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Agrobacterium-mediated genetic transformation of an interspecific grapevine

by

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Summary: Anther-derived somatic embryos (cv. Georgikon 28) were transformed with reporter gene encoding β-glucuronidase, using the *Agrobacterium tumefaciens* strain EHA101. Embryogenic calli were maintained and proliferated on MS medium containing plant growth regulators and 50 mg·l^{-1} kanamycin. Numerous somatic embryos were obtained by placing calli on hormone-free MS medium supplemented with 50 mg·l^{-1} kanamycin. Transgenic plants were regenerated from these embryos. Application of antioxidants during and after cocultivation reduced necrotic symptoms of plant cells, while in the presence of an antioxidant during cocultivation the frequency of transformation was decreased. Injuries caused by particle bombardment did not increase the transformation efficiency.

Key words: Agrobacterium tumefaciens, genetic transformation, somatic embryogenesis, antioxidant, biolistic bombardment.

Abbreviations: BA = 6-benzylaminopurine, 2,4D-2,4-dichlorophenoxyacetic acid.

Introduction

Grapevine is the most widely grown fruit in the world with a remarkable economic importance and menaced by a number of diseases. Conventional breeding of resistant cultivars is time consuming due to the long generation cycle. Thus, genetic transformation is a promising approach to overcome this difficulty. At present, *Agrobacterium*-mediated transformation systems seem to be the most successful tool to obtain transgenic grapevines. According to previous reports grapevine tissues are adequate for *Agrobacterium* transformation, however, several attempts failed due to the lack of plant regeneration from transformed cells (Baribault *et al.* 1989, 1990, Guellec *et al.* 1990, Berres *et al.* 1992). While direct plant regeneration from somatic tissues of grapevine is difficult regeneration through embryogenic culture is relatively easy and offers a solution for this deficiency. The first successful regeneration of transgenic grapevine using hypocotyls of somatic embryos was reported by Mullins *et al.* (1990). With the aid of somatic embryogenesis, transgenic grapevines were produced transforming embryogenic calli (Le Gall *et al.* 1994, Nakamo *et al.* 1994, Krastanova *et al.* 1995, Perl *et al.* 1996) or somatic embryos (Martinelli and Mandolino 1994, Scorza *et al.* 1995). In the later cases plant regeneration occurred via secondary embryogenesis. Kikkert *et al.* (1996) obtained transgenic grapevines from somatic embryos using the biolistic transformation method (Klein *et al.* 1987).

Previously we described an *in vitro* culturing protocol to obtain embryogenic calli from anthers, induction of secondary embryogenesis from somatic embryos and regeneration of plants from somatic embryos (Mozsár and Viczián 1996). Based on this system, we now report a procedure for genetic transformation of grapevine, establishing transformed embryogenic calli lines which continuously produce somatic embryos resulting numerous transgenic plants. In this study we also investigate the effect of antioxidants and injuries caused by biolistic particle bombardment on the efficiency of *Agrobacterium*-mediated transformation of grapevine embryos.

Material and methods

Anther-derived embryogenic culture from the hybrid grapevine Georgikon 28 (G28) *(Vitis berlandieri* x *V.riparia* T.K.5BB x *V. vinifera)* was obtained as described previously (Mozsár and Süle 1994). Briefly, dissected anthers were cultured on MSE medium (MS salts and vitamins, Murashige and Skoog 1962) containing 0.8 % Noble-agar (Difco), 20 g·l^{-1} sucrose, 5 æM 2,4D, and 0.9 æM BA for a month and then transferred onto fresh medium with the same hormones. Embryogenic calli appeared two months after dissection. Somatic embryos were formed when these calli were placed onto MS or half strength MS medium.

In the transformation experiments, the disarmed *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.* 1986) carrying the binary vector pGSGluc1 was used (Saito *et al.* 1991). T-DNA of this binary plasmid carried a *nptII* gene conferring resistance to kanamycin and a *uidA* gene encoding the enzyme β-glucuronidase. Both genes were driven by a dual promoter of mannopinesynthase (Velten *et al.* 1984). pGSGluc1 was introduced into EHA101 via triparental mating. Bacterium cultures were grown overnight at 28 °C in modified liquid YEB (0.5 g·l^{-1} sucrose in place of 5 g·l^{-1}, Süle *et al.* 1994) supplemented with 100 mg·l^{-1} ampicillin and 50 mg·l^{-1} kanamycin.

Embryogenic calli were immersed into suspension of EHA101/pGSGluc1 (OD_{600} = 0.6). After 30 min calli were rinsed twice with sterile, distilled water, blotted dry and transferred

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onto MS medium plus 20 g·l^{-1} sucrose with or without 0.4 % Polyclar (Serva). Polyclar (insoluble polyvinylpyrrolidone) is an antioxidant with phenol binding feature. Petri dishes were incubated in the dark at 28 °C for 48 h. After 2 d, calli were rinsed 3 times with sterile distilled water and placed onto MS, MS plus 0.4 % Polyclar, MS plus 15 mM N-acetylcystein, MS plus 0.1 % L-cystein or MS plus 0.3 % activated charcoal. All media were supplemented with 500 mg·l^{-1} cefotaxim. The control calli were immersed into a YEB medium for 30 min.

In preliminary experiments the transient expression levels of the *uidA* gene in embryogenic calli were low. In order to get more precise differentiation we used somatic embryos (5-10 mm in length) to investigate the influence of Polyclar on the transformation efficiency during cocultivation. We used the transformation protocol described above. After 2 d of cocultivation on MS or MS plus 0.4 % Polyclar, embryos were transferred onto MS containing 500 mg·l^{-1} cefotaxim. 4 d later GUS expression was detected by the histological and fluorimetrical method.

In another experiment we examined the effect of particle bombardment on the efficiency of *Agrobacterium*-mediated transformation prior to cocultivation. A commercially available gas-driven accelerator device (Gene-Booster) was used to bombard somatic embryos with 1 µm uncoated tungsten particles. Somatic embryos were placed onto MS medium. Petri dishes were put 10 cm away from the microcarrier launch point and were bombarded once. Sample chambers were evacuated to 0.5 bar and 28 bar gas pressure was applied to the acceleration tube. Embryos were immersed into a diluted overnight culture of EHA101/pGSGluc1 (OD_{600}= 0.6) immediately after bombardment. The transformation protocol was used as described above. Following a two-day cocultivation on MS, embryos were washed with sterile, distilled water three times and placed onto MS medium supplemented with 500 g·l^{-1} cefotaxim. Plates were incubated for 4 d in the dark at 28 °C. GUS expression was detected by histological staining.

To obtain transgenic plants we used the bacterium strain and protocol as described above. After a 4-day incubation on a cefotaxim containing medium embryos were placed onto the MSE medium with 50 mg·l^{-1} kanamycin, 500 mg·l^{-1} cefotaxim and 0.2 % Polyclar against active oxygen and phenolic substances released by dead cells.

Postcocultivation of embryos on a selective medium was in the dark at 28 °C for 60 d. Somatic embryogenesis from developed embryogenic calli was induced on MS amended with 50 mg·l^{-1} kanamycin and 250 mg·l^{-1} cefotaxim.

Plant regeneration from somatic embryos occurred in liquid MS medium as described previously (MOZSÁR and SÜLE 1994).

The histological detection of β-glucuronidase activity was carried out as described by LE GALL *et al.* (1994). For the fluorimetrical method, samples were homogenised in 2 ml Eppendorf tubes using a micropestle. For each sample 200 µl extraction buffer (50 mM Na_2PO_4, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1 % Na-Lauryl Sarcosine, 0.1 % Triton X-100) were added. Tubes were centrifuged at 10,000 *g* for 10 min. From each tube 50 µl of supernatant were used to measure the protein content according to LOWRY *et al.* (1951). Another 50 µl of each supernatant was transferred into tubes containing 200 µl assay buffer (1 mM 4-methylumbellyferil-glucuronide in extraction buffer). Tubes were incubated at 37 °C for 3 h. From each reaction mixture 100 µl samples were transferred into tubes containing 900 µl 0.2 M Na_2CO_3 to stop the reaction. The amounts of free 4-methylumbellyferil (4-MU) were measured with a Hoefer TKO 100 fluorimeter.

To prove the presence of foreign DNA sequences in plant cells, the polymerase chain reaction (PCR) was used. The reaction mixture in a total volume of 50 µl contained 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1 % Triton X-100, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate, 2 pmol of each primer, 2.5 µl template, 2.5 U Taq DNA polymerase (Pharmacia). PCR was performed in a DNA thermal cycler PDR1, using the following protocol: initial denaturation at 95 °C for 2.5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, additional extension at 72 °C for 7 min. After PCR, 5 µl was separated by electrophoresis in a 2 % agarose gel in TAE buffer and the amplified DNA fragment was visualised by ethidium bromide staining.

The primer pairs 5'-GAGGCGAGGCGGCTATGACTG-3' and 5'-ATCGGGAGCGGCGATACCGTA-3' (HOFFMANN *et al.* 1997) were used to amplify the *nptII* gene and VCF 5'-ATCA TTTGTAGCGACT-3', VCR 5'-AGCTCAAACCTGCTTC-3' (SAWADA *et al.* 1995) for the amplification of *virC*. The *nptII* primers amplify a 800 bp fragment of the *nptII* gene. The VCF-VCR primers amplify a 730 bp fragment of the *virC* gene and were used to verify the absence of *Agrobacterium* on or within putatively transformed plants.

Results and Discussion

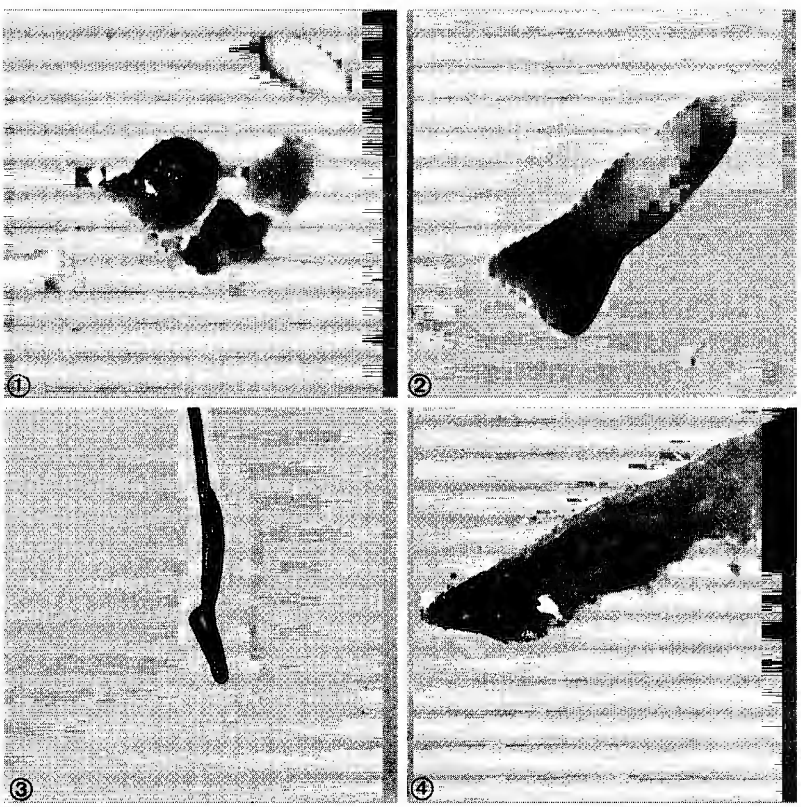
After cocultivation with *Agrobacterium,* when calli were placed onto MS, they became dark brown and exhibited severe necrotic symptoms. Necrotic calli did not grow further and died. Control calli immersed in bacterium-free YEB remained viable and maintained their yellow colour. One week after cocultivation calli on MS supplemented with either antioxidants or charcoal showed very reduced browning. After a month, colour of the calli on the medium with charcoal were very similar to the control. Those on media with different antioxidants were also healthy but a little bit more brownish. No differences between the antioxidant treatments were observed. In all cases, Polyclar improved condition of calli during cocultivation. Although charcoal was the most effective to reduce browning of calli it also strongly modified the impact of the plant growth regulators added to the medium (unpubl. results) probably by absorbing them. Therefore, in subsequent experiments we used Polyclar to reduce necrotic responses of explants. In addition, Polyclar is insoluble in water thus it can not interfere with biological processes in the cells.

In the next step we investigated the effects of Polyclar application during cocultivation on the transformation efficiency. Scoring histologically stained embryos cocultivated

on MS and on MS supplemented with 0.4 % Polyclar showed that in the presence of Polyclar the number of blue spots was decreased 10 times. The average blue spots per embryos detected histologically in MS and in MS with 0.4 % Polyclar were 2.63 and 0.27, respectively. The difference is statistically significant at P=0.05 according to Student's t test. Fluorimetrical measurements provided similar results. Average β-glucuronidase activity of extractions of embryos from MS and MS plus Polyclar were 115 and 47 unit per mg protein, respectively. One unit equals an enzyme activity, which produces one pmole 4-MU per min. During cocultivation bacteria grew more vigorously on the MS + 0.4 % Polyclar medium than on the MS medium. Accelerated growth of *Agrobacterium* was probably caused by the effect of Polyclar binding reactive oxygen and phenolic compounds released by infected plant cells. The higher number of *Agrobacterium* cells around the explants at the end of cocultivation could be the cause for different transformation efficiencies, although we can not explain, why vigorous bacterial growth causes lower levels of transformation. PERL *et al.* (1996) found that application of antioxidants during and after cocultivation prevents necrotic responses of embryogenic grapevine calli. In their report, the presence of Polyclar during cocultivation did not reduce the frequency of transformation events on tobacco leaf disks. Contrary to their observation we found that antioxidants had negative effects on the transformation efficiency when applied during cocultivation of somatic embryos of grapevine.

In the bombardment experiment the average number of blue spots was higher on control (2.45) than on injured embryos (1.56; statistically not significant). SCORZA *et al.* (1995) reported successful transformation of grapevine somatic embryos bombarded with uncoated tungsten particles before cocultivation with *A. tumefaciens*. In their experiment they had no control to check the influence of bombardment on the efficiency of transformation. Under our conditions particle bombardment had no positive effect on the number of transformation events. Perhaps instead of injuries other factors such as the applied vacuum decreased the transformation efficiency. MARTINELLI and MANDOLINO (1994) also found that wounding of grape somatic embryos by needle before cocultivation did not increase the frequency of transformation.

After 60 d of cultivation on selective media embryogenic calli were formed on the surface of embryos. These calli were detached and placed to a medium with the same components, only Polyclar was omitted and cefotaxim was reduced to 250 $mg \cdot l^{-1}$ and incubated in the dark at 28 °C. Under these conditions transformed embryogenic calli were maintained and proliferated, while non-transformed calli died if the medium contained this amount of kanamycin. Histological detection showed strong ß-glucuronidase activity in cells of transformed calli (Fig. 1). When embryogenic calli were placed to the MS medium supplemented with 50 $mg \cdot l^{-1}$ kanamycin and 250 $mg \cdot l^{-1}$ cefotaxim numerous somatic embryos appeared on the surface of the calli. These embryos were able to grow and developed on kanamycin-containing MS medium and were coloured dark blue after histological staining (Fig. 2). Roots and leaves of regenerated plants were also blue after histological staining (Figs. 3 and 4) while tissues of non-transformed plants did not show blue coloration. We found some unstained parts at the leaves of transformed plants, while roots became uniformly blue. MARTINELLI and MANDOLINO (1994) reported a similar obser-



Figs. 1-4: GUS expression **1** in transgenic calli; **2** in transgenic somatic embryo; **3** in root of transformed grapevine; **4** in sliced leaf of transformed grapevine.

vation. They explained uneven staining with wax layers at the leaf surface inhibiting the entry of substrate into the tissue.

No *Agrobacterium* growth was detected when transformed tissues were placed to the YEB medium.

PCR analysis of DNA with *nptII* primers from GUS positive calli and plants gave an 800 bp fragment of the *nptII* gene whereas non-transformed controls did not give DNA amplification (Fig. 5). The same DNA preparations failed to produce the 730 bp *virC* gene fragment, showing that the cultures were free of contaminating agrobacteria.

Here, we describe a protocol for transformation of grapevine. The maintenance of embryogenic calli and high frequency of embryo formation enabled us to gain a large number of transgenic grapevines in a single transformation experiment.

During cocultivation, Polyclar and charcoal as well as all applied antioxidants after cocultivation were effective to remarkably reduce necrotic symptoms of plant cells after elimination of bacteria.

However, when we applied Polyclar during cocultivation, the transformation efficiency decreased significantly. We also proved that wounding of grapevine somatic embryos by particle bombardment did not increase the efficiency of transformation.

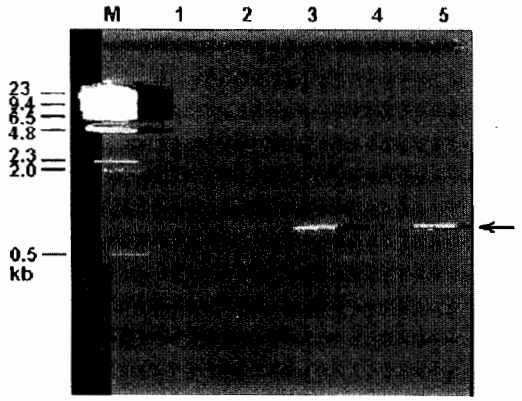


Fig. 5: Agarose gelelectrophoretic analysis of amplified DNA fragments by PCR. Lanes: M: lambda *Hind*III digest (bands from top to bottom: 23130, 9416, 6557, 4361, 2322, 2027, 564); **1**: untransformed grapevine with *nptII* primers; **2**: untransformed grapevine with *virC* primers (VCF-VCR); **3**: *Agrobacterium* (EHA101) with *virC* primers; **4**: transformed grapevine with *virC* primers; **5**: transformed grapevine with *nptII* primers.

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