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MOLECULAR BREEDING OF GRAPEVINE USING USEFUL GENE CONSTRUCTIONS

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INTRODUCTION

The foundation of genetic transformation is the existence of an effective regeneration and transformation system and gene useful constructions for practice. Different transformation systems are often based on somatic cultures. To induce somatic embryogenesis and to regenerate transgenic plants numerous methods can be used. Among these, essential differences are noticeable in the parts of plant used for induction, the medium with different combinations of plant growth regulators. Different types of explants have been tested for their ability to produce somatic embryos under induction conditions, such as anthers (Perrin et al., 2004), leaf discs (Harst 1995), ovaries (Kikkert et al., 2005) and petiole-derived callus (Martinelli et al., 1994). However, somatic embryogenesis remains genotype dependent (Maillot et al., 2006). To induce embryogenic callus approximately ten plant growth regulators (PGR) have been used in combinations; 2,4-D and BAP have been effectively adopted to induce embryogen callus in a wide range of grape cultivars (Lopez-Perez et al., 2005; Pinto-Sintra, 2007).

Crossbreeding to obtain frost tolerant and disease resistant grapevine varieties has been carried out in Hungary for decades. However, it has proved to be difficult to get newly processed varieties accepted by growers, wine industry and consumers, because of their preference for the well-known, traditional varieties. Therefore the new, cross-bred grapevine genotypes have little chance to become popular for growers and on the market. On the other hand, traditional breeding methods are time consuming and tedious procedures.

Molecular breeding offers the possibility of generating plants that contain one additional gene, but do not differ noticeably from their parental cultivars in normal conditions. Grapevine transformation experiments have already been started in Hungary as well to introduce novel genes into the rootstock variety ‘Georgikon 28’ (Mozsár et al., 1998). Based on these results, Oláh et al. (2003a) started the examination of grapevine regeneration via somatic embryogenesis and tested this transformation method on ‘Richter 110’ and ‘St. George’ genotypes with *nptII/GUS* genes. In their experiments they obtained genetically transformed grapevine plants from embryogenic cultures regenerating well.

Abiotic and biotic stresses have a significant effect on plant productivity. Under stress conditions rapid accumulation of reactive oxygen species (ROS) starts inside the cells. Production of the hydroxyl radical (OH·), which is the most harmful one, depends on the presence of free iron in living cells (Halliwell and Gutteridge, 1986). Through Fenton reaction hydrogen peroxide (H_2O_2) contacts with free Fe^{2+} resulting in the formation of hydroxyl

radicals: $H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^\cdot + Fe^{3+}$. The iron-mediated Fenton-oxidants can destroy all classes of biologically important macromolecules, especially nucleic acids (Henle and Linn, 1997). Since intracellular iron catalyzes oxidative reactions, the control of the concentration of free iron might be a potential way to reduce oxidative damage (Deák et al., 1998). Intracellularly most of the nonmetabolic iron is kept in a protein called ferritin. This iron-storage protein is widespread in living organisms from bacteria to mammals (Theil, 1987). The plant ferritins are mainly localized in the chloroplasts. Deák et al. (1998) managed to get tobacco plants transformed by ferritin gene deriving from *Medicago sativa* (*MsFerr*). These considerations supported the idea that overexpression of ferritin in grapevine plants makes them tolerant against oxidative damage.

Crown gall disease induced by *Agrobacterium tumefaciens* or *Agrobacterium vitis* causes serious damage worldwide on several plant species, e. g. on grapevine, fruit trees and raspberry. It is particularly important bacterial disease of grapevine in cool-climate production areas, such as the grape-growing regions of Hungary. Although the loss can be reduced by using *Agrobacterium*-free planting material and resistant rootstock varieties, there is no efficient method yet that can be routinely used by grape-growers to prevent this disease (Burr et al., 1998).

In the process of crown gall tumorigenesis pathogenic agrobacteria transfer a specific segment of their Ti (tumor inducing) plasmid, termed T-DNA into the plant chromosomal DNA resulting in tumorous growth and opine production (Weising and Kahl, 1996; Zupan and Zambryski, 1995). The T-DNA transfer process is determined by a set of virulence proteins (VirA-F). VirE1 strongly interacts with VirE2 and has been proposed to prevent the self-aggregation and binding of VirE2 molecules to the T-strand in the bacterium (Deng et al., 1999). Szegedi et al. (2001) assumed that in the case of VirE1 protein being already present in the plant before *Agrobacterium* infection will bind VirE2 protein transferred with the infection. If this interaction is strong enough, it may prevent tumor formation and cause resistance to crown gall disease.

Crown gall tumors result from overproduction of auxin and cytokinin in plant cells transformed by *Agrobacterium tumefaciens* (Winans, 1992). These abnormally high phytohormone levels result from expression of three genes transferred stably into the plant genome from the *A.tumefaciens* tumor-inducing (Ti) plasmid: *iaaM* (Trp mono-oxygenase), *iaaH* (indole-3-acetamide hydrolase) and *ipt* (AMP isopentenyl transferase). IaaM converts Trp into indole-3-acetamide, which IaaH converts into indole-3-acetic acid (Inzé et al., 1984). Loss of either enzyme prevents auxin production. Ipt converts AMP into isopentenyl-AMP, a

cytokinin (Winans 1992). Inactivation of *ipt* and either one of the two auxin biosynthesis genes abolishes crown gall formation (Lee et al., 2003). Posttranscriptional gene silencing (PTGS) or RNA interference of three integrated *Agrobacterium* oncogenes resulting crown gall tumors can be specially useful because the mRNA products of genes transferred by *Agrobacterium* appear in the transformed cells (Burr et al., 1998).

AIMS OF OUR STUDY

1. Induction and propagation of embriogenic callus in different culivars.
2. The increase of regeneration efficiency.
3. Genetic transformation experiments with different gene constructions to obtain crown gall and oxidative stress resistant plants:
   - To obtain 'Richter 110' transgenic plants overexpressing alfalfa ferritin gene using EHA105(pRok2Ferr) vector.
   - To obtain *Agrobacterium* resistant transgenic 'Richter 110' lines by overexpressing *Agrobacterium* VirE1 proteins using EHA101(pTd93VirE1) construction; and through posttranscriptional gene silencing applying EHA101(pJP17) vector.
4. Testing the expression and the effect of the different gene constructions in transformed plants.

MATERIALS AND METHODS

Induction and propagation of embryogenic callus

Induction of embriogenic callus was started from anthers collected before blooming from flower buds of different genotypes. The flower clusters in the bud stage were cut and disinfected in 10% sodium hypochloride solution (Clorox) for 15 min then rinsed three times in sterile distilled water. Collected inflorescences from 'Chardonnay' cultivar were surface sterilized by submersion in 7 % $Ca(OH)_2$ solution, too. Excised anthers together with filaments were placed on solid medium. We studied the embryogenic capacity of anther derived calli in 12 various grape genotypes. The embryogenic capacity of 'Arany sárfehér', 'Cabernet franc', 'Odysseus', 'Orpheus', Taurus' and 'Richter 110' was tested on two different media (MSE, MST); in case of 'Chardonnay', 'Kékfrankos', 'Korai Bíbor', 'Pannon frankos', 'Rajnai rizling' and 'Teleki 5C' cultivars four media (MSE, MST, MSE/2, NNE)

were used. MS based media (Table 1) contained Murashige and Skoog (1962) basal nutrients and vitamins, were supplemented with 20 g/l sucrose and 70 mg/l FeEDTA, and were solidified with 7 g/l Oxoid agar. The pH was adjusted to 5.8. Used (1.) MSE medium (Mozsár and Süle, 1994) contained 0.1 mg/l BAP and 1.1 mg/l 2,4-D, (2.) MSE/2 is a modified MSE medium with half amount of macroelements, (3.) MST (Oláh et al., 2003a) was supplemented with 0.05 mg/l TDZ and 1.1 mg/l 2,4-D. (4.) NNE medium is based on Nitsch and Nitsch (1969) medium, with 0.1 mg/l BAP and 1.1 mg/l 2,4-D hormon composition. The emryogenic callus induction experiments were repeated 6 times, with each plate holding 50 anthers. The anther cultures were incubated at 26 ºC in dark. The embryogenic capacity of the resulting calli was evaluated three months after the start of the experiment. Calli was transferred monthly to fresh MSE media, because Oláh et al. (2008) in their experiments found this medium to be the best for propagation of anther derived embryogenic callus. Besides embryogenic calli from 'Richter 110' rootstock were maintained on Chée and Pool (1987) medium supplemented with 1.1 mg/l 2,4-D and 0.1 mg/l BA (CPE medium, Table 1).

We aimed to homogenize the embryogenic material derived from 'Teleki 5C', 'Richter 110' and 'Chardonnay' anthers, applying MSNOA liquid medium (Table 1) in dark conditions. In our experiments we tested the effect of 5 μM NOA (2-naphtoxy-acetic acid) in the medium containing a certain amount of maltose and glycerin described by Mauro et al. (1995). The homogenous calli propagated in the suspension medium were placed onto solid MS/2 medium after 2 weeks. In control experiments embryogenic callus of the three cultivars from MSE medium was used. Their further growth was observed on solid MS/2 medium and was compared to the materials derived from suspension medium.

Table 1 All sort of the used media.

Media	Basal medium	Source of C	Macroelemens (conc.)	Microelemens (conc.)	PGR
MS/2	Murashige & Skoog	sucrose	half	total	-
MSE	Murashige & Skoog	sucrose	total	total	BA, 2,4-D
MSE/2	Murashige & Skoog	sucrose	half	total	BA, 2,4-D
MST	Murashige & Skoog	sucrose	total	total	TDZ, 2,4-D
MSNOA	Murashige & Skoog	maltose glycerin	total	total	NOA
NNE	Nitsch & Nitsch	sucrose	total	total	BA, 2,4-D
CPE	Chée &Pool	sucrose	total	total	BA, 2,4-D

Grapevine transformation experiments using *MsFerr*, *iaaM* and *virE1* genes

For transformation experiments the anther derived embryogenic culture of 'Richter 110' was used. Somatic embryos were induced on hormone-free solid MS/2 medium (Table 1). The embryogenic culture of 'Richter 110' was transformed with *Agrobacterium tumefaciens* of which T-DNA contained the different gene constructions.

To increase oxidative stress resistance of grapevine we applied EHA105(pRok2Ferr) gene construction. The EHA105(pRok2Ferr) construction contains neomycin-phosphotransferase (*nptII*) gene as a selection marker and alfalfa ferritin gene. The ferritin gene is under the control of CaMV 35S promoter. The alfalfa ferritin is transported and accumulates in the chloroplasts (Deák et al., 1998).

The EHA101(pTd93VirE1) construction contained *nptII* selection marker gene and a *virE1* gene derived from *A. tumefaciens* under the control of CaMV 35S promoter (Szegedi et al., 2001). The oncogene silencing construct EHA101(pJP17) was established from the T-DNA fragments of *A. tumefaciens* A348 sequences that contains an octopine Ti plasmid. pJP17 harbors a single copy of a partial *iaaM* and a full length *ipt* sequences placed under the control of CaMV 35S and FMV promoters that direct the production of self-complementary mRNAs for efficient silencing (Lee et al., 2003, Viss et al., 2003).

For the cocultivation we applied small volumes (20-30 µl) of bacterial suspension (approximately 10^8 cells/ml) placed onto the surface of embryogenic cultures kept on hormone-free solid MS/2 medium. Plant tissues (somatic embryos with size 1-2 mm) were

cocultivated for two days with agrobacteria, then they were transferred to same medium containing 20 mg/l kanamycin, 200 mg/l carbenicillin and 300 mg/l claforan, 4 g/l insoluble polyvinylpyrrolidone and 0.1 g/l dithioerythritol (Oláh et al., 2003b). Calli have been transferred monthly to fresh medium of the same composition.

Plant regeneration and acclimatization

Germinating embryos were isolated and transferred separately to new tubes containing MS/2 medium without antioxidants and they were exposed to light (16 h, 24°C) to induce shoot development.

During the selection we obtained a lot of putative transformed embryos showing developmental disorder. We exposed the abnormal embryos transformed with EHA105(pRok2Ferr) construction to various treatments in order to restore the normal shoot formation. In our experiments we have tested the MS/2 medium containing no selection agent (after two-year selection), applied both 0.22 mg/l BA and kanamycin (selection agent), and cut the abnormal embryos under the hypocotyl keeping them on selection medium further.

To propagate regenerated plants shoots were cut with two buds and transferred to tubes containing antioxidant-free MS/2 medium. For hardening microcuttings with two buds including the shoot tips were planted into jars filled with approximately 50 ml perlite, 4x4x4 cm Grodan rockwool (www.grodan.com) and with 3x3x4 cm pit-pot blocks (www.pit-pot.com) moisted with tapwater. In control experiments microcuttings were rooted in tapwater solified with 6 g/l agar. From this time the lids were gradually opened. After four to five weeks completely hardened, rooted plants were obtained that were ready to transfer into soil (peat:sand:perlit=1:1:1) for further growth in the greenhouse.

Detection of integrated DNA by PCR analysis

Plant DNA was isolated from young leaves of *in vitro* grown plants by Qiagen Easy Plant DNA Mini kit (Qiagen, Hilden, Germany) according to the supplier's instructions. A 700 bp region of the *nptII* gene was amplified by the *nptII*F (5'-ATCGGGAGCGGCGATACCGTA-3') and *nptII*R (5'-AGGCGAGGCGGCTATGACTG-3') primers (Hoffmann et al., 1997). To verify the absence of *Agrobacterium* vector in putatively transformed plants, DNA samples were also tested with the VCF (5'-ATCATTTGTAGCGACT-3') and VCR (5'-AGCTCAAACCTGCTTC-3') primers (Sawada

et al., 1995), which amplifies a 730 bp virulence region located on the Ti plasmid. A 513 bp region of the alfalfa ferritin gene was amplified by (5'-GTCACGGTGTGTGGCACTTTGA-3') and (5'-AGACAGAGCCAATTCCATGGCA-3') primers (Oláh 2005). The *iaaM* gene was detected with primers (5'-GAACCAAGCGGTTGATAACAGCC-3') and (5'-CTGCGACTCATAGTCCAGGAATAC-3') (Viss et al., 2003) which amplify a 150 bp fragment of the *iaaM* gene. The presence of *virE1* gene was detected by (5'-CCATCATCAAGCCGCA-3') and (5'-CTCCTTCTGACCAGCAAGA-3') primers (Szegedi et al., 2001).

Testing oxidative stress resistance of transgenic lines

Previous studies indicated that paraquat is related to oxidative stress, therefore leaf cuttings from EHA105(pRok2Ferr) transgenic and control lines were floated on 0,5 µM, 1 µM, 2 µM, 3 µM, 4 µM paraquat dichloride (1,1-Dimethyl-4,4-bipyridinium dichloride) solutions. All treatments have been carried out in dark conditions for three hours, than the leaves have been lightened with 35 µmol m^{-2} s^{-1} PPFD for one hour. Photochemical yield of photosynthesis in the leaf disks was measured before and after the above treatments with a pulse amplitude modulated chlorophyll fluorimeter (Imaging PAM or Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany), according to Schreiber et al. (1997).

Testing *Agrobacterium* resistance

To test the susceptibility of the selected 'Richter 110' transgenic lines harbouring the *virE1* construction to crown gall disease, *in vitro* grown plants were tested by *A. vitis* Tm4. The hardened pJP17 lines were infected with *A. tumefaciens* A348 and C58, with *A. vitis* Tm4, AT1 and S4. To this end stems of plants were inoculated at 3 points with 1 µl suspension (OD_{600} = 0.5-1.0) of an overnight bacterial culture. Non-transformed 'Richter 110' plants were used as positive controls. Results were scored after six weeks incubation.

We tested agrobacterium resistance of 'Pegazus', 'Csépi muskotály', 'Borostyán', 'Odysseus', 'Orpheus', 'Taurus', 'Korai bíbor' and 'Pannon frankos' cultivars bred at our Department using *A. tumefaciens* C58, *A. vitis* Tm4, AB3, AT1 and S4 strains. In control experiments 'Kékfrankos' and 'Szürkebarát' were used as positive controls, and 'Kunbarát' was used as negative control (Szegedi 1981).

RESULTS

Induction and propagation of embryogenic callus

The variability of embryogenesis among genotypes is important, so 12 genotypes were investigated for initation of embryogenic callus. After three months, in 11 genotypes we successfully obtained anther derived embryogenic callus with the range of 0,25-12 % (Table 2, 3). The embryogenesis was not sucessful in case of 'Arany sárfehér' on MSE medium. In 4 cultivars: 'Korai bíbor', 'Odysseus', 'Orpheus' and 'Pannon frankos' we reported first embryogenesis. The effectiveness of embryogenic callus induction was determined by counting the percentage of embryogenic callus. Different types of calli could be observed, but only the white-yellow, harder and showing globular stage proved embryogenic type. MSE medium proved the most successful in 9 cultivars, MST was positive in 4 cases, NNE in 5 cases and MSE/2 medium only in 1 case.

Table 2 Results of emryogenic callus induction (%) on MSE and MST media.

Cultivars	Results of emryogenic callus induction (%)		
	Medium	Average	Standard deviation
'Arany sárfehér'	MSE	-	-
'Cabernet franc'	MSE	7,5	5
'Odysseus'*	MSE	1,25	1,37
'Orpheus'*	MSE	6	7,2
'Richter 110'	MST	8	4,8
'Taurus'	MSE	3,33	3,81

* Cultivars for which embryogenicity was first reported in this work.

Table 3 Results of emryogenic callus induction (%) on four different media with averages and standard deviations.

Cultivars	MSE	MSE/2	NNE	MST
'Chardonnay'	4,46 ± 7,08	9,17 ± 5,84	1,5 ± 3,64	0,25 ± 0,79
'Kékfrankos'	7,8 ± 3,67	0	12 ± 6,02	1,25 ± 0,7
'Korai bíbor'*	1,25 ± 1,37	0	2 ± 3,26	0
'Pannon frankos'*	0	0	11,08 ± 7,81	0
'Rajnai rizling'	4,2 ± 3,75	0	2,5	0
'Teleki 5C'	5	0	0	6,67 ± 5,2

* Cultivars for which embryogenicity was first reported in this work.

Comparing the two tested sterilization treatments of flower buds for induction of embryogenic calli in case of 'Chardonnay' cultivar we concluded that the procedure with $Ca(OH)_2$ solution is as effective as the treatment with NaOCl. CPE medium proved applicable to propagate embryogenic callus in case of 'Richter 110'. On CPE medium the propagated calli differed in morphology from calli on MSE medium, which explicable with the selection and proliferation of embryogen structures and necrosis of non-embryogen structures.

Effectivity of plant regeneration is highly affected by the quality of starting material. Using liquid medium containing 5 μM NOA we achieved homogeneous 'Richter 110', 'Teleki 5C' and 'Chardonnay' embryogenic cultures propagated rapidly and contained cells with small vacuoles. The cultures after the two week period in MSNOA liquid medium regenerated more rapidly on hormone-free solid medium, than to the cultures kept on MSE medium earlier.

Grapevine transformation

We have carried out genetic transformation experiments using EHA105(pRok2Ferr) vector and have successfully obtained 'Richter 110' plants transformed with alfalfa ferritin gene. In the transformation experiments to introduce enhanced resistance to *Agrobacterium,* EHA101(pTd93virE1) and EHA101(pJP17) transformation vectors were used. The transgenic nature of regenerated plants was confirmed by PCR analysis. All of them contained the *nptII* selection marker gene and the useful genes. The result of PCR analysis with *virC* gene specific primers verified that the tested regenerated plants were free of contaminating *Agrobacterium* cells.

The pRok2Ferr transformant plants were tested by qPCR investigation and Western blot analysis in Biological Research Center, Szeged (Zok et al. 2009). QPCR investigation of ferritin gene expression revealed that the *Medicago* ferritin is highly expressed in the transgenic plants. Western blot results indicate that *Medicago* ferritin accumulated at high protein level in leaves of the transgenic grapevines, and the approximate molecular weight of the detected protein corresponded to the processed form of ferritin in transformants.

Plant regeneration and acclimatization

We carried out plant regeneration until we got enough plants for the further experiments. Shoot formation of pRok2Ferr transformed 'Richter 110' embryos was best restored by using

0.22 mg/l BA in the selection medium (Table 4) and cutting the abnormal embryos under the hypocotyl. Applying these methods we observed the recovery of shoot development of embryos that showed developmental disorder due to the transformation process. Plants with shoot and root were propagated and hardened to greenhouse conditions. In the experiment when microcuttings were tested using four different rooting media, both pit-pot blocks and perlite promoted rapid rooting of microcuttings, but proper rooting of 'Richter 110' microcuttings was observed when perlite was used.

Table 4 The result of induction of normal shoot formation using 1 μM BA in the selection medium in the case of EHA105(pRok2Ferr) transformed 'Richter 110'.

'Richter 110'	MS/2	MS/2+0.22 mg/l BA
1	0/38 (0%)	5/26 (19,2%)
2	0/30 (0%)	2/23 (8,7%)
3	3/33 (9%)	9/25 (36%)
4	0/32 (0%)	2/15 (13,3%)
5	1/17 (5,8%)	6/17 (35,3%)
6	0/28 (0%)	7/22 (31,8%)
Average (s. d.)	2,46% (3,95)	24,05% (11,87)

Oxidative stress resistance of transgenic lines

In case of two transgenic lines we found an increased tolerance to oxidative stress effects, which suggests the protective role of ferritin in grapevine leaves. Untransformed 'Richter 110' leaves lost about half of their maximum photochemical yield upon exposure to 1 μM paraquat with further decrease at higher concentrations. In F7 and F9 transgenic lines accumulating the alfalfa ferritin in their chloroplasts, however, the extent of this decrease was lower, and these leaf disks retained more than half of their F_v/F_m even at 4 μM paraquat concentration (Figure 1).

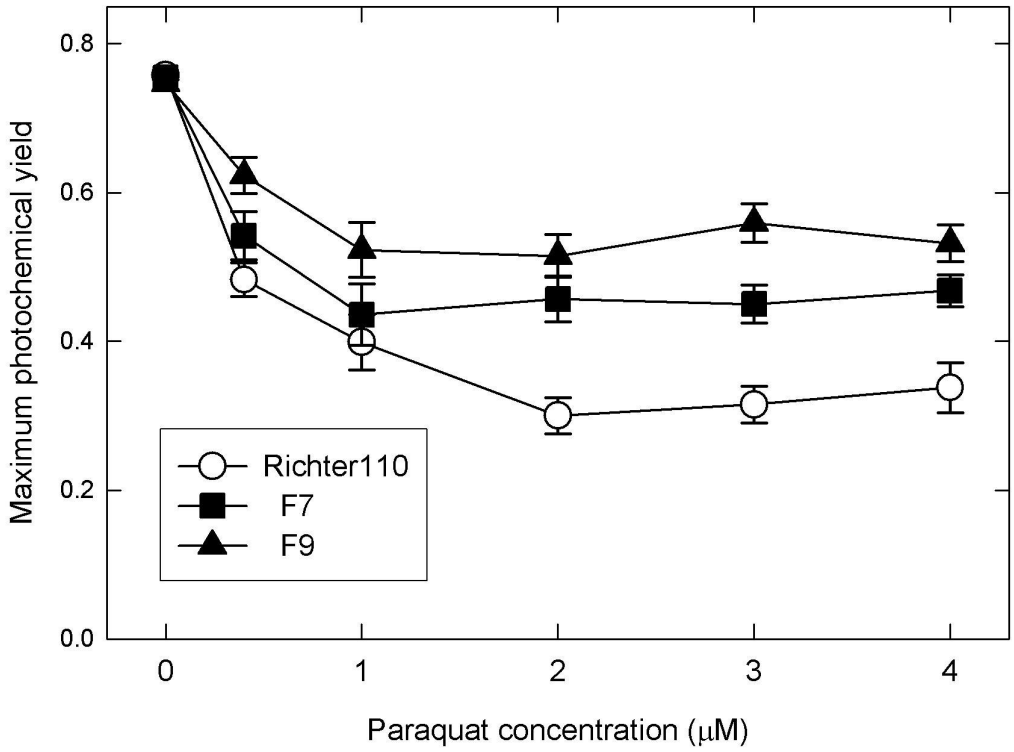

Figure 1 Changes in photochemical yield of grapevine leaf disks upon exposed to various concentrations of paraquat in the light. Richter110: 'Richter 110' untransformed rootstock cultivar; F7, F9: transgenic plants.

Agrobacterium resistance experiments

From 21 pJP17 transgenic lines eight showed resistance (no tumor formation) to *A. tumefaciens* A348 of which the silencing contruct was derived from. Three out of these lines showed resistance to *A. vitis* AT1 as well. All lines were susceptible to *A. tumefaciens* C58, *A. vitis* Tm4 and S4. Thus no line showing resistance to all of the agrobacteria tested was found, which shows that crown gall resistance induced by the oncogene silencing construct pJP17 is highly specific to the strain the hormone genes are derived from.

9 of the 17 tested VirE1 transgenic lines were resistant to *A. vitis* Tm4 strain since they did not form tumors after six weeks of incubation as compared to the control plants. With these results we have confirmed on grapevine plants that the v*irE1* gene is able to reduce crown gall sensitivity. Of the new cultivars bred at our Department 'Korai bíbor' and 'Orpheus' showed resistance to *A. tumefaciens* C58; 'Odysseus' and 'Pannon frankos' to *A. tumefaciens* C58, *A. vitis* AT1 and S4; while 'Taurus' was resistant to all of the five tested *Agrobacterium* strains.

NEW SCIENTIFIC RESULTS

1. We reported first embryogenesis in case of ‘Korai bíbor’, ‘Odysseus’, ‘Orpheus’ and ‘Pannon frankos’.
2. CPE medium (based on the medium described by Cheé and Pool (1987)) supplemented with 1.1 mg/l 2,4-D and 0.1 mg/l BA was first used to propagate ‘Richter 110’ embryogenic callus and proved applicable.
3. We concluded, that perlite has a good effect on rooting of ‘Richter 110’ microcuttings during hardenig process.
4. The use of 0.22 mg/l BA in the selection medium is applicable to restore shoot formation of transformed ‘Richter 110’ embryos showing developmental disorder.
5. We obtained transgenic ‘Richter 110’ plants harbouring pRok2Ferr and pRok2FerrFLAG vector constructions in our experiments to enhance oxidative stress resistance of grapevine.
6. Two pRok2Ferr transgenic ‘Richter 110’ lines were found to have an increased tolerance to oxidative stress effects.
7. In the transformation experiments to introduce enhanced resistance to *Agrobacterium,* transgenic lines were obtained harbouring EHA101(pTd93virE1), EHA101(pJP17) and EHA101(pJP17-S4) transformation vectors
8. We concluded that pJP17 gene construction provides resistance only to *A. tumefaciens* A348 of which the silencing contruct was derived from.
9. Of the new cultivars bred at our Department ‘Korai bíbor’ and ‘Orpheus’ showed resistance to *A. tumefaciens* C58; ‘Odysseus’ and ‘Pannon frankos’ to *A. tumefaciens* C58, *A. vitis* AT1 and S4; while ‘Taurus’ was resistant to all of the five tested *Agrobacterium* strains.

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