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OPTIMIZING *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF GRAPEVINE

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Summary

A translational fusion between the enhanced green fluorescent protein (EGFP) and neomycin phosphotransferase (NPTII) genes was used to optimize parameters influencing *Agrobacterium*-mediated transformation of *Vitis vinifera* L. cv. Thompson Seedless. The corresponding bifunctional protein produced from this EGFP/NPTII fusion gene allowed for a single promoter to drive expression of both green fluorescence and kanamycin resistance, thus conserving promoter resources and eliminating potential promoter–promoter interactions. The fusion gene, driven by either a double cauliflower mosaic virus 35S (CaMV 35S) promoter or a double cassava vein mosaic virus (CsVMV) promoter, was immobilized into *Agrobacterium* strain EHA 105. Somatic embryos capable of direct secondary embryogenesis were used as target tissues to recover transgenic plants. Simultaneous visualization of GFP fluorescence and kanamycin selection of transgenic cells, tissues, somatic embryos, and plants were achieved. GFP expression and recovery of embryogenic culture lines were used as indicators to optimize transformation parameters. Preculturing of somatic embryos for 7 d on fresh medium prior to transformation minimized *Agrobacterium*-induced tissue browning/necrosis. Alternatively, browning/necrosis was reduced by adding 1 g l^{-1} of the antioxidant dithiothreitol (DTT) to post co-cultivation wash media. While combining preculture with antioxidant treatments did not result in a synergistic improvement in response, either treatment resulted in recovery of more stable embryogenic lines than did the control. A 48 h co-cultivation period combined with 75 mg l^{-1} kanamycin in selection medium was optimal. DNA analysis confirmed stable integration of transgenes into the grape genome; 63% had single gene insertions, 27% had two inserts, and 7 and 3% had three and four inserts, respectively. Utilizing optimized procedures, over 1400 stable independent transgenic embryogenic culture lines were obtained, of which 795 developed into whole plants. Transgenic grapevines have exhibited normal vegetative morphology and stable transgene expression for over 5 yr.

Key words: Agrobacterium tumefaciens; EGFP; preculture; preconditioning; transgene expression; transgenic plants; *Vitis*.

Introduction

In recent years, significant progress has been made in grapevine transformation (see review of literature by Gray et al., 2005a). Use of organogenic cultures as target tissues for *Agrobacterium*-mediated transformation has been met with limited success (Mullins et al., 1990; Levenko and Rubtsova, 2000; Mezzetti et al., 2002), thus leading to widespread adoption of embryogenic culture systems. Biolistic bombardment resulted in intense transient GUS expression in somatic embryos of *Vitis vinifera* cv. Thompson Seedless (Gray et al., 1993). This was subsequently used to develop transgenic plants from embryogenic suspension cultures of *V. vinifera* cvs. Chardonnay and Merlot and *Vitis* interspecific hybrid Chancellor (Kikkert et al., 1996, 2000). However, *Agrobacterium*-mediated transformation of embryogenic cultures has been used to transform the largest number of species, including several varieties of *V. vinifera* (Nakano et al., 1994; Mauro et al., 1995; Scorza et al., 1995, 1996; Perl et al., 1996; Hoshino et al., 1998; Xue et al., 1999; Yamamoto et al., 2000; Iocco et al., 2001; Das et al., 2002). However, necrosis of *Agrobacterium*-infected embryogenic tissues has been a major obstacle in improving the transformation efficiency of elite *V. vinifera* cultivars. Perl et al. (1996) reported that adding antioxidants to the selection medium would improve recovery of transgenic plants. However, such a treatment rendered transfected explants insensitive to kanamycin selection. Despite this progress, actual yields of transgenic plant lines remained low and well below the large number of independent lines required for screening and selecting for improved phenotypic traits (Kaniewski and Thomas, 1999).

We have developed procedures to facilitate transformation of grapevine, that include well-established methods of embryogenic culture initiation, maintenance, and plant recovery (Gray and Mortensen, 1987; Gray, 1989, 1995; Jayasankar et al., 2003). A fusion reporter marker construct composed of the enhanced green fluorescent protein (EGFP) and kanamycin resistance (NPTII) genes was developed for effective monitoring of transformation of grapevine (Li et al., 1999, 2001). In this report, we use this fusion gene to investigate and optimize parameters for *Agrobacterium*-mediated transformation of grapevine. We also propose

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modifications in explant preculture and antioxidant treatments to reduce necrosis and promote recovery of a large number of transgenic plants from *V. vinifera* cv. Thompson Seedless. Using the procedures described herein has contributed to routine transformation of a number of *Vitis* species and cultivars in our laboratory, and has allowed for detailed studies of transgene insertion and expression in grape (Li et al., 2003, 2004; Gray et al., 2005b).

Materials and Methods

Plant materials. Somatic embryos (SEs) used as targets for transformation were initiated from *in vitro*-grown leaves of Thompson Seedless as previously described (Gray, 1995). Cultures were maintained on a plant growth regulator (PGR)-free X6 medium (Li et al., 2001). This medium consists of MS (Murashige and Skoog, 1962) lacking glycine, and modified to contain 3.033 g l^{-1} KNO_3 and 0.364 g l^{-1} NH_4Cl as sole nitrogen sources, 60.0 g l^{-1} sucrose, 1.0 g l^{-1} *myo*-inositol, 7.0 g l^{-1} TC agar (Phytotechnology laboratories, LLC, Shawnee Mission, KS, USA) and 0.5 g l^{-1} washed activated charcoal. The pH of the medium was adjusted to 5.8 prior to autoclaving. The cooled medium, 30 ml, was dispensed into each 100 × 15 mm plastic Petri plate and plates were sealed with several layers of Parafilm™, and maintained in the dark at 26°C. Cultures were transferred to fresh X6 medium at 6–8 wk intervals.

Transformation vector and bacterial strain. A bifunctional marker gene composed of an in-frame translational fusion between the EGFP gene from *Aequorea victoria* and a neomycin phosphotransferase (NPTII) gene was utilized (Li et al., 2001). The fusion marker gene was controlled by a double-enhanced cauliflower mosaic virus 35S (CaMV 35S) promoter (−419 to −90 enhancer fragment duplicated) followed by the Ω leader sequence of TMV and a termination site and polyadenylation signal of the CaMV 35S transcript (Mitsuhara et al., 1996). This gene expression unit was introduced into the T-DNA region of a pBIN19-derived binary vector (Datla et al., 1991), resulting in the transformation vector pSGN. Alternatively, a double-enhanced cassava vein mosaic virus (CsVMV) promoter was used in the transformation vector pCGN to drive the fusion gene in the same binary backbone (Li et al., 2001). These vectors were introduced into *A. tumefaciens* strain EHA105 (Hood et al., 1993) by the freeze–thaw method (Burrow et al., 1990) and used for transformation.

Preparation of bacterial cells. *Agrobacterium* harboring the transformation vector was cultured overnight at 26°C on an orbital shaker at 185 rpm in liquid Luria broth (LB) medium containing 20 mg l^{-1} rifampicin and 70 mg l^{-1} kanamycin. Prior to transformation, the bacterial culture (OD_{600} = 0.8–1.0) was mixed with an equal volume of a liquid medium containing DKW basal salts (Driver and Kuniyuki, 1984), 0.3 g l^{-1} KNO_3, 1.0 g l^{-1} *myo*-inositol, 2.0 mg l^{-1} each of thiamine-HCl and glycine, 1.0 mg l^{-1} nicotinic acid, 30.0 g l^{-1} sucrose, 5.0 μ*M* 6-benzyladenine (BA), 2.5 μ*M* each of naphthoxyacetic acid (NOA), and 2,4-dichlorophenoxyacetic acid (2,4-D), at pH 5.7 (hereafter designated DM medium), and cultured continuously under the same conditions for 4 h.

Transformation of somatic embryos. SEs were submerged in bacterial culture for 10 min, blotted on sterile paper towels, and transferred onto DM medium solidified with 7.0 g l^{-1} TC agar for co-cultivation. To determine the optimum time for co-cultivation, each treatment consisted of 90–120 SEs (replicates) grown in the dark for 0, 1, 2, or 4 d. SEs with at least 10 GFP-positive cells were scored as positive for transient expression. After co-cultivation, SEs were then washed three times in sterile water, incubated for 10 min in liquid DMcc medium (DM medium containing 200 mg l^{-1} each of carbenicillin and cefotaxime) and incubated on solidified DMcc medium (containing 7.0 g l^{-1} TC agar) for 4 d. For selection of transgenic cells, SEs were transferred to DMcck medium (solidified DMcc medium supplemented with kanamycin). To determine the optimum concentration of kanamycin for selecting stably transformed callus, each treatment consisted of 120 SEs (replicates) grown on medium supplemented with 25, 50, 75, 100, or 150 mg l^{-1} kanamycin in the dark. After 20 d, SEs with callus growth were determined. SEs and attached callus were transferred onto X6cc medium (X6 medium containing 200 mg l^{-1} each of carbenicillin and cefotaxime), and maintained with a 20-d subculture interval to fresh medium. After 60 d, the number of embryos with at least one GFP-positive callus were determined. Such calli were considered to be stably transformed. Transgenic SEs derived from a primary SE explant were isolated and designated as an independent transgenic line.

Plant regeneration was carried out by culturing transgenic mature SEs on modified MS medium lacking $CaCl_2$ and supplemented with 709 mg l^{-1} Ca $(NO_3)_2 \cdot 4H_2O$, 0.845 mg l^{-1} $MnSO_4 \cdot H_2O$, 1.0 g l^{-1} *myo*-inositol and 1.0 μ*M* BA. SEs were grown in the dark for 2 wk, and then transferred to light conditions (65 μmol s^{-1} m^{-1}) with a 16-h photoperiod for another 2 wk. Shoots excised from germinated SEs were placed on rooting medium composed of the above-mentioned modified MS medium lacking BA, and supplemented with 0.4 μ*M* naphthaleneacetic acid (NAA). Rooted plants were transplanted into 7 cm pots containing potting mix (Promix BX, A.H. Hummert Seed Co., St Louis, MO, USA), and grown in the greenhouse.

Preculture treatments. Preliminary evaluation suggested that freshly transferred cultures exhibited less browning during subsequent transformation. Therefore, an experiment was conducted to determine whether SE transfer to fresh medium immediately prior to *Agrobacterium* co-cultivation might uniformly reduce browning and enhance transformation efficiency. Cultures were grown for 6 wk prior to the experiment. Each treatment consisted of 90–120 SEs (replicates) placed on fresh X6 medium for 0, 4, or 7 d. These SEs were subjected to the transformation routine. After 10 d, SEs with at least 10 GFP-positive cells were scored positive for transient expression. After 8 wk, the number of embryos with at least one GFP-positive callus were considered to be stably transformed.

Antioxidant treatments. SEs subjected to *Agrobacterium* transformation and 2 d co-cultivation on DM medium were transferred to 125 ml Erlenmeyer flasks containing 25 ml liquid DMcck15 medium (DMcc with 15 mg l^{-1} kanamycin) supplemented with 0, 0.5, or 1.0 g l^{-1} dithiothreitol (DTT). These cultures were maintained at 25°C in dark on a shaker at 110 rpm for 3 d before SEs were transferred onto solidified DMcck70 medium for selection. An additional treatment that incorporated 1 g l^{-1} DTT in both co-cultivation and wash media was included. Each treatment consisted of 90 SEs (replicates). The level of tissue browning was estimated by measuring the absorbance value at 420 nm of spent medium after the wash period, with fresh medium serving as zero control. The effect of antioxidant treatments following transformation was determined by evaluating transient GFP expression 10 d after transformation. SEs with at least 10 GFP positive cells were recorded. Stable proliferating GFP positive callus and SE lines were evaluated after 8 wk and SEs with at least a single proliferating GFP positive callus or SE line were scored.

Data analysis. Data were analyzed with the statistical analysis system using categorical modeling with analysis of standard errors (SAS Institute, 2004) to evaluate the response of SEs to experimental treatments.

Analysis of transgene expression. GFP expression in transformed SE was monitored using a stereomicroscope equipped for epi-fluorescence illumination (Leica MZFLIII). Transformation frequency, based on transient GFP expression, was calculated as the percentage of GFP-expressing SEs of the total number of inoculated SEs. Stable transformation frequencies were estimated based on the percentage of GFP-expressing SEs or kanamycin-resistant callus. In most experiments, 30 SEs were placed in each Petri dish, with three replicate dishes per treatment. Experiments were repeated two to three times.

Polymerase chain reaction (PCR) and Southern blot hybridization analyses. Total DNA was isolated from young leaves according to the method of Lodhi et al. (1994), but EDTA concentration was increased (100 m*M*) in the extraction buffer. Total DNA was isolated from a non-transformed Thompson Seedless plant (as negative control), 11 Thompson Seedless plants transformed with pSGN, and five Thompson Seedless plants transformed with pCGN and subjected to PCR using the EGFP gene-specific primers EG-1 and EG-2. DNA of pSGN also was incorporated as a positive control for the PCR reaction. PCR was performed to detect specific DNA sequences corresponding to the EGFP transgene. A forward primer EG-1 (5′-AGA GGA TCC CCG GGT ACC GGT CGC-3′) and a reverse primer EG-2 (5′-GCC CTG CAG TCC CTT GTA CAG CTC GTC-3′) were used to amplify a 757 bp DNA fragment from the EGFP/NPTII fusion gene. Conditions for PCR reactions were identical to those previously described (Murai et al., 1991). Briefly, 100 ng of total genomic DNA from each sample were mixed with PCR reaction solution prepared according to the manufacturer's instructions (Promega) and then subject to PCR amplification. The PCR program used consisted of 40 cycles with cycle 1 being performed at 93°C for 3 min, 55°C for 1 min, and 72°C for 1 min, cycles 2–39 at 93, 55, and 72°C for 1 min each and cycle 40 at 72°C for 5 min. Amplified DNA products were analyzed via electrophoretic separation in 0.8% agarose gels.

Southern blot hybridization was performed as previously described (Li et al., 1997). Ten micrograms of DNA were digested with *Eco*RI, separated by electrophoresis in a 0.8% (w/v) agarose gel, and transferred onto nylon membrane (Schleicher and Schuell, Inc., Keene, NH, USA). A 1.5 kb DNA fragment corresponding to the EGFP/NPTII fusion gene was obtained by digestion of plasmid DNA, labeled with digoxigenin and used as a probe for hybridization according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). The hybridized membrane was washed, subjected to chemiluminescent development using CDP-Star substrate and then exposed to film (Kodak BioMax Light-1).

Results

Influence of co-cultivation duration on transformation efficiency. The GFP marker allowed real-time monitoring of transgene expression during co-cultivation. GFP expression was not observed in SEs after 1 h of co-cultivation with *Agrobacterium* (Fig. 1). After 24 h of co-cultivation, about half of the SEs showed transient GFP expression. Almost all SEs had transient GFP expression when the co-cultivation period was extended to 48 h. Most SE explants turned dark brown and their growth ceased when co-cultivation time was extended to more than 96 h, presumably due to the overgrowth of *Agrobacterium* on the surface of SE explants. These results indicated that a 48 h co-cultivation period with *Agrobacterium* was optimal.

Development of transgenic SE lines. The number of GFP-expressing epidermal cells on hypocotyls and cotyledons of SEs increased up to 10 d following co-cultivation (Fig. 2*A*). Transient GFP expression gradually decreased in both intensity and frequency over time as cultures were subjected to kanamycin selection. By 8 wk, stable embryogenic callus and proliferating SE lines were commonly observed (Fig. 2*B*). GFP-expressing SEs that were isolated and placed on X6cc medium, a standard practice in our laboratory, continued to proliferate normally (Fig. 2*C*). Such GFP-expressing culture lines have been reliably proliferated for over 4 yr by careful selection and transfer of young SE to fresh medium at 6–8 wk intervals.

Effect of kanamycin concentration on transformation efficiency. Initiation and subsequent proliferation of GFP-expressing SE-derived callus of Thompson Seedless transformed with pSGN demonstrated that 75 mg l^{-1} kanamycin was optimal for selection (Fig. 3). Transgenic callus recovered following selection on 25 mg l^{-1} kanamycin was often chimeric, whereas the amount of transgenic callus obtained after selection on 150 mg l^{-1} kanamycin was reduced, and growth was slower during the selection period.

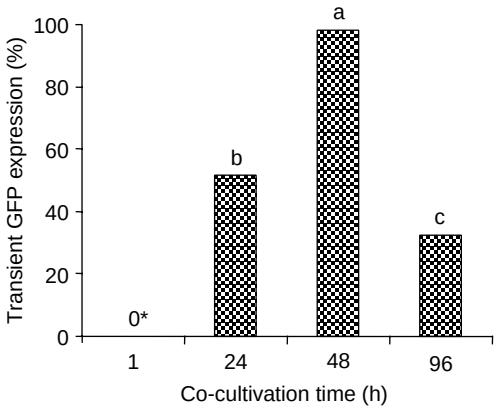


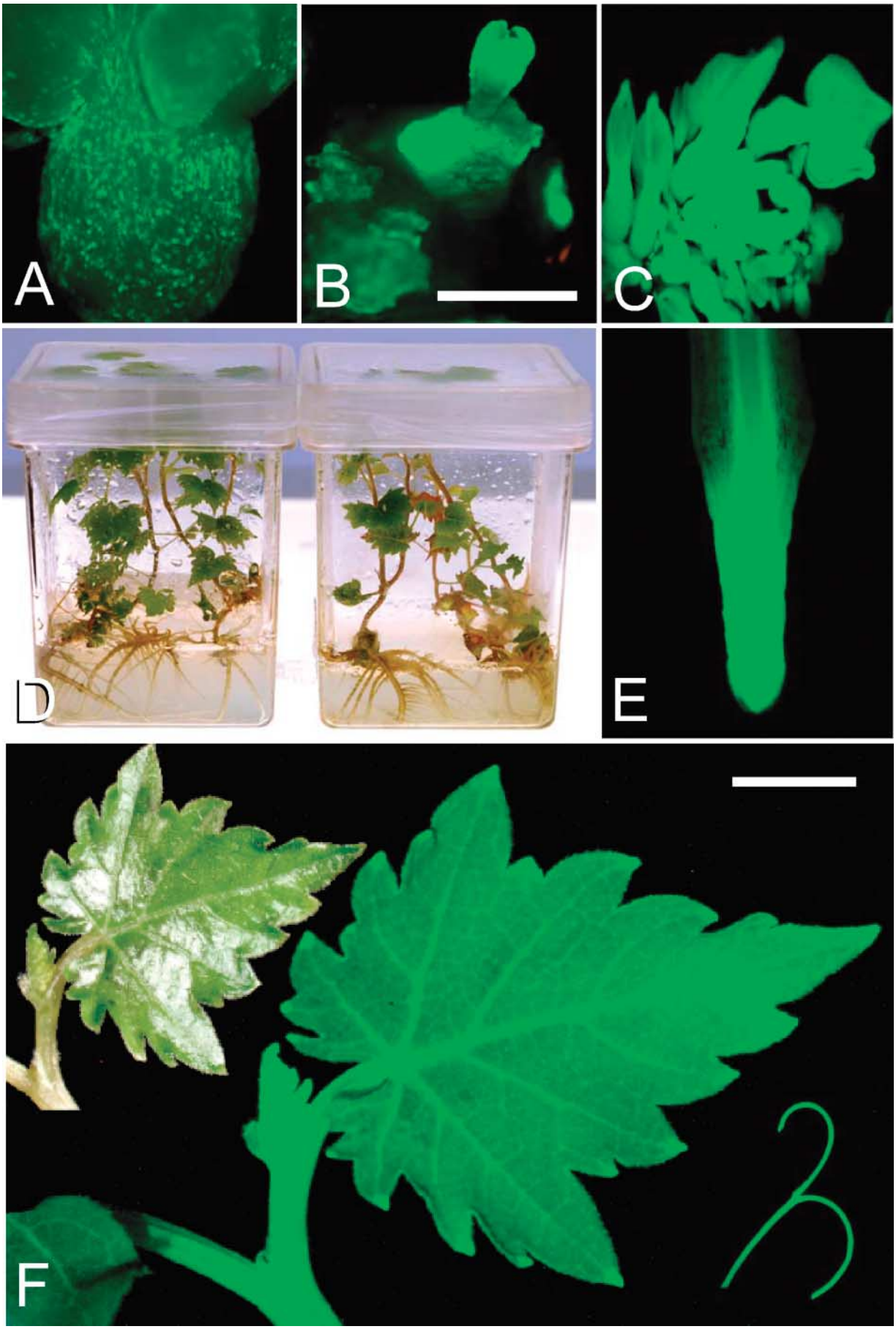
FIG. 1. Effect of co-cultivation duration on transient GFP expression in SE of *V. vinifera* cv. Thompson Seedless grapevine. SEs were inoculated with *Agrobacterium* harboring the plasmid pSGN. The percentage of GFP-expressing explants was determined 10 d after transformation. Mean separation between treatments was by analysis of standard errors from categorical modeling, $P = 0.05$. *Observations without variation cannot be assigned a confidence estimate.

Effects of preculture and antioxidant treatments. SEs were precultured on X6 medium for 0, 4, and 7 d and then subjected to transformation using *Agrobacterium* containing pSGN. After *Agrobacterium* inoculation, most SE without preculture became brown, whereas precultured SEs grew without browning and produced callus. Although there was no difference in the frequency of transient GFP expression between SEs with or without preculture, stable transformation frequencies increased dramatically with preculture (Fig. 4). For example, up to 62% of SEs exposed to a 7 d preculture treatment produced GFP-expressing callus. Transformation frequency was lower when the preculture treatment was longer than 10 d, presumably as the medium nutrients became depleted (data not shown). Accordingly, a 7 d preculture treatment of SE on X6 medium prior to *Agrobacterium* exposure was used for subsequent experiments.

The effect of a 3 d wash with 0, 0.5, or 1.0 g l^{-1} DTT on browning of *Agrobacterium*-treated SEs demonstrated that all of the antioxidant treatments reduced brown exudates in the spent wash solution, such that all treatments exhibited 3.5–6-fold less browning as measured by absorbance spectroscopy (Table 1). There was no difference between 0.5 and 1.0 g l^{-1} DTT wash treatments and from the control treatment in transient GFP expression. Adding 1.0 g l^{-1} DTT to both co-cultivation and wash medium had an inhibitory effect (Table 1). However, stable callus production was enhanced by addition of 0.5 or 1.0 g l^{-1} DTT. DTT at 1.0 g l^{-1} produced more GFP-positive embryo lines than the control treatment (Table 1). Addition of DTT to the co-cultivation medium inhibited recovery of stable callus and embryo lines (Table 1).

Production of transgenic plants and transgene expression. Preculture/DTT treatments and modified conditions for co-cultivation and selection improved *Agrobacterium*-mediated transformation of grapevine. This allowed transformed SEs to be obtained within 8 wk after *Agrobacterium* inoculation. Transgenic plants were regenerated from mature transgenic SEs within 6–8 additional wk (Fig. 2*D*). Plants were established in potting mix and acclimated to greenhouse conditions. To date, over 1400 stably transformed embryogenic culture lines have been recovered. Of 795 transgenic plant lines produced and grown in the greenhouse for at least 1 yr, all exhibit vegetative morphological characteristics identical to non-transformed control plants. Thompson Seedless plants do not flower under our greenhouse conditions, so that floral and fruit characteristics will not be evaluated until field trials are established.

Evaluation of transgenic plants expressing the EGFP/NPTII fusion marker demonstrated the expected fluorescence throughout all organs and organ systems, including the root (Fig. 2*E*), shoot, leaf, and shoot tip (Fig. 2*F*) and tendril (Fig. 2*F*, lower right insert), which are modified inflorescences in grapevine. Fluorescent emission was evenly expressed throughout all tissues in selected plants, allowing very fine discrimination of tissue types (note vascular tissue in Fig. 2*E*), especially when compared with images obtained with conventional incident light illumination (Fig. 2*F*,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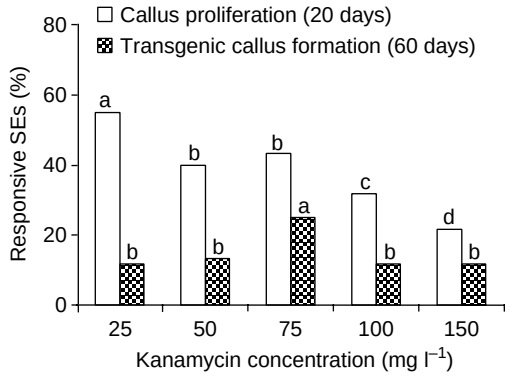

FIG. 3. Effect of kanamycin concentration on growth and recovery of transgenic *V. vinifera* cv. Thompson Seedless grapevine callus after *Agrobacterium*-mediated transformation. SEs were transformed with pSGN-containing *Agrobacterium*. The numbers of SEs producing callus growth and those with GFP-expressing callus were determined 20 and 60 d after transformation, respectively. Mean separation between treatments was by analysis of standard errors from categorical modeling, $P = 0.05$.

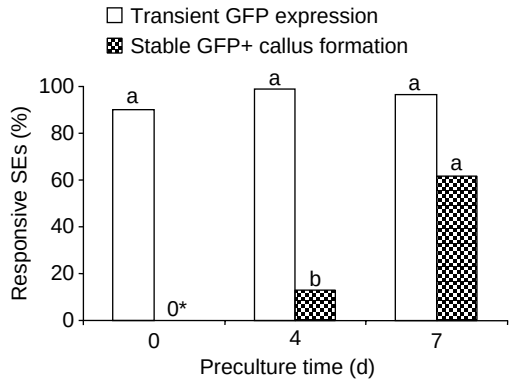

FIG. 4. Influence of preculture treatments on transformation of *V. vinifera* cv. Thompson Seedless grapevine. SEs cultured for 6 wk were transferred to fresh X6 medium for 0, 4, or 7 d and then transformed with pSGN-containing *Agrobacterium*. Transient GFP expression (based on counting individual embryos with more than 10 GFP-positive cells) and stable GFP-expression (based on the number of embryos that produced at least one GFP-positive callus) were determined 10 d and 8 wk after bacterial inoculation, respectively. Mean separation between treatments was by analysis of standard errors from categorical modeling, $P = 0.05$. *Observations without variation cannot be assigned a confidence estimate.

upper left insert). These findings were in agreement with observations reported previously (Li et al., 2001).

Comparison of GFP emission between non-transformed and transformed leaves demonstrated the high level of expression due to integration of the EGFP/NPTII fusion gene. Non-transformed leaves emit a red color due to autofluorescence of chlorophyll, although a light green non-specific autofluorescence (not indicative of GFP expression) is sometimes present. In comparison, leaves expressing GFP emit a bright green fluorescence that often completely obscures chlorophyll autofluorescence (Fig. 2*F*).

Molecular analysis of transgene integration. PCR analysis confirmed integration of transgenes into the grape genome. The results (Fig. 5*A*) indicated that a 0.75 kb DNA fragment corresponding to the EGFP gene sequence within the EGFP/NPTII fusion gene was present in the positive control sample containing pSGN (Fig. 5*A*, *lane 2*) but absent in the negative control sample containing DNA from a non-transformed plant (*lane 3*). All transgenic plants analyzed produced the EGFP gene-specific 0.75 kb fragment after PCR amplification (Fig. 5*A*, *lanes 4–10* and *12–20*).

DNA samples from randomly selected transgenic Thompson Seedless plants were digested with a single restriction enzyme and subjected to Southern blot hybridization to estimate the copy number of transgenes, thus indicating whether multiple insertions were occurring during transformation. DNA from a non-transformed (control) plant and from five transgenic plants was hybridized with a 1.5 kb DNA probe corresponding to the EGFP/NPTII fusion gene. No hybridization was observed in the negative control plant (Fig. 5*B*, *lane 1*). Plants TSGN-1-1 and TSGN-1-3, derived from transgenic line TSGN-1, produced two hybridization fragments each (10.4 and 11.6 kb), suggesting the integration of two independent copies of the fusion gene (Fig. 5*B*, *lanes 2* and *3*). Transgenic plant TSGN-3-2 showed a single hybridization fragment (9.6 kb), indicating the insertion of a single copy of the fusion gene (Fig. 5*B*, *lane 4*). The remaining two sister plants TCGN-3-1 and TCGN-3-4 derived from the transgenic line TCGN-3 generated two hybridization fragments each (6.6 and 7.7 kb), suggesting the integration of two independent copies of the fusion gene (Fig. 5*B*, *lanes 5* and *6*). All transgenic plants analyzed thus far contained hybridization fragments with a molecular size larger than the total length of T-DNA of both plasmids pSGN and pCGN (2.79 and 2.65 kb, respectively). This suggested that hybridization signals detected were derived from intact transgene (T-DNA) sequences that had been integrated into the grape genome. In addition, all sister plants from the same transgenic line displayed an identical hybridization pattern.

Transgenic plants TSGN-1-1 and TSGN-1-3 produced hybridization fragments with greater signal intensities and with molecular sizes similar to that of the linearized plasmid pSGN (Fig. 5*B*, *lanes 7* and *8*). To clarify whether these signal fragments were derived from possible contamination with residual *Agrobacterium* DNA harboring plasmid pSGN, PCR was performed using primers ChvA F1 and ChvA R1 (Bond and Roose, 1998). Both primers ChvA F1 and ChvA R1 were expected to anneal to the chromosomal *Vir*A gene-specific sequences from *Agrobacterium* and generate a 0.65 kb fragment after PCR amplification. However, no such ChvA-specific PCR product was generated from either plant, indicating that no

FIG. 2. Production of transgenic *V. vinifera* cv. Thompson Seedless grapevine and comparative GFP expression obtained using plasmid pSGN. *A*, Transient GFP expression in epidermal cells of SE hypocotyl 10 d after *Agrobacterium* transformation treatment. *B*, Stable GFP expressing embryogenic callus 8 wk after transformation with pSGN-containing *Agrobacterium* and selection with 75 mg l^{-1} kanamycin. *C*, Stably transformed GFP-expressing transgenic SEs obtained after transformation with pSGN-containing *Agrobacterium* and selection with 75 mg l^{-1} kanamycin. *D*, Rooting of *in vitro* shoots derived from germinated transgenic SEs. *E*, Root from GFP-expressing transgenic plant. Note intense fluorescence in vascular tissue (× 25). (Figs. *A–C*), *E*, *bar* = 0.7 mm, 1 mm, 2.8 mm, 1 mm, respectively). *F*, GFP expression in shoot, leaf and shoot tip. Fluorescent emission is evenly diffused throughout all tissues (*bar* = 3 mm). Insert (*upper left*) details the same shoot illuminated by incident light (*bar* = 4.8 mm). Insert (*lower right*) shows GFP-expressing tendril (*bar* = 12 mm).

TABLE 1

EFFECT OF DTT TREATMENTS ON CULTURE BROWNING AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF *V. VINIFERA* THOMPSON SEEDLESS[z]

Treatment	Browning of wash medium[y]	Transient expression[x]	Stable callus lines	Stable embryo lines
0 DTT	0.249	65 a	31 b	20 b
0.5 g l^{-1} DTT wash	0.065	70 a	42 a	28 ab
1.0 g l^{-1} DTT wash	0.054	65 a	42 a	36 a
1.0 g l^{-1} DTT co-cultivation and wash	0.059	51 b	20 c	4 c

[z] Each treatment contained 90 somatic embryos (SEs). SE with at least 10 green fluorescent protein (GFP) positive cells 10 d after *Agrobacterium* treatment were scored as positive. SE with at least one proliferating GFP positive callus and/or SE line after 8 wk were scored as positive.

[y] Browning was estimated using absorbance spectroscopy (OD_{420}).

[x] Mean separation within columns by analysis of standard errors from categorical modeling, $P = 0.05$.

residual *Agrobacterium* contamination was present (data not shown). Hence, the higher signal intensity observed from these transgenic plants was likely due to the loading of excessive amounts of genomic DNA during the electrophoresis/DNA transfer process.

Subsequently, an additional 27 independent transgenic lines were subjected to transgene integration analysis. Among all 30 independent transgenic plant lines analyzed (27 + 3 shown in Fig. 5*B*), 63% contained a single copy, 27% contained two copies, 7% had three and 3% had four copies, respectively, of the transgene in their genome (Fig. 6). These results show that the transformation procedure generated a preponderance of transgenic plants with a single copy of genome-integrated transgene.

Discussion

We report a simplified procedure for production of large numbers of transgenic plants from *V. vinifera* 'Thompson Seedless.' Use of the EGFP/NPTII fusion gene, previously developed in our laboratory, facilitated continuous monitoring of transgene expression in grapevine cells following *Agrobacterium*-mediated transformation through all developmental stages of transgenic grapevine. The EGFP fusion marker enabled early identification of non-chimeric transformants and permitted the recovery of transgenic embryos within a short period of time. Based on GFP expression, factors limiting efficiency of transformation were identified and overcome. The optimized protocol consisted of preculturing SE for 7 d on fresh culture medium, co-cultivation with *Agrobacterium* for 48 h, and selection of transgenic cells on medium containing 75 mg l^{-1} kanamycin. As an alternative to preculture, rinsing cultures for 3 d in liquid medium containing 1 g l^{-1} DTT was equally effective. The EGFP fusion marker should facilitate identification of optimal transformation conditions for grapevine varieties that respond differently than our model grapevine, Thompson Seedless.

DNA analysis indicated that 90% of transgenic plants contained one-to-two copies of a stably integrated transgenic construct in their genome. We demonstrated that multiple transgenic embryos recovered from a specific location on a particular explant actually resulted from a single transformation event, since they had an identical transgene integration profile. Thus, from a particular transgenic cell line, multiple identical sister embryos and plants were isolated.

Somatic embryos of grapevine proliferate via direct secondary embryogenesis and, based upon microscopic observations, it generally is assumed that new embryos originate from single epidermal or sub-epidermal cells (Gray and Mortensen, 1987; Gray, 1992, 1995; Jayasankar et al., 2003). Thus, SEs are ideal targets for transformation because the regenerative cells are accessible to *Agrobacterium*, and the single cell origin of secondary SEs results in non-chimeric transformants. Most previous reports on successful recovery of transgenic grapevines also utilized somatic embryos as explants (Mauro et al., 1995; Scorza et al., 1995, 1996; Perl et al., 1996; Yamamoto et al., 2000; Iocco et al., 2001). In most of these studies, tissue browning and necrosis following *Agrobacterium* inoculation were the major obstacles. We observed that tissue browning often increased noticeably when *Agrobacterium*-infected SE were subcultured on antibiotic-containing transformant selection medium. Tissue necrosis often produced an inhibitory effect on further cell growth and led to cell death. Tissue necrosis resulted in the apparent production and accumulation of phytotoxic phenolic compounds in other parts of the explant. This ultimately killed both transformed and non-transformed cells. Consequently, the transformation efficiency, i.e. the number of transgenic lines and plants recovered for *V. vinifera*, were relatively low when compared to those obtained for other *Vitis* species such as *V. rupestris* (Krastanova et al., 1995).

Perl et al. (1996) described tissue browning and necrosis to be a hypersensitive response of grapevine embryogenic cells to *Agrobacterium* infection and demonstrated that onset of browning was correlated with elevated levels of peroxidase activity. Use of antioxidants such as DTT or polyvinylpolypyrrolidone (PVPP) reduced tissue browning. These antioxidants might have simultaneously contributed to the inability of kanamycin at high levels (up to 500 mg l^{-1}) to inhibit cell growth of non-transformed grapevine cells, thus rendering the kanamycin-based selection system ineffective (Perl et al., 1996). Das et al. (2002) utilized a similar antioxidant treatment to obtain transgenic plants from embryogenic leaf discs, but did not report a problem with kanamycin selection. Scorza et al. (1995, 1996) did not report browning as a problem, but they incorporated high levels of cysteine, a sulfur-containing amino acid with antioxidant properties, into the culture medium.

We consider induced browning to be a natural plant response in the host/pathogen interaction between *Agrobacterium* and grapevine. Plant cells are capable of generating active oxygen species (AOS) in response to microbial infection as a part of their defense mechanism (Bolwell and Wojtaszek, 1997). The accumulation of large amount of AOS in grapevine cells following their inocu1ation

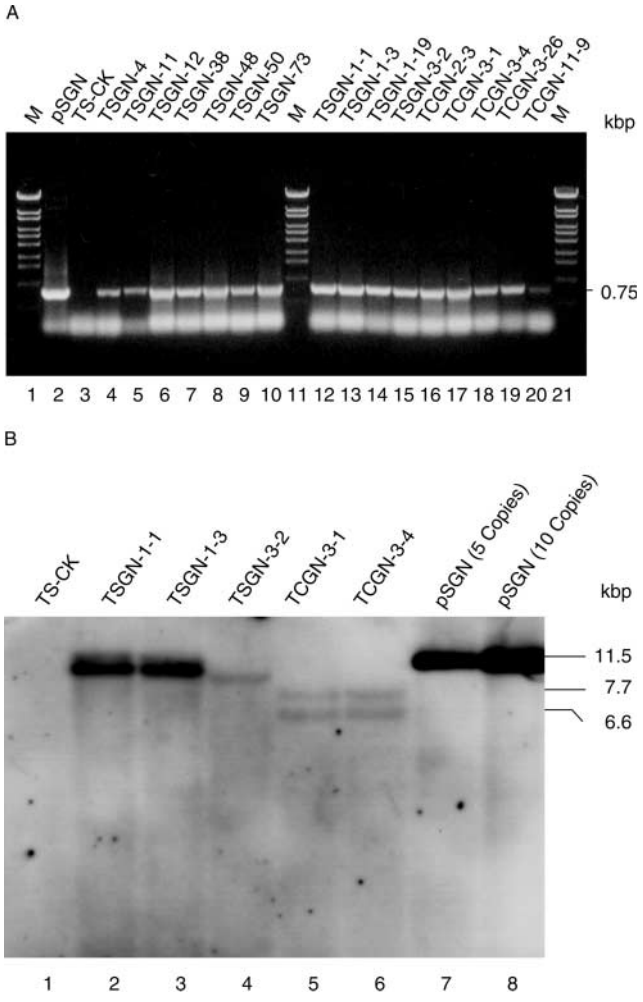

FIG. 5. Molecular analysis of transgenic *V. vinifera* cv. Thompson Seedless grapevine. *A*, PCR analysis of DNA isolated from leaf tissue of non-transformed and transgenic Thompson Seedless plants. A 0.75 kbp DNA fragment amplified from the EGFP-specific gene sequences was indicated: pSGN, a binary vector containing the EGFP/NPTII fusion gene under control of a double CaMV 35S promoter; TS-CK, non-transformed Thompson Seedless; TSGN, transgenic Thompson Seedless plants transformed with pSGN; TCGN, transgenic Thompson Seedless plants transformed with a binary vector pCGN containing the containing the EGFP/NPTII fusion gene under control of a double CsVMV promoter. *B*, Southern hybridization analysis of total DNA from leaf tissue of a non-transformed (*lane 1*) and five randomly selected transgenic plants (*lanes 2–6*). *Lanes 2–4*, DNA from three transgenic plants obtained after transformation with pSGN-containing *Agrobacterium*. *Lanes 5 and 6*, DNA from two transgenic plants obtained from transformation using pCGN-containing *Agrobacterium*. *Lanes 7 and 8*, copy number standards representing 5 and 10 copies, respectively, per diploid grapevine genome. Standards were reconstructed using DNA of binary vector pSGN.

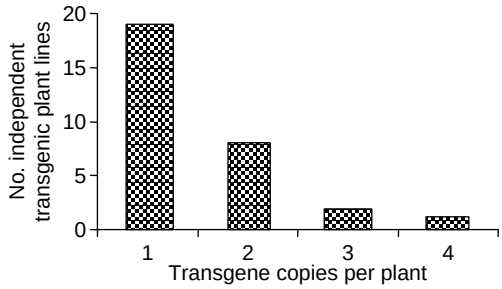

FIG. 6. Number of *V. vinifera* cv. Thompson Seedless grapevines with one or more transgene copies after *Agrobacterium*-mediated transformation.

with *Agrobacterium* during the transformation process likely contributes to the onset of cell death and tissue necrosis. However, plant cells also have the ability to produce antioxidants to inactivate AOS, thus protecting themselves from further oxidative damage (Fernandez and Videla, 1996). Production of antioxidants by plant cells is influenced by both genetic and environmental factors. For example, a significant increase in the activity of antioxidant enzymes occurred in plants grown in an elevated CO_2 environment (Schwanz and Polle, 2001), whereas plants subjected to nutrient deficiency showed a dramatic reduction in antioxidant enzyme activity (Yu and Rengel, 1999).

Several antioxidants, including ascorbic acid, L-cysteine, DTT and PVPP were evaluated in preliminary experiments (data not shown). We found that DTT reduced browning and also exhibited the least toxicity. We demonstrated that preculturing of SE on fresh culture medium for a relatively short period of time and/or application of the antioxidant DTT reduced the level of tissue browning and the necrotic response. As a result, significantly higher transformation frequencies were obtained. We did not observe insensitivity to kanamycin selection with use of antioxidants; however, applying $1\,g\,l^{-1}$ DTT during culture wash was considerably less than the $2\,g\,l^{-1}$ DTT and $5\,g\,l^{-1}$ PVPP applied by Perl et al. (1996) during the entire selection period. Iocco et al. (2001) reported that rinsing *Agrobacterium* with liquid plant culture medium and utilizing a low bacterial density (0.3 OD_{500}) greatly reduced its phytotoxic effects. However, we found that no special bacterial treatments were needed prior to transformation. Kanamycin-based selection was fully functional. The beneficial effects of such preculture and antioxidant treatments deserve further investigation.

During the course of this study, over 1400 independent transgenic embryogenic cultures were produced. Due to the logistical difficulties of effectively screening large numbers of plants in the greenhouse, we have limited the production of independent plant lines to 795. Although this study specifically details the methodology to produce transgenic SE lines of Thompson Seedless, we have recovered transgenic SE and plants from several other *Vitis* genotypes, including scion varieties and rootstocks.

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