Genetic Transformation of Embryogenic Cultures and Recovery of Transgenic Plants in *Vitis vinifera*, *Vitis rotundifolia* and *Vitis* Hybrids

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Abstract

Factors influencing *Agrobacterium*-mediated transformation of 15 *Vitis rotundifolia*, *Vitis rupestris*, *Vitis vinifera* and *Vitis* hybrids were explored. A green fluorescent protein/neomycin phosphotransferase II (GFP/NPT II) fusion gene was used to measure transient and stable transgene expression levels. Embryogenic cell cultures at different developmental stages were co-cultivated with *Agrobacterium* to determine the best stage for transformation. The effect of antioxidant treatment on recovery of transgenic embryo and plant lines was also studied. Transient and stable GFP expression varied widely among the species. For example, 1-81% of *V. vinifera* somatic embryos exhibited transient GFP expression, depending on the cultivar. Similarly, 0-60% of co-cultivated embryos produced stable transgenic lines. Proembryonal masses and cotyledonary stage somatic embryos were best for transformation of *V. rotundifolia*, *V. rupestris* and certain *Vitis* hybrids, whereas only cotyledonary stage somatic embryos were best for transformation of *V. vinifera*. *Agrobacterium*-induced browning/necrosis was reduced by adding 1 g L^{-1} dithiothreitol (DTT) antioxidant to the post co-cultivation wash medium. Transgenic plants have been recovered from *V. rupestris* 'St. George', *V. rotundifolia* 'Alachua' and 'Carlos', *V. vinifera* 'Cabernet Franc', 'Chardonnay', 'Merlot', 'Pinot Noir', 'Sauvignon Blanc', 'Shiraz', 'Superior Seedless', 'Thompson Seedless' and 'Zinfandel', and *Vitis* hybrids 'Conquistador', 'Seyval Blanc' and 'Freedom'. Research to optimize transformation conditions of additional *Vitis* species and hybrids is ongoing.

INTRODUCTION

Genetic improvement of *Vitis* has been carried out by hybridization and clonal selection (Olmo, 1942; Rantz, 1995). However, breeding in *Vitis* is limited by extreme heterozygosity and inbreeding depression (Winkler et al., 1974), which makes back crossing and recurrent selection difficult. F1 hybrids are of intermediate quality compared to parental lines and the long juvenile period of vines makes screening of new selections tedious and time consuming (Alleweldt and Possingham, 1988). As an alternative, genetic transformation might allow modification of popular grape cultivars by adding single traits without changing desirable characteristics (Gray et al., 2005). Transformation of embryogenic cultures has become routine only for a few cultivars and rootstocks, mostly using embryogenic cell cultures and *Agrobacterium tumefaciens* to effect gene transfer (Gray et al., 2005). Roadblocks to genetic transformation include poor embryogenic potential and low transformation efficiency due to severe browning/necrosis of cultures following co-cultivation with *Agrobacterium* (Perl et al., 1996). Initiation and maintenance of embryogenic cultures for a range of *Vitis* species and cultivars have been well documented (Gray and Mortensen, 1987; Gray, 1989, 1992, 1995). In the present study, the effects of developmental stage of embryogenic tissue and antioxidant treatment on *Agrobacterium*-mediated transformation were compared for two *Vitis rotundifolia* cultivars, *Vitis rupestris* 'St. George', nine *Vitis vinifera* cultivars and three *Vitis* hybrids.

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MATERIALS AND METHODS

Induction of Embryogenic Cultures

Embryogenic cultures of *V. rupestris* 'St. George', *V. vinifera* cultivars 'Cabernet Franc', 'Chardonnay', 'Merlot', 'Pinot Noir', 'Sauvignon Blanc', 'Shiraz', and 'Zinfandel', and *Vitis* hybrids 'Conquistador' and 'Freedom' were induced from anthers and ovaries as previously described (Gray and Mortensen, 1987). Embryogenic cultures of *V. rotundifolia* 'Alachua' and 'Carlos' (Robacher, 1993), *V. vinifera* 'Thompson Seedless' and 'Superior Seedless', and *Vitis* hybrid 'Seyval Blanc' (Gray, 1995) were initiated from in vitro grown leaves as previously described. Cultures were then maintained on growth regulator-free X6 medium (Li et al., 2001) for somatic embryo (SE) development and proliferation.

Embryogenic liquid suspensions (Jayasankar et al., 1999) were produced from nine of the cultivars (see Table 3) in order to increase culture mass. Liquid suspension culture-derived PEMs and SEs were transferred to X6 medium and maintained as described above before use in transformation experiments.

Transformation Vector

A reporter-marker fusion gene consisting of an enhanced green fluorescent protein (GFP) and the neomycin phosphotransferase (NPTII) gene, driven by a double Cassava Vein Mosaic Virus (CsVMV) promoter was introduced in the T-DNA region of a pBIN119 derived binary vector (Li et al., 2001). This construct was introduced into *Agrobacterium tumefaciens* strain EHA 105 using the freeze-thaw method (Burrow et al., 1990).

Preparation of *Agrobacterium* Cultures for Transformation

Agrobacterium strain EHA 105 was cultured overnight at 26°C on an orbital shaker at 185 rpm in liquid MG/L medium (Garfinkle and Nester, 1980) containing 100 mg L^{-1} kanamycin and 20 mg L^{-1} rifampicin. Prior to transformation, the bacterial culture was pelleted by centrifugation at 6000 rpm for 8 min, resuspended in 25 ml liquid X2 medium (the same composition as X6 medium with 20 g L^{-1} sucrose) and adjusted to an OD value of 0.6-0.8. The resuspended *Agrobacterium* was further cultured for an additional 3 h before use in transformation experiments.

Transformation of Embryogenic Cultures

PEMs and SEs were used for *Agrobacterium* co-cultivation as described earlier (Li et al., 2005). Embryogenic cultures were incubated in the bacterial solution for a period of ten minutes. Excess bacterial solution then was removed and the cultures were transferred to DM medium. This medium consisted of DKW basal salts (Driver and Kuniyuki, 1984), supplemented with 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, 7.0 g L^{-1} TC agar, 5.0 μM BA, 2.5 μM each of NOA and 2,4-D, at pH 5.7. Cultures were co-cultivated in the dark for two days and then washed for 3 days in liquid DMcck15 medium (DM medium containing 200 mg L^{-1} each of carbenicillin and cefotaxime, and 15 mg L^{-1} kanamycin). Cultures were then transferred to solid DMcck medium for callus formation and PEM proliferation. For selection of transgenic cells, *V. rupestris*, *V. vinifera* and *Vitis* hybrid cultures were maintained on DM medium for a period of one month, whereas *V. rotundifolia* cultures were maintained for a period of 3 months. Cultures were then transferred to X6cck70 medium for development of somatic embryos.

Antioxidant Treatment

The effect of the antioxidant dithiothreitol (DTT) on culture browning following co-cultivation with *Agrobacterium* was studied. SEs of different species and cultivars were co-cultivated with *Agrobacterium* for two days and then transferred to liquid DMcck15 medium supplemented with 0 or 1.0 g L^{-1} DTT. SEs were then cultured for

three days in darkness on a shaker at 110 rpm and transferred to solid DMcck medium for transgenic culture formation and proliferation. The extent of browning in wash medium was estimated by measuring the absorbance values of spent medium with a spectrophotometer at 405 nm wavelength.

Analysis of Transient and Stable Gene Expression

Transient GFP expression in co-cultivated PEMs and SEs was determined with a stereomicroscope equipped with epi-fluorescence illumination (Li et al., 2005). Percent transient expression was recorded as the number of SEs exhibiting 10 or more GFP-expressing cells vs. the total number of SEs co-cultivated with *Agrobacterium*. Stable transformation frequency was estimated based on the number of SEs producing GFP expressing callus vs. the total number of co-cultivated SEs. Transgenic plant lines were confirmed by observing GFP expression of leaves, stems, tendrils and/or roots.

Plant Regeneration

Transgenic SEs were transferred to germination medium, which consisted of MS medium (Murashige and Skoog, 1962) lacking $CaCl_2$ and supplemented with 709 mg L^{-1} $Ca(NO_3)_2$ $4H_2O$, 0.845 mg L^{-1} $MnSO_4$ H_2O, 1.0 g L^{-1} myo-inositol and 1.0 μM BA. SEs were placed in light (65 μmol s^{-1} m^{-1}) with a 16 h light photoperiod for 4-6 weeks. Plants with a shoot and root system were acclimatized to ex vitro conditions in a growth chamber and then transferred to the greenhouse.

RESULTS AND DISCUSSION

Developmental Stage Suitable for Genetic Transformation

The optimum stage of embryogenic cultures for *Agrobacterium*-mediated transformation differed among *Vitis* species (Table 1). For *V. vinifera*, SEs were the best targets for co-cultivation with *Agrobacterium* as indicated by GFP expression. PEMs failed to exhibit transient and stable GFP expression when used for co-cultivation. A similar result was observed with *Vitis* hybrids 'Conquistador' and 'Seyval Blanc'. The results confirm those of Li et al. (1999, 2001, 2005). In contrast, both PEMs and SEs of *V. rotundifolia* cultivars, *V. rupestris* 'St. George' and *Vitis* hybrid 'Freedom' exhibited high transient GFP expression following co-cultivation with *Agrobacterium*. Thus, the developmental stage of culture amenable to genetic transformation is species-dependent.

Antioxidant Treatment

The extent of browning of embryogenic cultures following co-cultivation with *Agrobacterium* varied among the *Vitis* species and cultivars. *V. rotundifolia* cultivars 'Alachua' and 'Carlos', *V. vinifera* cultivars 'Shiraz', 'Superior Seedless', 'Thompson Seedless' and 'Zinfandel' and *Vitis* hybrids 'Seyval Blanc', 'Conquistador' and 'Freedom' showed a significant reduction in browning following the use of DTT in the post co-cultivation medium wash (Table 2). However, *V. vinifera* cultivars 'Merlot', 'Chardonnay' and 'Cabernet Franc' did not show such a reduction. Browning of embryogenic cultures is attributed to a hypersensitive response of plant cells to the bacteria (Perl et al., 1996). Tissue browning after infection with *Agrobacterium* is a defense response, which results in the production of reactive oxidative species and is referred to as an oxidative burst (Wojtazek, 1997). Thus, the addition of an antioxidant to the culture medium following co-cultivation disrupts the defense response, subsequently improving recovery of transgenic tissue. Similar results were obtained by the use of DTT in *V. vinifera* (Perl et al., 1996), *Dioscorea esculenta* (Okpuzor and Omidiji, 1998), and *Glycine max* (Olhoft et al., 2001) to reduce culture browning. In contrast, no positive effect of DTT was observed in reducing browning of *Sorghum bicolor* embryogenic cultures following *Agrobacterium*-mediated transformation (Carvalho et al., 2004). Thus, response of embryogenic cultures to DTT is species- and cultivar-dependent.

Development of Transgenic SE Lines

Transient GFP expression was observed 48 h after co-cultivation and varied from 0-81% among the different species and cultivars (Table 3). The highest transient expression was observed in *V. vinifera* 'Thompson Seedless' (81%) followed by 'Superior Seedless' (76.7%) and 'Shiraz' (72.2%), *Vitis* hybrid 'Seyval Blanc' (72.2%) and *V. rotundifolia* 'Carlos' (70%). Among rootstocks, *V. rupestris* exhibited the best transient GFP expression (84%) followed by *Vitis* hybrid 'Freedom' (83%). Transient expression began to decrease after 10 days following co-cultivation. Transfer of explants to DMcck medium resulted in formation of GFP stable embryogenic cultures four weeks after co-cultivation. Stable transgenic SEs were observed two months following transfer of embryogenic cultures to X6cck70 with *V. rupestris*, *V. vinifera* cultivars and *Vitis* hybrids. In contrast, embryogenic cultures of *V. rotundifolia* 'Alachua' and 'Carlos' required three months on DMcck medium to produce transgenic cultures and PEMs. GFP stable SEs were observed following transfer of cultures to X6cck70 medium. The number of transgenic SE lines produced varied among species and cultivars (0 to 60%). Iocco et al. (2001) observed a similar differential response among cultivars of *V. vinifera*.

Transgenic Plant Regeneration and Transgene Expression

Transgenic plants were regenerated from SEs five weeks following transfer to germination medium. Plants were placed in potting mixture, acclimatized in a growth room and then transferred to the greenhouse. Transgenic plant lines have been recovered from *V. rotundifolia* 'Alachua' and 'Carlos', *V. rupestris* 'St. George', *V. vinifera* 'Cabernet Franc', 'Chardonnay', 'Merlot', 'Pinot Noir', 'Sauvignon Blanc', 'Shiraz', 'Superior Seedless', 'Thompson Seedless', 'Zinfandel', and *V.* hybrids 'Freedom', 'Seyval Blanc' and 'Conquistador' (Table 3). Uniform GFP fluorescence was observed in all plant organs as previously described (Li et al., 2001; Li et al., 2005). Efforts are ongoing to improve transformation efficiency in recalcitrant cultivars and to increase transgenic plant recovery.

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Tables

Table 1. Developmental stage amenable to *Agrobacterium*-mediated genetic transformation.

Species	Developmental stage
V. rotundifolia	PEM, Cotyledonary stage SE
V. rupestris	PEM, Cotyledonary stage SE
V. vinifera	Cotyledonary stage SE
Vitis hybrid ‘Conquistador’, ‘Seyval Blanc’	Cotyledonary stage SE
Vitis hybrid ‘Freedom’	PEM, Cotyledonary stage SE

Table 2. Effect of DTT on browning of embryogenic cultures following *Agrobacterium*-mediated transformation in *Vitis* species and cultivars.

Cultivar	Culture browning[1]	
	No DTT	DTT (1.0 g L^{-1})
	Vitis rotundifolia	
Alachua	0.45 ± 0.006	0.14 ± 0.003
Carlos	0.56 ± 0.001	0.23 ± 0.008
	Vitis vinifera	
Cabernet Franc	0.40 ± 0.005	0.41 ± 0.007
Chardonnay	0.99 ± 0.007	0.96 ± 0.008
Merlot	0.36 ± 0.001	0.36 ± 0.002
Shiraz	0.50 ± 0.003	0.45 ± 0.000
Superior Seedless	0.12 ± 0.001	0.08 ± 0.002
Thompson Seedless	0.25 ± 0.002	0.05 ± 0.001
Zinfandel	0.36 ± 0.005	0.22 ± 0.002
	***Vitis* hybrids**	
Conquistador	0.13 ± 0.006	0.08 ± 0.003
Freedom	0.23 ± 0.006	0.08 ± 0.003
Seyval Blanc	0.99 ± 0.006	0.42 ± 0.002

[1]Browning was measured as optical density (OD) value of post co-cultivation wash medium in a spectrophotometer at 405 nm wavelength.

Table 3. GFP expression in *Vitis* species and cultivars.

Cultivar	Proliferation in liquid medium	Transient expression (%)[1]	Stably transformed embryo lines (no.)[1]	Stably transformed plant lines (no.)[1]
		Vitis rotundifolia		
Alachua	Yes	58.0	72	21
Carlos	Yes	70.0	6	1
		Vitis rupestris		
St. George	Yes	84.0	74	3
		Vitis vinifera		
Cabernet Franc	No	72.0	1	1
Chardonnay	Yes	44.4	8	4
Merlot	Yes	68.8	102	39
Pinot Noir	No	10.0	19	5
Sauvignon Blanc	No	24.7	46	4
Shiraz	No	72.2	7	6
Superior Seedless	Yes	76.7	28	1
Thompson Seedless	Yes	81.0	1101	623
Zinfandel	No	52.2	5	1
		***Vitis* hybrids**		
Conquistador	No	68.8	20	10
Freedom	Yes	83.0	8	1
Seyval Blanc	Yes	72.2	35	5

[1]Based on GFP expression.