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

M. Dutt [1], 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

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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.

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*)

* Corresponding author. Tel.: +1 407 884 2034; fax: +1 407 814 6186.
E-mail address: djg@ufl.edu (D.J. Gray).
[1] Current address: Citrus Research and Education Center, University of Florida/IFAS, 700 Experiment Station Road, Lake Alfred, FL 33850, USA.

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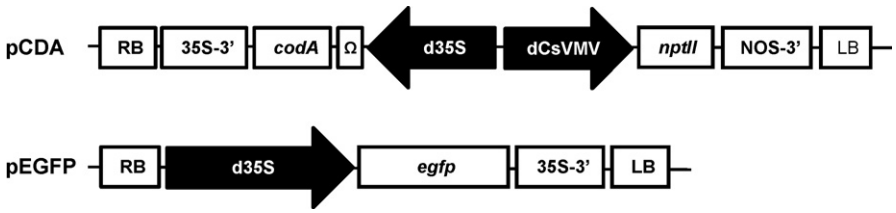

Fig. 1. Physical map of T-DNA region of the binary plasmids used for co-transformation. d35S, double enhanced (2× −419 to −90) CaMV 35S promoter with the Ω leader sequence of TMV; dCsVMV, double enhanced (2× −443 to −123) CsVMV promoter; 35S-3′and NOS-3′, termination site and polyadenylation signal of the CaMV 35S and NOS transcript; RB, right border; LB, left border.

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 *cod*A 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′ATGGTACCATGTCGAATAACGCTTTACAAA3′ was developed to change the *E. coli* GTG start codon to ATG and introduce a KpnI site immediately upstream of the translation start site. A reverse primer (CDA-32) 5′ATGCGGCCGCCGTCAACGTTTGTAATCAATGGCTTC3′ 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 pGCodA. The cloned gene was verified first by restriction enzyme analysis and then by DNA sequencing.

2.2. Plasmid construction

The *codA* gene was excised from pGCodA 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 pdCodA. 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 pBIN19-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 with shrimp alkaline phosphatase (SAP) for 5′ dephosphorylation 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-cotyledonary 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 meaasured using a Sunrise Optical Microplate Reader (Phenix Research Products, CA, USA) with a filter set at 620 nm wavelength. The OD_{620} 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 carbenicillin 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 DMcck100 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 X6cck70 medium (X6 medium containing 200 mg l^{-1} each of carbenicillin 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