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Thidiazuron-induced regeneration and genetic transformation of grapevine rootstock varieties

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Summary

***In vitro* regeneration from cell to plant, a technique considered to be an important precondition of gene transfer, was attempted in 12 grapevine rootstocks starting with anthers of the plants as inocula. Embryogenic callus was induced in solid Murashige-Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and thidiazuron (TDZ). Five of the tested rootstock cultivars (Börner, Richter 110, SO 4, St. George and Teleki 5 C) yielded embryogenic callus lines that regenerated entire plants. From these cultures Richter 110 and St. George varieties produced transgenic plants following *Agrobacterium*-mediated transformation with an *nptII/gusA* gene construct using a simple, but effective cocultivation protocol. The putative transgenic lines expressed the *gusA* gene. The T-DNA transfer and integration was confirmed by PCR analysis.**

Key words: thidiazuron (TDZ), somatic embryogenesis, *Vitis*, *Agrobacterium tumefaciens*, *nptII* gene, *gusA* gene.

Introduction

Grape rootstock varieties show proper resistance to different fungal diseases, but most of them are susceptible to abiotic stresses and to bacterial or virus diseases. To overcome these difficulties molecular breeding methods have recently been started to improve the stress- and disease resistance of grapevine cultivars (Perl and Eshdat 1998, Vivier and Pretorius 2000, Colova-Tsolova *et al*. 2001, Kikkert *et al*. 2001, Martinelli and Mandolino 2001). Transgenic rootstocks have been obtained in the last decade (Mullins *et al*. 1990, Le Gall *et al*. 1994, Krastanova *et al*. 1995, Mozsár *et al*. 1998, Xue *et al*. 1999), however routine regeneration protocols are known only for a few important genotypes.

A basic prerequisite of genetic transformation of somatic cells is an efficient protocol of cell-to-plant regeneration. First results on successful somatic embryogenesis of grape were reported by Mullins and Srinivasan (1976), and on adventitious organogenesis by Rajasekaran and Mullins (1981). For the induction of embryogenic structures from anthers most frequently combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BA) were used (Martinelli and Gribaudo 2001). Thidiazuron [TDZ, 1-phenyl-3-(1,2,3-thidiazol-5-yl)-urea], a synthetic compound, has been found to substitute for the hormonal requirement of somatic embryogenesis and morphogenesis in many plant species (Murthy *et al*. 1998). This compound has been successfully applied to replace BA on several *Vitis* genotypes as well, although its effect highly depended on the grapevine tissue culture, and on the *Vitis* genotype used for experiments. For somatic embryogenesis from leaf discs and from protoplasts of Seyval blanc 0.88-0.90 mg l^{-1} TDZ was used (Harst 1995, Reustle *et al*. 1995). Similar concentrations (0.88-1.1 mg l^{-1}) were applied for induction of secondary embryos in suspension cultures (Bornhoff and Harst 2000) and for initiation of embryogenic lines from anthers (Iocco *et al*. 2001). Nakano *et al*. (1997) used 2.2 mg l^{-1} TDZ to induce adventitious embryogenesis from leaf explants of different genotypes. In microspore cultures 0.2-2.0 mg l^{-1} TDZ was used for embryogenesis of different *Vitis* genotypes (Sefc *et al*. 1997) and 2.2 mg l^{-1} were applied in subculture after a two-weeks treatment with BAP of anthers of cv. Riesling (Harst and Alleweldt 1993). For maintenance of long-term embryogenic lines of *Vitis x Labruscana* cultivars 0.044 mg l^{-1} TDZ was sufficient (Motoike *et al*. 2001).

Grapevine transformation experiments have already been started in Hungary to introduce novel genes into the rootstock variety Georgikon 28 (Mozsár *et al*. 1998). We have tested a modified regeneration and transformation method on several additional rootstock varieties. Our objective was to study the effect of TDZ on induction of embryogenic calli from rootstock cultivars to extend the range of *Vitis* genotypes for genetic transformation.

Material and Methods

Production of embryogenic calli and somatic embryos: Induction of embryogenic calli was started from 100-200 anthers collected just before bloom from flower buds of 12 rootstock cultivars (Tab. 1.). The flower clusters in the bud stage were cut and disinfected in sodium hypochloride solution (Clorox, 10 %) for 15 min then rinsed three times in sterile distilled water. Excised anthers together with filaments were placed on solid medium containing the Murashige and Skoog (1962) basal salts supplemented with 20 g l^{-1} sucrose, 70 mg l^{-1} FeEDTA solidified with 7 g l^{-1} Oxoid agar. The pH was adjusted to 5.8. MST media were

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Table 1

The genetic origin of the tested rootstock varieties

Cultivars	Genetic origin
140 Ruggeri	*V. berlandieri* x *V. rupestris*
Börner	*V. riparia* x *V. cinerea*
Fercal	(*V. berlandieri* x *V. vinifera*) x EM333
Georgikon 28	*V. berlandieri* x *V. riparia* x *V. vinifera*
Kober 125 AA	*V. berlandieri* x *V. riparia*
Kober 5 BB Cr. 2	*V. berlandieri* x *V. riparia*
Richter 110	*V. berlandieri* x *V. rupestris*
Riparia Gloire	*V. riparia*
SO 4	*V. berlandieri* x *V. riparia*
St. George	*V. rupestris*
Teleki 5 C	*V. berlandieri* x *V. riparia*
Teleki 5 C Wed.	*V. berlandieri* x *V. riparia*

supplemented with various concentrations of 2,4-dichlorophenoxy-acetic acid (2,4-D) and thidiazuron (TDZ) (Tab. 2). After inoculation, the cultures were incubated in darkness at 26-28 °C. After induction calli were maintained with monthly transfer to MSE medium (MOZSÁR and SÜLE 1994). Somatic embryos were induced on hormone-free solid mediφν. Υθε ηεσνιξαυιξη ενβσζοτ ψεσε ιτομαυεδ αξδ υσαξτ-ζεσσεδ τεπασαωεμς υοξεψ υφβετ γοξυαιξιξη 10 νμ θαμζ-τυσεξηυθ, θοσνοξε-ζσεε τομιδ νεδιφν Υθεξ υθες ψεσε εωποτεδ υο α πθουοπεσιοδ οζ 16 θ (80 ™νομ ν^{-2} s^{-1}). After 3-6 weeks the plantlets with shoots were individually transferred onto the same medium. Experiments were repeated in two consecutive years.

Table 2

Hormone concentrations in the MST media

MST media	TDZ (mg l^{-1})	2,4-D (mg l^{-1})
MST1	0.05	1.1
MST2	0.1	1.1
MST3	0.1	2.2
MST4	0.2	1.1

Co-cultivation with *Agrobacterium* and selection of transformed lines: The embryogenic cultures of Börner, Richter 110, St. George and Teleki 5 C were transformed with *Agrobacterium tumefaciens* EHA101(pTd33). The T-DNA of pTd33 binary vector plasmid harbours an *nptII* gene conferring resistance to kanamycin, and a *gusA* gene, which encodes the ß-glucuronidase enzyme. Both genes are driven by the CaMV35S promoter (TINLAND *et al*. 1995). For the co-cultivation instead of immersing the whole plant material into a large volume of bacterial suspension, we applied small volumes (20-30 µl) of bacterial suspension (10^8 cells ml^{-1}) placed onto the surface of embryogenic cultures kept on hormone-free solid medium. Plant tissues (somatic embryos, length: 1-5 mm) were co-cultivated for 2 d with agrobacteria, then they were transferred to the same medium containing 20 mg l^{-1} kanamycin, 200 mg l^{-1} carbenicillin and 300 mg l^{-1} claforan, 4 g l^{-1} insoluble polyvinylpyrrolidone (PERL *et al*. 1996, MOZSÁR *et al*. 1998) and 0.1 g l^{-1} dithioerythritol (BORNHOFF and HARST 2000). Calli were transferred monthly to fresh medium of the same composition. From 50 to 100 germinating embryos were isolated and transferred separately to new tubes containing the same medium without antioxidants and they were exposed to light to induce shoot development.

Biochemical and molecular analysis of transgenic plants: The activity of the *gusA* gene was tested by histochemical assays during plant regeneration as described by JEFFERSON *et al*. (1987). For PCR analysis plant DNA was isolated from young leaves with Qiagen DNeasy Plant Mini Kit according to the supplier's protocol. A 700 bp region of the *npt*II gene and a 703 bp region of the *gusA* gene was detected using published primers (HOFFMANN *et al*. 1997). To verify the absence of the *Agrobacterium* vector in putatively transformed plants DNA samples were also tested with the *vir*C gene specific primers VCF and VCR, which amplifies a 730 bp virulence region located outside the T-DNA (SAWADA *et al*. 1995). PCR reactions were carried out in 25 µl reaction volume containing 1x *Taq* buffer, 1.5 mM $MgCl_2$, 5 % DMSO, 200 µM of each dNTPs, 0.5 µM of both primers, 1 µl template DNA (25 µg ml^{-1}) and 1.25 unit *Taq* polymerase. Reactions were started at 94 °C for 1 min, followed by 30 cycles of at 92 °C for 1 min, at 54 °C for 1 min and at 72 °C for 1 min 30 s. Reactions were terminated at 72 °C for 3 min. Samples were stained with ethidium bromide and analysed by electrophoresis in 1.5 % agarose gel.

Results and Discussion

Embryogenic callus or embryo cultures were reproducibly obtained on MST containing 2,4-D and TDZ in 5 of the 12 varieties tested. Teleki 5 C produced embryogenic calli on all hormone combinations, while Börner, SO 4 and St. George did only on MST1. Exceptionally, Richter 110 yielded exceptionally high amounts of embryogenic calli on MST4 (Tab. 3.). On the other hand, embryogenic callus induction on the MSE medium was unsuccessful except for cv. Richter 110 of which about 1 % of anthers generated embryogenic lines. Cells of the embryogenic rootstock cultivars maintained their embryogenic capacity during the transfers. For further studies the MST1 medium was used that yielded the most reproducible results for embryogenic callus induction (Tab. 4.). These calli regenerated plants from 17-37 % (Tab. 5) although irregular shoot development was also observed in these *Vitis* genotypes.

These embryo cultures were used for *Agrobacterium*-mediated transformation experiments. Transformed somatic embryos and regenerated plants were obtained from the cultures of. St. George and Richter 110 via secondary embryogenesis similarly as described for Georgikon 28 (MOZSÁR *et al.* 1998). On average, 2 and 7 putative transgenic plants were regenerated from 4 g of Richter 110 and St. George inocula, respectively.

Table 3

Embryogenic callus induction on excised anthers*

Cultivar	MST1	MST2	MST3	MST4
140 Ruggeri	0	0	0	0
Börner	2	0	0	0
Fercal	0	0	0	0
Georgikon 28	0	0	0	0
Kober 125 AA	0	0	0	0
Kober 5 BB Cr. 2	0	0	0	0
Richter 110	2	0	0	7
Riparia Gloire	0	0	0	0
SO 4	1	0	0	0
St. George	1	0	0	0
Teleki 5 C	4	3	3	3
Teleki 5 C Wed	0	0	0	0

* (Number of anthers producing embryogenic callus/number of anthers) x 100.

Table 4

Embryogenic callus induction on excised anthers on MST1 media

Cultivar	Average*	Standard deviation
140 Ruggeri	0	0
Börner	2.05	0.04
Fercal	0	0
Georgikon 28	0	0
Kober 125 AA	0	0
Kober 5 BB Cr. 2	0	0
Richter 110	1.65	0.25
Riparia Gloire	0	0
SO 4	0.9	0.07
St. George	1.65	0.46
Teleki 5 C	8.5	3.18
Teleki 5 C Wed	0	0

* (Number of anthers producing embryogenic callus/number of anthers) x 100.

Table 5

Frequency of plant regeneration from germinated embryos of rootstock cultivars

Cultivar	Average*	Standard deviation
Börner	19.5	2.25
Richter 110	35.5	1.25
SO 4	25.0	3.50
St. George	31.0	2.50
Teleki 5 C	27.5	0.75

* (Regenerated plants/somatic embryos) x 100.

The putative transformants (embryos and plants) expressed the *gus*A gene in histochemical assays. As shown by the *gus*-test, the ratio between chimerical and non-chimerical plants was approximately 1:1. The T-DNA transfer and integration was further confirmed by PCR analysis of non-chimerical St. George and Richter 110 lines. The results of these experiments showed that the *gus*A gene was present in the DNA samples of the putative transformants tested. The same DNA preparations failed to produce the 730 bp *vir*C gene-specific fragment, therefore the regenerated plants were free of contaminating *Agrobacterium* cells that may cause false positive results (data not shown). The *npt*II gene was similarly detected in all lines (data not shown).

Using a regeneration protocol based on the application of TDZ as a cytokinin we have successfully initiated embryogenic callus cultures from anthers of 5 of the 12 rootstock genotypes tested of which the regeneration of Börner and SO 4 has not yet been published. On the other hand, on BA containing medium only one cultivar (Richter 110) responded positively, although, in previous studies Georgikon 28 generated embryogenic callil on this hormone combination as well (Mozsár *et al.* 1998). The reason of our failure with Georgikon 28 is unknown. In our studies the 0.05 mg l^{-1} concentration of TDZ was more efficient than the previously published 0.88-2.2 mg l^{-1} (for literature see Introduction). Two of the 5 embryogenic cultures produced transgenic plants after co-cultivation with *Agrobacterium*. Therefore it is possible to transfer economically useful traits into additional rootstock cultivars of grapevine.

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Erratum

In the paper

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the text on page 134, lines 6-10 was printed in Greek letters and should be replaced by

"Somatic embryos were induced on hormone-free solid medium. The germinating embryos were isolated and transferred separately to new tubes containing 10 ml half-strength, hormone-free solid medium. Then they were exposed to a photoperiod of 16 h (80 µmol m^{-2} s^{-1})."

The publishers apologize for this error.