A co-transformation system to produce transgenic grapevines free of marker genes

M. Dutt, Z.T. Li, S.A. Dhekney, D.J. Gray*

Mid-Florida Research and Education Center, University of Florida/IFAS, 2725 Binion Road, Apopka, FL 32703, USA

Abstract

A co-transformation system was developed to produce grapevines free of selectable marker genes. This was achieved by transforming Vitis vinifera L. 'Thompson Seedless' somatic embryos with a mixture of two Agrobacterium strains. The first strain contained a binary plasmid with an egfp gene of interest between the T-DNA borders. The second strain harbored the neomycin phosphotransferase (nptII) gene for positive selection and the cytosine deaminase (codA) gene for negative selection, linked together by a bi-directional dual promoter complex. Our technique included a short positive selection phase on medium containing 100 mg l\(^{-1}\) kanamycin before subjecting cultures to prolonged negative selection on medium containing 250 mg l\(^{-1}\) 5-fluorocytosine. We regenerated 25 stable EGFP expressing transgenic lines. PCR analysis confirmed 18 lines contained only the egfp gene, whereas the remaining contained both egfp and codA/nptII genes. Presumably, the 18 monogenic lines arose through cross protection by being in close proximity to cells that expressed nptII and thus detoxified kanamycin in the immediate vicinity. This is the first report for grapevine using a combination of positive and negative selection to produce transgenic plants that do not contain marker genes.

Keywords:
Vitis vinifera  
codA  
nptII  
Negative selection  
Vegetatively propagated crops

1. Introduction

Genetic transformation requires a method to separate transformed cells from non-transformed cells and typically is achieved by expression of a marker gene that provides a growth advantage to transformed cells in a selection medium [1]. In absence of a selectable marker gene, transformed cells tend to be at a competitive disadvantage compared to non-transformed cells and die out as a consequence [2]. The marker gene is needed only for selection of transgenic cells and typically is linked to an actual gene of interest. Thus, selection for ability to proliferate in the presence of a selective agent results in isolation of transgenic cells containing both the gene of interest and the marker gene [3]. However, once a desired plant is selected from transgenic cells, the marker gene is no longer needed. The presence of marker genes may complicate future commercialization due to concerns regarding their effects on ecosystems and/or human health [4,5]. For example, one concern is that selectable marker genes could become transferred to other organisms, leading to the creation of antibiotic resistant bacterial strains (from antibiotic resistance markers) or new, aggressive weedy plant species (from herbicide resistance markers) [6]. Development of transgenic plants without marker genes alleviates such concerns. Using Agrobacterium tumefaciens, several strategies have been proposed to generate marker gene-free transgenic plants, including use of the following: co-transformation, transposable elements, site-specific recombination or intrachromosomal recombination [7–12]. Amongst them, co-transformation, using a mixture of two Agrobacterium strains [10] or a single Agrobacterium strain containing two T-DNAs [13] could be readily integrated into existing transformation protocols. Marker gene-free tobacco plants have been produced using a co-transformation system that incorporated a negative selectable marker gene [4].

In the present study, we investigated whether a co-transformation system incorporating both negative and positive selectable marker genes could be used to produce marker gene-free grapevines (Vitis vinifera L.). The premise was that cells with transient marker gene expression could cross protect adjacent cells that contain only the gene of interest from the suppressive effect of the selection environment [14]. In this system, both negative and positive selectable marker genes were not physically linked to the gene of interest. Instead, they were placed into T-DNAs of two separate Agrobacterium strains [4,15]. One strain contained a neomycin phosphotransferase (nptII) gene and a cytosine deaminase (codA)
gene from *Escherichia coli* linked by a bi-directional dual promoter [16] to serve as a dual positive and negative selection system. The second strain contained an enhanced green fluorescent protein (EGFP) gene (used in this study as the “gene of interest”) controlled by a double enhanced (2 × −419 to −90) cauliflower mosaic virus 35S (CaMV 35S) promoter [17]. A mixture of the two strains was used to transform grapevine somatic embryos (SE). Following co-transformation, marker gene-free transgenic SE were identified based on EGFP expression and reactions to positive and negative selection conditions. Polymerase chain reaction (PCR) and quantitative real-time PCR analyses were utilized to confirm transgene insertion and copy number of selected plants.

2. Materials and methods

2.1. Cloning of the codA gene

Genomic DNA of *E. coli* strain XL-1 Blue (Stratagene, CA, USA) was used as template for isolation of the codA gene. The codA coding sequence ranging from start codon at nucleotide position 1642 to stop codon at nucleotide position 2923 in the codBA operon region [18] was amplified by using PCR and codA-specific oligonucleotide primers. A forward primer (CDA-51), 5’ATGTTACAATGTCGAATGGCTTC3’ was designed to introduce a KpnI site immediately upstream of the GTG start codon to ATG and introduce a KpnI site immediately upstream of the translational start site. A reverse primer (CDA-32) 5’ATGCGCCGCGCCGTCACAGTTTGAATCGGCTTC3’ was designed to introduce a NotI site 2 bp downstream of the TGA stop codon. Primers were purchased from Integrated DNA Technologies, IA, USA. Following PCR, the 1299 bp codA gene fragment was isolated from PCR products via electrophoresis gel separation and cloned into pGEM-T Easy plasmid (Promega) resulting in the plasmid pGCDa. The cloned gene was verified first by restriction enzyme analysis and then by DNA sequencing.

2.2. Plasmid construction

The codA construct was excised from pGCDa as a KpnI/NotI fragment and ligated into a KpnI/NotI cloning site between the double enhanced CaMV 35S promoter (d35S) and a CaMV 35S terminator (35S-3’) in a pUC18-derived plasmid pDR to form plasmid pCDa. A 2.4 kb HindIII DNA fragment containing the expression cassette d35S-codA-35S-3’ was isolated and cloned into the unique HindIII site of a pB1919-derived binary vector pLCN. Plasmid pLCN contains a nptII gene cassette driven by a double enhanced (2 × −443 to −123) Cassava Vein Mosaic Virus (CsVMV) promoter and a nopaline synthase gene terminator (NOS-3’) [16]. The binary plasmid pLCN was linearized with HindIII and treated to prevent self-ligation. After ligation with the codA cassette fragment, a binary vector containing a divergent arrangement of codA and nptII expression cassettes was selected and designated as pCDA (Fig. 1). A second binary plasmid was constructed by replacing a gus/nptII fusion gene from p35G [16] with an egfp gene (Clontech Laboratories Inc., Mountain View, CA) resulting in plasmid pEGFP (Fig. 1). E. coli strain DH5α was used for the cloning of all plasmids. Both binary plasmids were introduced into *A. tumefaciens* strain EHA105 [19] by the freeze–thaw method [20].

2.3. Plant materials

SE were initiated from *in vitro*-grown leaves of *V. vinifera* L. ‘Thompson Seedless’. The youngest leaves were placed abaxial side down in Nitsch and Nitsch [21] based NB2 medium in dark for a month before being switched to a 16 h light/8 h dark cycle using cool white fluorescent lights for initiation of pro-embryonic masses (PEM) as previously described by Gray [22]. The PEM were transferred to X6 medium [23] for development of SE. Embryogenic cultures were maintained by subculture onto fresh X6 medium every 2–3 months. SE at the mid-cotyledonal stage of development were used for transformation studies.

2.4. Grapevine transformation procedure and establishment of codA selection protocol

To test activity of the codA gene in grapevines, *Agrobacterium*-mediated transformation of SE was carried out according to Li et al. [23] with modifications. *Agrobacterium* EHA105 containing pCDA was grown overnight in liquid YEP medium containing 100 mg l⁻¹ kanamycin. Cultures were grown for approximately 24 h with shaking (185 rpm) at 26 °C. After centrifugation at 6500 × g for 8 min at 25 °C, the bacteria were resuspended in 25 ml of liquid X2 medium. The resulting cell suspension culture was then incubated for 3 h at 26 °C prior to use in transformation. Values of optical density (OD) of cultures were measured using a Sunrise Optical Microplate Reader (Phenix Research Products, CA, USA) with a filter set at 620 nm wavelength. The OD₆₂₀ value of the bacterial suspension was adjusted to 0.8 before co-cultivation.

Grapevine SE were submerged in bacterial solution for 10 min, blotted on sterile paper towels and transferred onto filter papers wetted with liquid DM medium [23] for co-cultivation in dark at 26 °C for 3 days. SE were subsequently placed in 50 ml of liquid DMcc medium (DM medium containing 200 mg l⁻¹ each of carbencillin and cefotaxime) to inhibit *Agrobacterium* growth and cultured on an orbital shaker at 120 rpm overnight. For positive selection of transgenic cells, SE were cultured on DMcco100 medium (DMcc medium solidified with 0.7 g l⁻¹ TC agar [Phytotechnology Laboratories, LLC, Shawnee Mission, KS, USA] supplemented with 100 mg l⁻¹ kanamycin) in dark for 30 days. The callus was then transferred onto solidified X6cco70 medium (X6 medium containing 200 mg l⁻¹ each of carbencillin and cefotaxime and 70 mg l⁻¹ kanamycin) and maintained for 30–60 days with a 30-day subculture interval until secondary embryogenesis was observed. Transgenic SE from a particular primary SE explant were isolated and designated as an independent transgenic line. To confirm transgene integration, embryos...
from each test line were germinated on MS1B (MS medium supplemented with 1 μM N\textsubscript{6}-benzylaminopurine [BAP]) and grown into plants. Genomic DNA was isolated from leaf tissues and evaluated to confirm presence of the codA gene by PCR.

Five randomly selected independent transgenic lines proliferating after 2 subcultures on kanamycin-containing medium were used in experiments to determine a 5-fluorocytosine (5-FC) response curve. Cytosine deaminase activity was determined by response of transgenic SE to exogenously supplied 5-FC. SE were placed on DM medium containing either 0, 50, 100, 250 to 500 mg l\textsuperscript{-1} 5-FC and supplemented with carbencillin and cefotaxime as above (DMcc) for 30 days. Each 5-FC treatment consisted of 30 SE, replicated three times (i.e., 90 total embryos per independent line per treatment). The percentage of embryos that proliferated on medium containing each 5-FC concentration was determined.

2.5. Optimizing kanamycin treatment for co-transformation

 Cultures of two Agrobacterium EHA105 strains containing binary plasmids pCD\textscript{A} and pEGFP, respectively, were grown as described above. Prior to co-transformation, OD\textsubscript{620} value of each culture was adjusted to 0.8. Agrobacterium strains were mixed in a ratio of 1:1 and used to treat grapevine SE as described above. The effect of kanamycin treatment time on obtaining of stably transformed callus after subsequent 5-FC selection was determined by placing co-cultivated embryos on solidified or in liquid DMcck100 medium for 3, 5, or 7 days plus a 0 day control, resulting in seven treatments. Medium treatments were accomplished by placing SE in 50 ml of liquid DMcc100 medium as outlined above or into 100 mm × 15 mm Petri dishes (Fisher Scientific, PA, USA) containing 25 ml of DMcc100 medium solidified with 7.0 g l\textsuperscript{-1} TC agar (Phytotechnology Laboratories LLC, KS, USA) for each time period. After the positive selection treatment, negative selection was carried out in DMcc medium supplemented with 250 mg l\textsuperscript{-1} 5-FC for 30 days, as described above. The controls consisted of SE that were directly transferred to negative selection (DMcc medium containing 250 mg l\textsuperscript{-1} 5-FC). Resulting embryogenic callus from treatments and controls was transferred to X6c medium for SE proliferation. The presence of GFP positive callus was determined 30 days after transfer to DMcc containing 250 mg l\textsuperscript{-1} 5-FC as described above. Each treatment contained 100 SE replicated four times. Percentage of SE that produced GFP-expressing callus was determined after 30 days.

2.6. Optimizing the ratio of pCD\textscript{A} to pEGFP

 Cultures of Agrobacterium EHA105 strains containing pCD\textscript{A} and pEGFP, were mixed in ratios of 1:0, 0:1, 1:1, 1:2 and 2:1, respectively. Grapevine SE were submersed in bacterial mixture, co-cultivated for 3 days and processed through the steps including selection on 250 mg l\textsuperscript{-1} 5-FC as described above. Each treatment contained 100 SE replicated four times. Percentage of SE that produced GFP-expressing callus was determined after 30 days.

2.7. Selection and regeneration of transformants

EGFP-specific fluorescence was evaluated using a Leica MZFLIII stereomicroscope equipped for epi-fluorescence with an HBO 100 W Mercury lamp illuminator and a GFP filter set composed of an excitation filter (470/40 nm), a dichromatic beam splitter (485 nm) and a barrier filter (525/50 nm) (Leica Microscopy System Ltd., Heerbrugg, Switzerland). Frequency of transient EGFP expression was obtained after 3 days of co-cultivation on filter paper soaked with DM medium as outlined earlier, while that of stable EGFP expression was determined based on the formation of GFP expressing calli proliferating in X6 medium for at least 45 days. The number of stably transformed calli was determined by counting those with at least one EGFP-positive multicellular area. EGFP-positive calli were transferred to fresh X6 medium for further proliferation and induction of transgenic SE. For germination, mature transgenic SE were placed in MS1B and maintained at 25 °C with a 16 h light/8 h dark cycle using cool white fluorescent lights. Well-developed transgenic plants with an actively growing root system were subsequently moved into plug trays containing Promix BX potting medium (A.H. Hummert Seed Co., MO, USA). Plants that had produced three or more leaves were subsequently transferred to a greenhouse and maintained in 17 cm × 16 cm pots filled with Promix.

2.8. Detection of transgenes via PCR and quantitative real-time PCR

Genomic DNA was extracted from young leaves of putative transgenic plants based on the cetyltrimethylammonium bromide (CTAB) method [24]. PCR amplification was carried out to confirm genomic integration of the egfp gene by using egfp-specific oligonucleotide primer pairs including a forward primer (EG-51), 5’ATGTTGACGAGCGCCAGCGTGTG3’ and a reverse primer (EG-32) 5’ CTGTACACGCTGTCATGCGGAA3’. PCR cycling was as described earlier. Amplified products were size fractionated on a 0.8% agarose gel in TAE buffer. Gel electrophoresis was carried out at 80 volts for 40 min before DNA bands were visualized with a UV transilluminator.

Real-time PCR assays were performed in a LightCycler 480 instrument equipped with a 96-well plate Thermo-base and a software release v.1.5 (Roche Molecular Biochemicals, Indianapolis, IN, USA). All reactions were carried out in a 20 μl final volume containing 2 μl sample DNA (a total of 6 ng), 10 μl of 2 μ SYBR Green I Master PCR buffer (Cat No. 04707516001, Roche), 2 μl of each primer (0.5 μM) and 4 μl sterile water. Samples were replicated three times for each run and experiments were repeated twice. External controls representing 1–5 copies of the EGFP gene were prepared by diluting EcoRI linearized EGFP gene-containing plasmid pU203 to a given concentration and adding the DNA to reaction mixtures that contain 6 ng of DNA from non-transformed V. vinifera ‘Thompson Seedless’. The amount of plasmid DNA corresponding to a single copy of the EGFP gene per reaction was calculated as 6 ng (genomic DNA amount) × 5.352 × 10\textsuperscript{3} bp (size of pU203)/4.75 × 10\textsuperscript{8} bp (1C genome size of grape) = 0.0676 pg. Oligonucleotide primers for amplification of a 340-bp target fragment from the EGFP gene included a forward primer ERT-51: 5’CCATCTGGTGGCAGTGCAGACG3’ and a reverse primer ERT-32: 5’TTCAGCTCTGATGCCGTTTAC3’. Real-time PCR conditions were as follows: 95 °C for 10 min followed by 45 thermal cycles of 95 °C for 5 s, 56 °C for 10 s and 72 °C for 40 s, with a ramping rate of 4.4, 2.2 and 4.4 °C/s, respectively. The level of SYBR-specific fluorescence (483–533 nm) at the end of each cycle was measured and recorded via instrument CCD camera and software.

Following thermal cycling, melting curve analysis procedures consisted of heating up the sample plate to and incubating at 95 °C for 5 s at a ramp rate of 4.4 °C/s, lowering the temperature to 50 °C (with ramp rate at 2.2 °C/s) and maintaining the same temperature for 1 min, and then heating the sample plate to 95 °C with a ramp rate of 0.11 °C/s. Fluorescence change profiles in relation to thermal dynamic dissociation of DNA were recorded during the last heating step via continuous data acquisition process (5 acquisition/s).

Fluorescence signals were analyzed using instrument software-enabled “Absolute Quantification Analysis Using the Second
Derivative Maximum Method™. This analysis method utilizes crossing point (Cp) values to extrapolate initial concentration of target DNA in each sample ([25]; Roche instrument manual v. 1.5, pp. 167–169). Transgene copy number (EGFP gene) in each transgenic plant analyzed was determined based on comparison of Cp-extrapolated concentrations with in-run standard curves derived from EGFP gene-containing plasmid.

3. Results

3.1. *codA*-specific deaminase activity in grapevines

Thompson Seedless SE were transformed with pCDA and a total of twenty independent transgenic SE lines were generated. SE from five randomly selected lines proliferating after two rounds of subculture in kanamycin-containing X6 medium were transferred onto MS1B. PCR analysis of genomic DNA from SE-derived plantlets confirmed presence of the *codA* gene in all kanamycin resistant lines (Fig. 2). SE from each of these lines grown on media with increasing concentrations of 5-FC exhibited a decrease in callus formation with complete growth inhibition at 250 and 500 mg l⁻¹ 5-FC (Table 1). Morphologically, SE exposed to the lower concentrations of 5-FC (50 and 100 mg l⁻¹) exhibited growth inhibition and necrosis, but callus production eventually occurred (Fig. 3). Thus, 250 mg l⁻¹ of 5-FC was considered to be the lowest concentration at which complete inhibition of growth could be achieved in *codA* expressing transgenic SE and was thus used in subsequent experiments.

3.2. Co-transformation of grapevine SE

The effect of kanamycin selection time using liquid or solidified DMcck100 medium on the subsequent recovery of stably-transformed embryogenic callus after 5-FC selection revealed that there was little significant difference between 3, 5 or 7 days in liquid or solidified medium. In general, co-transformed embryos subjected to 3 or 5 days of positive selection in liquid medium treatments had a higher percentage of EGFP expression than those from solid medium treatments. However, kanamycin selection yielded significantly more stably transformed calli when compared to the no selection control (Table 2). Typically, embryogenic calli and SE grew from necrotic explants (Fig. 4A) and could be identified by EGFP expression (Fig. 4B).

A significant difference in GFP expression obtained with 0:1 pCDA/pEGFP (25%) compared to those with pCDA included (55–74%) demonstrates the effectiveness of *codA* gene-based 5-FC selection in this system. That the mixture ratio of 2:1 pCDA:pEGFP resulted in less GFP expressing calli compared to 1:1 and 1:2 ratios suggests that an insufficient amount of *egfp*-containing plasmid is rate limiting (Table 3). However, after prolonged negative selection, no transgenic SE expressing GFP were regenerated from cultures transformed only with pCDA or pEGFP. A total of 25 transgenic lines that expressed GFP were generated from the mixed *Agrobacterium* strain/plasmid treatments. This included 13 from the 1:1 treatment, 10 from the 1:2 treatment and 2 from the 2:1 treatment (Fig. 5).

3.3. Molecular analysis of transgene integration

Transgenic SE were transferred to fresh X6 medium for proliferation. PCR analysis of genomic DNA of all 25 lines showed that 7 contained both *egfp* and *codA/nptII* genes, whereas 18 lines contained only the *egfp* gene and not the *codA* gene (Fig. 6). Plants regenerated from marker-free lines exhibited high EGFP expression in all tissues and organs (Fig. 4C).

A set of reaction mixtures composed of one to five copies of EGFP gene equivalence, respectively, established a standard curve for evaluation of transgene copy number. These mixtures were prepared by adding EGFP gene-containing plasmid DNA to reactions containing non-transformed plant genomic DNA as described by Song et al. [26]. Cp-extrapolated target sequence concentrations in these standard samples matched, with high accuracy, the corresponding calculated concentrations of external plasmid DNA in each reaction (Table 4, compare mean concentrations from plasmid 1c to 5c). Accuracy of derived concentrations also was verified by amplifying DNA from a transgenic grape plant previously shown to harbor a single copy of the EGFP gene via
Southern hybridization [27] (data not shown). Extrapolated target sequence concentrations derived from 5 randomly selected marker-free transgenic plants revealed that MF-1, MF-2 and MF-3 contain a single copy, MF-4 contains two copies and MF-5 has up to 6 copies (Table 4). Results from repeated experiments were identical (data not shown). No amplification was detected from a non-transformed control plant (data not shown).

Melting curve analysis of real-time PCR products revealed that all amplified DNA produced a single-peak profile, indicating that fluorescence signals from all samples were derived from target-specific amplification. These results were also confirmed by electrophoresis of PCR products in which only a single band with a molecular size of 340 bp was obtained (data not shown).

4. Discussion

Co-transformation of unlinked DNA fragments provides a quick and easy method for introduction of non-selectable transgenes into the plant genome [28]. Co-transformation via a negative selection marker system has been successfully utilized to produce marker gene-free tobacco plants through segregation of progeny [4]. However, the technique is not suitable for use in vegetatively propagated crops like grapevine where it is not desirable to generate T1 lines for gene segregation. The current technique adapts co-transformation to vegetatively propagated crops by including a temporary positive selection phase before subjecting cells to negative selection. The assumption is that plant cells receiving either or both T-DNAs will be able to survive a temporary selection phase by virtue of transient expression of the positive selection gene (in this case nptII, which provides resistance to the antibiotic kanamycin). We believe that some cells harboring only the gene of interest may be cross-protected by adjacent nptII expressers or internal transient expression of a non-integrated nptII gene. When selection pressure is removed, such cells would have had the opportunity to integrate T-DNA containing the gene of interest independently from T-DNA containing the marker gene, thus offering stable marker free gene integration [14]. The E. coli codA gene has earlier been used successfully as a negative selection marker in plant transformation [4,8]. To inhibit cell proliferation, 5-FC concentrations ranging from 50 to 500 mg l⁻¹ have been shown to be effective [29]. We determined that 250 mg l⁻¹ was optimum for efficient negative selection. Our results demonstrated production of transgenic grapevines without genomic integration of an nptII gene by the addition of a temporary positive selection phase coupled with prolonged negative selection.

It has been speculated that co-transformation with different bacterial cultures and T-DNAs is facilitated by formation of a successful microbe-plant cell interface [30], since co-transformation relies on an effective interaction between the plant cell and Agrobacterium strains. Therefore, relative amount of bacterial cells from each strain during co-cultivation can have an impact on co-transformation efficiency [4,30]. In tobacco, Park et al. [4] observed highest co-transformation efficiency with a negative selection protocol using an equal mixture of two Agrobacterium strains. However, we did not observe significant differences in EGFP expression between the 1:1 pCDA/pEGFP and most of the 1:2 and 2:1 treatments. We also recovered stable GFP-expressing calli with the 0:1 pCDA/pEGFP treatment. This is a result of a low frequency of selection escape using our system. It is interesting to note that, even though transgenic GFP expressing calli was produced from the 0:1 treatment, we did not regenerate any stable somatic embryos from the said treatment (Fig. 5).

An important criterion during the initial positive selection phase is to provide an optimum amount of time that will neither promote proliferation of non-transformed cells nor result in high levels of marker gene integration [14]. Liquid medium culture has advantages over solid medium due to absence of nutrient gradients developing in the medium [31]. The present procedure extends that of Park et al. [4] by adding a short positive selection phase in order to select grapevine cells. We utilized the discovery that an initial short kanamycin selection phase effectively leads to arrest of cell proliferation and then combined it with prolonged negative selection to successfully generate stable GFP expressing cells. Among transformed cells, genomic integration of the egfp gene, independent of the marker genes, clearly occurred and was likely due to random T-DNA integration events. Ability of these cells to grow following a temporary positive selection phase was likely due to the influence of adjacent transformed cells that contained and expressed the selection marker gene nptII. Subsequent negative selection

**Table 2**  
Comparison of an initial positive treatment in liquid or solid medium containing 100 mg l⁻¹ kanamycin for 3, 5 or 7 days on recovery of stably transformed callus after negative selection on medium containing 250 mg l⁻¹ 5-FC.

<table>
<thead>
<tr>
<th>Duration of positive selection</th>
<th>Medium</th>
<th>Solid</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>68ab²</td>
<td>70a</td>
</tr>
<tr>
<td>5 days</td>
<td>73a</td>
<td>69ab²</td>
</tr>
<tr>
<td>7 days</td>
<td>62ab²</td>
<td>51b</td>
</tr>
<tr>
<td>Control</td>
<td>31c</td>
<td></td>
</tr>
</tbody>
</table>

¹ Grape SE were transformed with a 1:1 ratio mixture Agrobacterium strains containing pCDA (containing the nptII gene to confer resistance to kanamycin for use in positive selection, and the codA gene to confer sensitivity to 5-FC for use in negative selection) and pEGFP (containing the egfp gene as the gene of interest). Co-cultivation was carried out on filter papers wetted with liquid DM medium in dark at 26 °C for 3 days.

² Positive selection for the specified time interval was carried out in DMcck100 medium. Controls consisted of SE transferred directly into negative selection medium without an initial positive selection treatment.

Data represents the mean percentage of 100 SE replicated four times that produced GFP-expressing callus. Means followed by the same letter were not different at α = 0.05 using Student’s t-test.
pressure using 5-FC resulted in the death of cells expressing the nptII gene because it was linked to codA in the plasmid. Following co-transformation of tobacco, McKnight et al. [32] regenerated 11 plants, of which 3 contained both T-DNAs. Integration of two distinct T-DNAs at a same genomic locus after co-transformation has also been reported in rapeseed [33]. This would also lead to production of stable transgenic lines that contain both sets of T-DNA as was observed in seven of the transgenic grapevine lines. Transgenic plant lines containing both pCDA and pEGFP were recovered, which suggests that some SE lines overcame negative selection despite insertion of the codA gene. It also might be attributed to insertion of this marker in a non-actively transcribing region of the genome, thereby resulting in gene silencing and lack of cytosine deaminase synthesis and activity.

This study demonstrated the successful production of transgenic grapevines free of the selectable marker gene(s) that are typically required only for initial identification and selection of

### Table 3

<table>
<thead>
<tr>
<th>Ratio pCDA to pEGFP</th>
<th>GFP expressing callus (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>0d</td>
</tr>
<tr>
<td>0:1</td>
<td>25c</td>
</tr>
<tr>
<td>1:1</td>
<td>73a</td>
</tr>
<tr>
<td>1:2</td>
<td>74a</td>
</tr>
<tr>
<td>2:1</td>
<td>55b</td>
</tr>
</tbody>
</table>

*a* Grapevine SE were co-transformed with different ratios of Agrobacterium strains pCDA and pEGFP. After co-cultivation for 3 days, SE were subjected to 5 days of positive selection in liquid DM medium containing kanamycin. SE were then placed on DM medium containing 5-FC for 30 days. The number of GFP expressing callus was determined at the end of the negative selection period.

*y* Data represents the mean percentage of 100 SE replicated four times that produced GFP-expressing callus.

*z* Means followed by the same letter were not different at \( \alpha = 0.05 \) using Student’s t-test.

![Fig. 4](image1.png)

Fig. 4. Marker-free GFP-positive SE and plant. (A) Development of transgenic SE from necrotic explant on medium containing 250 mg l\(^{-1}\) 5-FC. (B) Same image as A illustrating GFP-expression from SE. (C) GFP expression from transgenic grapevine shoot-tip of MF2 line. AB, axillary bud; AM, apical meristem (enclosed by appendages); LF, leaf; TL, tendril.

![Fig. 5](image2.png)

Fig. 5. Total number of transgenic plant lines exhibiting EGFP epi-fluorescence obtained after co-transformation with different ratios of Agrobacterium strains containing pCDA and pEGFP from the experiment presented in Table 3.
transgenic cells. As shown by analyses of transgene integration, we produced transgenic *V. vinifera* 'Thompson Seedless' plants containing only a single stably integrated gene of interest, although examples of plants with multiple transgene copies were also recovered. We demonstrated a similar preponderance of plants with single copy transgene insertions in a previous study [23]. Our system utilizing somatic embryogenesis to produce marker gene-free plants provides the possibility to produce plants that contain only a gene of practical use and prevents the formation of genetic chimeras, which have been observed in other systems [27]. The technique can be used readily to produce grapevines with a range of other non-selectable genes and may be adapted for use in other vegetatively propagated crops as well.

Acknowledgements

This research was supported by the Florida Agricultural Experiment Station and the Florida Department of Agriculture and Consumer Services’ Viticulture Trust Fund. D.J. Gray and Z.T. Li own stock in Florida Genetics LLC, which may license resulting patents and, as such, may benefit financially as a result of the outcome of research reported in this publication.

References


