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The use of phosphinothricin resistance as selectable marker for genetic transformation of grapevine

R. Jardak-Jamoussi[1)], B. Bouamama[1)], A. Mliki[1)], A. Ghorbel[1)] and G. M. Reustle[2)]

[1)] Laboratoire Physiologie Moléculaire de la Vigne, Centre de Biotechnologie de Borj Cédria (CBBC), Hammam-Lif, Tunisia
[2)] RLP AgroScience GmbH, AlPlanta - Institute for Plant Research, Neustadt, Germany

Summary

A transformation procedure with the *bar* gene as a selectable marker was established via *Agrobacterium*-mediated transformation using strain LBA4404 harbouring the vector pPZP200-bar-gus-intron. Recreation of embryogenic cells from transformation stress in PPT free medium for four weeks improved viability and number of GUS expressing cells. Concentration of 2.5 mg·l^{-1} PPT yielded highest selection efficiency. Transgenicity of the regenerated grapevine plants was confirmed by histochemical GUS assay and *bar* specific PCR and RT/PCR. With the described procedure, 20 % of regenerated embryos could be converted into transgenic grapevines.

K e y w o r d s : *Vitis vinifera*, genetic transformation, *Agrobacterium tumefaciens*, *bar*, *gus*, PPT.

Introduction

To use transgenic approaches in grapevine breeding, efficient protocols for transformation, regeneration and appropriate selection systems are required. Genes coding for neomycin phosphotransferase (*npt*II), hygromycin phosphotransferase (*hph*) and phosphinothricin-*N* acetyl transferase (*bar*) confering resistance to kanamycin, hygromycin and phosphinothricin respectively, are frequently used as selection markers. Because of its efficiency, kanamycin resistance is the most frequently used selection system for genetic engineering of grapevine (Gambino *et al.* 2005). However, over the past years, controversial debates concerning possible ecological impact and harm to human health by transgenic plants expressing antibiotic resistance have been raised.

The phosphinothricin acethyl transferase (PAT) protein is encoded by the bialaphos resistance gene (*bar)* from *Streptomyces hygroscopicus*. This gene confers resistance to PPT by catalyzing the addition of an acetyl group to the free amino group (D'Halluin *et al.* 1992).

Recently, Hérouet *et al.* (2005) showed that no harm is resulting from the inclusion of this protein in food. Thus, the use of PPT resistance as selectable marker gene for genetic transformation of grapevine can mitigate public concerns.

The present work describes the establishment of an *Agrobacterium*-mediated genetic transformation system by using embryogenic material of Tunisian table grape variety 'Arich dressé' and the *bar* gene as selection marker.

Material and Methods

P l a n t m a t e r i a l : Initiation and maintenance of embryogenic cell cultures from anthers of *Vitis vinifera* L. 'Arich dressé' were realized as described by Bouamama *et al.* (2007).

P P T s e n s i t i v i t y o f e m b r y o g e n i c c e l l s : Toxicity of DL-PPT ($C_5H_{15}N_2O_4P$; *Mr*: 198.2; DUCHEFA) on embryogenic tissue was evaluated on MS (Murashige and Skoog 1962) medium with different concentrations of PPT (0, 1.0, 2.5, 5.0, 7.5 and 10 mg·l^{-1}). Each treatment included three replicates and was repeated twice (5 callus explants per plate). Effects of the different treatments were controlled every two weeks using a microscope.

G r a p e v i n e t r a n s f o r m a t i o n : *Agrobacterium tumefaciens* strain LBA 4404 (Hoekema *et al*. 1983) harbouring the binary vector pPZP200-*bar-gus-intron* was used for transformation experiments.

Agrobacteria were grown overnight by shaking (250 rpm) at 28 °C in 8 ml of liquid YEM medium (Vincent, 1970) supplemented with 15.0 mg·l^{-1} Rifampicin, 200 mg·l^{-1} Streptomycin and 200 mg·l^{-1} Spectinomycin to an OD = 1.0 at 600 nm.

For infection, the embryogenic callus was immersed for 30 min in 15ml bacteria suspension of liquid MS medium containing 200 µM Acetosyringone and 2.5 g·l^{-1} PVPP (Polyvinylpolypyrrolidone). Embryogenic callus was carefully plated onto solid MS medium (pH 5.8) consisting of the same components as in the infection medium. Per plate 10 callus explants were placed and co-cultivated for 48 h at 22 °C in the dark. After rinsing with liquid MS medium containing 500 mg·l^{-1} Carbenicillin and 2.5 g·l^{-1} PVPP, callus was incubated on growth regulator free solid MS medium (fresh medium transfer in 4 week intervals) containing 500 mg·l^{-1} Carbenicillin, 3 g·l^{-1} activated charcoal (AC) and different PPT concentrations (0, 2.5 or 5 mg·l^{-1}). Twelve weeks post co-culture, selection was continued for another 16 weeks period on MS medium supplemented with growth regulators (1.0 mg·l^{-1} NOA, 0.5 mg·l^{-1} 2,4D, and 0.2 mg·l^{-1} BAP). Subsequently, explants were transferred

Correspondence to: Dr. G. M. Reustle, RLP-AgroScience GmbH, AlPlanta - Institute for Plant Research, Breitenweg 71, 67435 Neustadt/Weinstraße, Germany. Fax: +49-6321-671-1313. E-mail: goetz.reustle@agroscience.rlp.de

on growth regulator free MS medium containing reduced PPT (1 mg·l^{-1}) and Carbenicillin (250 mg·l^{-1}) concentrations. For embryo conversion and regeneration, mature embryos were transferred into glass tubes on MS medium and cultivated at 24-25 °C under a 16 h photoperiod with a light intensity of 70 µmol m^{-2} s^{-1} for micropropagation and gradually exposition to greenhouse conditions.

GUS assays: Transformation efficiency was confirmed by histochemical GUS assays according to JEFFERSON *et al.* (1987) and assessed by recording GUS expression at different stages of somatic embryo development and in leaves of regenerated plants.

Molecular analysis: Detection of the *bar* gene (*bar* fragment*:* 324 pb) was performed by PCR using specific primers at 62.4 °C (5' TCT GCA CCA TCG TCA ACCACT ACA 3'; 5'GCA GCC CGA TGA CAG CGA CCA C 3'). DNA was extracted from non transgenic and putative transgenic grapevines leaves following the method of LODHI *et al.* (1994).

For reverse transcription/PCR**,** total RNA was extracted from leaf material of non-transformed and transformed, PCR positive grapevine plants using the plant RNeasy kit (Qiagen). Per sample 100 ng aliquots of RNA were used as templates for one RT-PCR amplification reaction (One step RT-PCR system, GibcoBRL).

Results

Effect of PPT on embryogenic callus of grapevine: Application of 7.5 and 10 mg·l^{-1} of PPT to embryogenic callus of grapevine resulted in a strong reduction of growth and a total necrosis of the callus within 2 weeks of culture. Necrosis of embryogenic callus was accompanied by a complete browning of the medium. Similar results were reported by PERL *et al.* (1996) for *Vitis vinifera* 'Superior seedless'. Using 1 mg·l^{-1} PPT during 4 weeks, formation of embryo clusters on most of the callus explants was observed as already found for *Vitis* sp. L. with PPT concentrations lower than 1.0 mg·l^{-1} (HÉBERT-SOULE *et al.* 1995). Application of 2.5 and 5 mg·l^{-1} PPT resulted in moderate necrosis, however, embryo maturation and conversion was completely inhibited. Addition of PVPP (2.5 g·l^{-1}) or AC (3.0 g·l^{-1}) could diminish the process of necrosis. Furthermore, PPT treatment (2.5 mg·l^{-1}) and the addition of growth regulators (1.0 mg·l^{-1} NOA, 0.5 mg·l^{-1} 2.4D, 0.2 mg·l^{-1} BAP), could induce formation of new embryo clusters. Combination of PVPP or AC and growth regulators with higher concentration of PPT (7.5 to 10.0 mg·l^{-1}) did not reduce the necrotic process and did not allow formation of new embryo clusters. Considering additional stress by the *Agrobacterium* treatment, for subsequent transformation experiments, PPT concentrations of 2.5 and 5.0 mg·l^{-1} were chosen for selection.

Establishing the PPT selection system for grapevine transformation: Transient GUS expression in the embryogenic callus following co-cultivation indicated successful infection events. Regarding the strong toxic effect of PPT on 'Arich dressé' embryogenic tissue, two strategies were tested for selection: i) transfer of callus directly to the selection procedure and ii) recreation of callus from transformation stress on PPT free MS medium for 4 weeks before starting the selection process with PPT. In the presence of PPT, embryogenic callus was markedly affected by an accentuated necrosis. In contrast, callus maintained on PPT free MS medium could retain its ability to proliferate. Additionally, the number of blue spots assayed on 5 callus explants, 4 weeks post co-cultivation (Experiment 1: 39.60 ± 2.15; Experiment 2: 51.00 ± 0.03) was significantly higher (at $p < 0.01$, by Duncan's multiple range test of STATISTICA software) on callus cultivated on PPT free medium than on callus cultivated on MS medium supplemented with 2.5 mg·l^{-1} PPT (Experiment 1:18.80 ± 3.71; Experiment 2: 15.00 ± 3.16).

After additional eight weeks under selection on 2.5 mg·l^{-1} and 5 mg·l^{-1} PPT, new creamy-white cell clusters started to develop. At this stage, the number of blue areas on 5 assayed clusters selected with 2.5 mg·l^{-1} was significantly (at $p < 0.01$) higher (33 ± 2.82) than on those selected with 5 mg·l^{-1} (10 ± 1.73). This demonstrates that recreation from stress of the *Agrobacterium* infection process before starting PPT selection could improve transformation efficiency. A recreation period was also reported by HOSHINO *et al.* (1998) for co-cultivated embryogenic callus of *Vitis vinifera* L. 'Koshusanjaku'. Addition of growth regulators (1.0 mg·l^{-1} NOA, 0.5 mg·l^{-1} 2,4D, 0.2 mg·l^{-1} BAP) at that point could improve the proliferation of newly developed embryogenic clusters.

During additional 16 weeks of cultivation on MS medium, containing growth regulators as described above, from 30 co-cultivated callus explants, 27 proliferating embryogenic clusters were obtained on medium with 2.5 mg·l^{-1} PPT, whereas only 7 clusters developed on medium with 5 mg·l^{-1} PPT.

From each initial co-cultivated callus explant one new embryogenic cluster was transferred to maturation medium consisting of growth regulator free MS medium with PPT (1 mg·l^{-1}) and Carbenicillin (250 mg·l^{-1}). In contrast, for selection of transformed suspension cells of sugarbeet, KISHCHENKO *et al.* (2005) increased PPT concentration from 5.0 to 10 mg·l^{-1} however, shoot regeneration failed under these conditions. Beside changing conditions for selection, reduction of antibiotic (Carbenicillin, Cefotaxim) targeting *Agrobacteria* during selection was also reported to be efficient for regeneration of transformed grapevine plants (BORNHOFF *et al*. 2005).

Conversion of embryos was realised on MS medium without growth regulators, PPT and Carbenicillin (rooting medium) within 8 weeks. Transgenicity of converting embryos was monitored by GUS expression. Continued selection with 2.5 and 5.0 mg·l^{-1} PPT during embryo maturation and conversion resulted in browning of the shoot meristem and the developing cotyledons.

In total 202 mature embryos were obtained by selection on 2.5 mg·l^{-1} PPT, whereas only 28 embryos developed from selection with 5 mg·l^{-1} PPT. In addition, selection with 5.0 mg·l^{-1} PPT strongly reduced ability of regenerated embryos for conversion into plants.

Plant regeneration of grapevines was performed on the rooting medium. GUS assay of regenerated plants showed 20 out of 100 regenerants being non-chimeric (Fig. 1) when selection was performed with 2.5 mg·l^{-1} PPT, whereas only 2 out of 19 regenerated plants were identified by GUS assay as non-chimeric when selected with 5 mg·l^{-1} PPT. After acclimatisation in the greenhouse, transformed plants showed normal vegetative growth and appeared healthy with a high similarity in leaf morphology to non-transgenic grapevines of 'Arich dressé.'

Fig. 1: GUS expression in a transgenic grapevine plant (*Vitis vinifera* 'Arich dressé').

Molecular analyses: PCR amplifications using primers specific to the *bar* gene yielded the expected product of 324bp in 20 PPT selected grapevine lines (Fig. 2 A). Genomic DNA of non-transformed grapevines gave no PCR product. RT-PCR using the same *bar* specific primers confirmed presence of the *bar* transcript in grapevines regenerated via selection with 2.5 mg·l^{-1} PPT (Fig. 2 B).

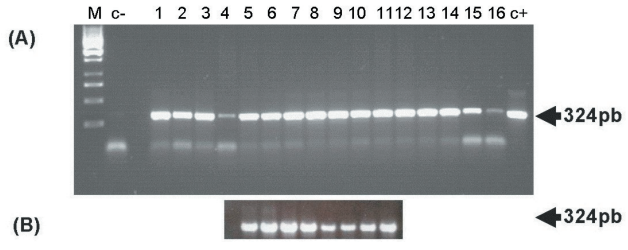

Fig. 2: (**A**) Ethidium bromide stained agarose gel of *bar* PCR amplified products with a size of 324 pb from 15 PPT-selected clones. Lanes: M Marker 1Kb DNA ladder (GibcoBRL); c-: DNA of non transformed grapevine (negative control). c+: plasmid vector pPZP200 bar-gus-intron (positive control). Lanes: 1; 2; 3; 4; 5; 6;7; 8; 9; 10; 11; 12; 13; 14, 15 and 16: DNA of transformed grapevine plants. (**B**): RT-PCR *bar* specific product of 324 pb amplified from RNA transgenic grapevine plants.

With the presented experiments, a protocol for regeneration of transgenic grapevines using herbicide resistance as selectable marker was established. In this system, recreation of infected callus from transformation stress, determination of suitable selective agent concentration, conditions and duration of selection lead successfully to stable transformed grapevines. Molecular analyses confirmed the validation of the proposed system. This is the first report on genetic transformation of a Tunisian grapevine cultivar using the *bar* selection system.

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