifuged (5000× *g*, 10 min) and resuspended in a medium consisting of MS salts, 20 g·L^{-1} sucrose, 100 μM acetosyringone, and 1.0 mM betaine phosphate and shaken for about 6 h at 20 °C before use. After bombardment, or in the nonbombarded treatment, somatic embryos were immersed in the resuspended *A. tumefaciens* culture. After 15 to 20 min, the *A. tumefaciens* culture was removed and the somatic embryos were placed onto cocultivation medium (ERP medium containing 100 μM acetosyringone). Somatic embryos were cocultivated for 2 days and then washed with liquid ERP medium (without charcoal) containing 300 μg·mL^{-1} cefotaxime and 200 μg·mL^{-1} carbenicillin. Somatic embryos were plated on agar-solidified ERP medium (0.75% ultrapure agar) with the antibiotics as above. All somatic embryo cultures were allowed to proliferate for two passages (3 weeks each) before being placed onto selection medium. Selection was carried out on ERP medium (containing the above specified amounts of cefotaxime and carbenicillin) with 40 μg·mL^{-1} kan.

Transformation confirmation. Transformation of somatic embryos and shoots produced after somatic embryo germination was assayed by growth on kan-containing medium and through a histological GUS assay (Jefferson 1987). Leaves sampled from plants growing in vitro were cultured for 1 week in liquid LB medium to assay for the presence of contaminating *A. tumefaciens*. After rooting and transfer to the greenhouse, plants were subjected to PCR and Southern analyses.

PCR amplification was conducted on DNA isolated from leaves of putatively transformed grape plants. Specific oligonucleotide primers for TomRSV-CP and Shiva-1 gene sequences were used to identify the presence of these genes in DNA from the

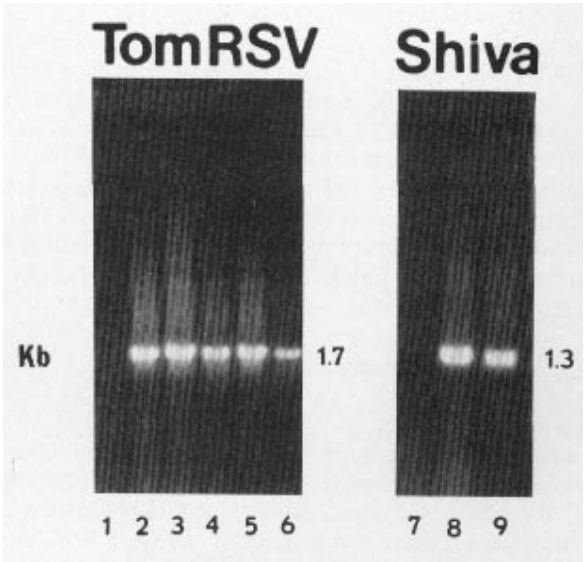

Fig. 2. PCR amplified TomRSV-CP or Shiva-1 fragments from transgenic 'Thompson Seedless' grape plants. TomRSV-CP primers; lane 1, pGA482GG transformant (without the TomRSV-CP gene); lane 2, transformant 3-2; lane 3, 3-3; lane 4, 3S-2; lane 5, 3S-3; lane 6, 3SB-X. Shiva-1 primers; lane 7 untransformed 'Thompson Seedless' plant; lane 8, transformant 4-3; lane 9, 4S-2. Transgenic plants 3-2, 3-3, and 4-3, were obtained from *Agrobacterium tumefaciens* infection alone. Plants 3S-2, 3S-3, 3SB-X, and 4S-2 were obtained from *A. tumefaciens* infection after microprojectile bombardment.

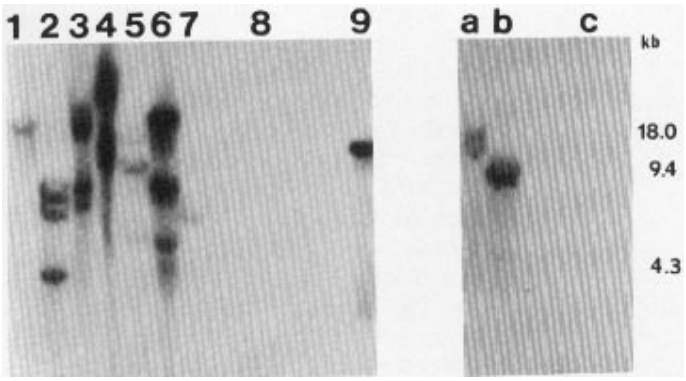

Fig. 3. Southern analysis of transgenic 'Thompson Seedless' grape plants. Lanes 1 through 9, TomRSV-CP transformants. DNA digested with EcoR1, probed with NOS/NPTII fragment. Lane 1, pGA482GG transformant (control without the TomRSV-CP gene); lane 2, transformant 3-2; lane 3, transformant 3-3 from tissue culture; lane 4, transformant 3-3 from greenhouse leaves (DNA runs slower on gel); lane 5, transformant 3S-2; lane 6, 3S-3; lane 7, 3SB-X; lane 8 untransformed control 'Thompson Seedless'; lane 9, pGA482GG/cpTomRSV plasmid. Lanes a through c Shiva-1 transformants. DNA digested with BamH1, probed with NOS/NPTII fragment. Lane a, transformant 4-3; lane b, transformant 4S-2; lane c, untransformed control 'Thompson Seedless'. Transgenic plants 3-2, 3-3, and 4-3, were obtained from *Agrobacterium tumefaciens* infection alone. Plants 3S-2, 3S-3, 3SB-X, and 4S-2 were obtained from *A. tumefaciens* infection after microprojectile bombardment.

different clones. For TomRSV-CP, these sequences were the 5' primer 5'-GGTTCAGGGCGGGTCCTGGCAAG-3' and 3' primer 5'-GTAAAAGCTAATTAAGAGGCCACC-3'; for Shiva-1, the sequences were the 5' primer 5'-ATCAAACAGGGTATCCTGCG-3' and 3' primer 5'-TTCCCACCAACGCTGATC-3'. PCR reactions were run using the GeneAmp kit components (Perkin-Elmer, Norwalk, Conn.) with the following cycle parameters: 1 min at 94 °C, 1.5 min at 65 °C, and 2 min at 72 °C. The first cycle used an additional 3 min melt at 95 °C and the last five cycles had 4-min extension times at 72 °C. After 35 amplification cycles, the PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

Southern analysis was carried out using a PCR-generated 1.1-kb NOS/NPTII probe. Digestion with EcoR1 was used to test for unique insertion events that would include segments of grape DNA in pGA482GGcpTomRSV transformants. BamH1 was used for the pBPRS1 transformants. DNA extraction followed the procedures of Callahan et al. (1992). Conditions for Southern analysis were as described by Scorza et al. (1990). The NOS/NPTII probe was radioactively labeled using random primers according to the instructions with the BioRad Random Primer DNA Labeling Kit (BioRad, Hercules, Calif.).

Results and Discussion

Transgenic grape somatic embryos were produced with and without microprojectile bombardment before cocultivation with *A. tumefaciens*. Leaf samples of the plants that survived kan selection produced the characteristic blue GUS positive reaction indicating the presence and activity of the GUS gene in these plants. Leaves from untransformed control plants showed no blue staining. Excised leaves from putative transformants cultured in liquid LB medium were negative for the presence of contaminating *A. tumefaciens*. PCR analysis using TomRSV-CP and Shiva-1 primers suggested that the 13 plants that survived kan selection after being exposed to Shiva-1 or TomRSV-CP transformation treatments contained the predicted gene sequences (Fig. 2). Southern analyses using the NPTII gene as a probe (Fig. 3) demonstrated incorporation of the foreign genes into the grape genome. While the Southern analysis directly shows only the incorporation of the NPTII gene into the genomes of the assayed grape plants, the close linkage of the TomRSV-CP or the Shiva-1 genes to the NPTII gene (Fig. 1) coupled with the positive PCR assays for the presence of these genes leads to the conclusion that these plants also contain the TomRSV or Shiva-1 gene. Southern analyses indicated that most TomRSV-CP transformants contained multiple copies of the gene insert. Both Shiva-1 transformants appeared to contain a single insert. Plasmid pGA482GG was used for transferring the TomRSV-CP gene. Our previous work using plasmid pGA482GG for transforming grape and other species suggested that multiple copy transformants are common (Scorza et al., 1994, 1995).

Since the embryogenic line used for this work was several years old, precise phenotypic evaluation will be necessary to eliminate off-type plants that may have resulted from somatic mutation in vitro. In this respect, it is promising to note that, at this point, all transgenic plants appear to be phenotypically normal (Fig. 4).

In our previous report (Scorza et al., 1995) we used microprojectile bombardment with *A. tumefaciens* to produce transgenic grape plants. In the work presented here we used both methods. Although with one *A. tumefaciens*/plasmid combination microprojectile bombardment before *A. tumefaciens* infection improved the yield of transformants, the numbers of transformants obtained in this study were too low to compare methodologies. It is apparent, however, that both microprojectile bombardment followed by exposure to *A. tumefaciens* and *A. tumefaciens* alone

Fig. 4. Transgenic 'Thompson Seedless' grape plant 4 months after transfer to the greenhouse.

Table 1. Production of transgenic 'Thompson Seedless' grape lines.

Treatment	Somatic embryos (no. treated)	Putative transformants (no.)	Transformation (%)
Agrobacterium tumefaciens alone			
Control plasmid[z]	100	1	1.00
TomRSV-CP	300	2	0.67
Shiva-1	300	2	0.67
Particle bombardment plus *A. tumefaciens*			
Control plasmid1	100	1	1.00
TomRSV-CP	300	7	2.30
Shiva-1	300	2	0.67

[z]Control plasmid was pGA482GG containing the NPTII and gusA genes but without Shiva-1 or TomRSV-CP genes.

are effective for transforming grape somatic embryos. In this and our previous report (Scorza et al., 1995) the overall transformation rate in terms of transgenic plants produced per somatic embryos treated was about 1% (Table 1).

The current report differs from our previous work in that we now report transforming grape from somatic embryos derived from leaves, while previously we reported producing transgenic plants from somatic embryos derived from zygotic embryos. The genes transferred include a viral coat protein gene and a lytic peptide gene. To date there have been few reports of transgenic grapevine production. Mullins et al. (1990) produced a transgenic *V. rupestris* Scheele plant after cocultivating 240 somatic embryo hypocotyls with *A. tumefaciens*, but transgenic 'Cabernet Sauvignon' and 'Chardonnay' (240 explants each) were not produced. Other workers have successfully produced transgenic shoots (Baribault et al., 1990; Nakano et al., 1994). Recently, Kikkert et al. (1995) produced transgenic plants of 'Chancellor', a complex *Vitis* interspecific hybrid. Krastanova et al. (1995) produced transgenic plants of two important grape rootstocks, 'Vitis rupestris du Lot' and 110 Richter (*V. berlandieri* x *V. rupestris*), expressing the coat protein of grapevine fanleaf virus. There has been, however, little information concerning the transformation of *V. vinifera* scion cultivars. We demonstrate the transformation of 'Thompson Seedless', a major *V. vinifera* scion cultivar. Disease resistance and trueness-to-type testing is underway.

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