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Study of different factors of grapevine regeneration systems and genetic transformation

Novák E., Zok A., Pedryc A. & Oláh R.

Department of Genetics and Plant Breeding, Faculty of Horticultural Science, Corvinus University of Budapest, H-1118, Budapest, Villányi 29–43, Hungary

Summary: The most limitating factor for successful transformation is the absence of high-yielding regeneration protocols. However, the anther-derived embryogenic culture is an optimal technique for genetic transformation and it has been widely applied in many important cultivars, but the necessity of further development of regeneration systems has been proved. We attempted to produce somatic embryos on a wide range of genotypes from various tissues; leaves, petioles, stem segments. We started the examination of grapevine regeneration via organogenesis, succeeded in inducing shoot from the meristematic tissue of the base of bud by testing induction medium contained different concentrations of two types of hormones. To optimize the conditions of the *Agrobacterium*-mediated transformation, we studied the effectiveness of different *Agrobacterium*-treatments, the use of antioxidants and the sufficient quantity of kanamycin for selection of transformed cells.

Key words: *Agrobacterium tumefaciens*, organogenesis, somatic embryogenesis, transgenic plant

Introduction

The application of new results of plant biotechnology enables to produce transgenic grape plants more and more efficiently, permits the introduction of useful agronomic traits into traditional, valuable cultivars without altering the features of the plants. Molecular breeding methods of grapevine have recently been widely used to improve stress tolerant, disease resistant of genotypes, to produce high yielding and quality cultivars adapted to urgent enviromental challenges as well as to the impacts of climate change. In addition, genetic transformation will allow identification and isolation of novel genes e.g. resistance genes from *Vitis* species utilizing reverse genetics as previously reported for *Arabidosis*. Succesful application of gene technology requires an efficient genetic transformation and reliable regeneration systems. To date, the regeneration of grape has been obtained by both embryogenesis and organogenesis.

Embryogenic cultures have been established for some grape species, e.g. *Vitis rupestris* (*Martinelli* et al., 1993), *V. rotundifolia* (*Dhekney* et al., 2008), *V. riparia* (*Mozsar & Sule*, 1994), and *V. vinifera* cultivars (*Perrin* et al., 2004). However, somatic embryogenesis remains genotype dependent (*Maillot* et al., 2006). Among the methods, the essential differences are noticeable in the parts of grapevine used for induction and the medium with different combinations of plant grow regulators. Different types of explants have been tested for their ability to produce somatic embryos under induction conditions, such as anthers (*Vidal* et al., 2003; *Perrin* et al., 2004), leaf discs (*Li* et al., 2001; *Das* et al., 2002) and ovaries (*Kikkert* et al., 2005; *Gambino* et al., 2007). To induct embryogen callus approximately ten plant grow regulators have been used in combinations but two of them; 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) have been effectively adopted in most of the cases. Shoot regeneration has been successfully applied to several grape species: *V. amurensis*, *V. armata* (*Martinelli* et al., 1996), *V.* rupestris 'St. George' (*Stamp* et al., 1990) and *V. vinifera* cultivars (*Péros* et al., 1998). Grape plants have been regenerated from juvenilis leaf blades (*Torregrosa & Bouquet* 1996), from anthers (*Popescu* et al., 1995). Meristematic tissue culture derived from buds was described to produce adventitious shoots (*Mezetti* et al., 2002).

Transformation of grapevine has been reported using the effective *Agrobacterium*-mediated system (*Iocco* et al., 2001). Basically antibiotic resistance has been applied for the selection of transformed cells, and the selection agent is usually kanamycin (*Torregrosa* et al., 2000). Several antagonisms can be found about the application of optimal kanamycin in the literature. The use of antioxidants has been kept necessary to reduce necrosis symptoms after *Agrobacterium*-treatment (*Perl* et al., 1996).

In this study we aimed at improving methods of grape regeneration, optimizing the conditions of transformation, as well as the different factors influencing selection and the efficiancy of regeneration.

Materials and methods

Plant regeneration via somatic embryogenesis was studied on tissues obtained from leaves, leafbases, stem segments and pistils. Tests were carried out in many different grape genotypes; *Vitis vinifera* cv. 'Chardonnay' and 'Rajnai rizling', rootstocks 'Georgikon 28', 'Richter 110',

interspecific hybrids 'Orpheus', 'Odysseus', 'N82', 'V9', 'Korai bibor'. Embryogenic culture induction medium consisted of MS (*Murashige & Skoog*, 1962) basal salts and vitamins, supplemented with 20 g/l saccharose, 70 mg/l FeEDTA, 1.1 mg/l 2,4-D, 0.2 mg/l BAP and were solidified with 7 g/l Oxoid agar. The pH was adjusted to 5.8. For somatic embryogenesis about 50 segments from young leaves, leafbases and shoots of different genotypes were placed on the medium. The method was repeated 3 times in darkness at 26–28 °C. The initial explants were pistils in the case of 'Katta kurgan', 'Kismis csornij' cultivars and *V. amurensis*, cultures were maintained at the same temperature and conditions as described previously. Besides BAP the suitability of TDZ (thidiazuron) was verified, which has a citokinin and auxin effect, is a good initiator of callus development. We used the same induction medium supplemented with 0.05 mg/l TDZ in several grape cultivars; such as 'Chardonnay' and 'Korai bibor'. We examined the regeneration via organogenesis of 'Chardonnay', 'Korai bibor' and 'Orpheus', 200 slices from young leaves, petioles and stems were set on MS solid medium supplemented with 10g/l saccharose and 3 mg/l BAP. In vitro cultures were incubated under long period of light at 26–28 °C. Shoot regeneration was attempted from the base of buds of 'Chardonnay', 'Korai bibor' and 'Richter 110'. To get regenerated plants, we used *in vitro* green cuttings with one bud, after removing the buds the cuttings were controlled in microscope. The apical dome of the shoots was eliminated and the micro-cuttings maintained for several months on MS medium supplemented different concentrations of two types of hormones (BAP, TDZ) to differentiate adventitious shoots (*Table 1*). According to the results we tested the most effective concentrations of these two hormons together for organogenesis of 'Richter 110'rootstock.

Table1 Experiments of regeneration via organogenesis

genotype	initial explants	hormon (mg/l)	
		TDZ*	BAP*
Chardonnay	base of bud	0,0; 0,25; 0,5; 1,0	0,0;0,1; 0,5; 1,0
Korai bibor	base of bud	0,0; 0,25; 0,5; 1,0	0,0; 0,1; 0,5; 1,0
Orpheus	leaf, petiole, stem segment	-	3,0
Chardonnay	leaf, petiole, stem segment	-	3,0
Korai bibor	leaf, petiole, stem segment	-	3,0

*TDZ=thidiazuron or BAP=6-benzylaminopurin

The embryogenic callus of 'Chardonnay', 'Richter 110', as well as the EHA101(pTd33) vector construction were used to adjust optimal conditions of genetic transformation. Our goals were to moderate the death of transformed cells and tissues after transformation process, which can decrease the cells of the chance of surviving. Firstly, tolerance to antibiotic of *Agrobacterium* vector was experienced, EHA101(pTd33) strain was inoculated on half strenght MS medium contained carbenicillin antibiotic used in different concentration (0–800 mg/l). Secondly, the cultures of embryogenic calli were controlled at the same conditions. The results of evaluation was happened visually by observing of relative growing and the rate of necrotisation. In order to optimize the treatment with *Agrobacteria*, studied different methods, including the way of transferring of *Agrobacterium* to plant cells, and the way of removing them from the culture. These investigations (*Table 2*) essentially differed from transferring bacteria to calli. In some cases we shaked the somatic embryogenic calli in 20 ml sterilized MSE liquid medium (Mozsar et al., 1994) contained 0.1 mg/l BAP and 1.1 mg/l 2,4-D with 1 ml of bacterium suspension (10^8 cell/ml) which had been grown in AB minimal medium overnight. Cultures were treated for different time (30, 60, 120 minutes). In the other cases small amount of bacterial suspension (20–30 µl) were applied onto the surface of calli (10^8 cells/ml) in 5–10 mm diameter. The embryogenic calli from the liquid cultures co-cultivated on solid MSE or hormonfree MS medium for 48 hours. After treatment the plant material was tested to different concentrations of carbenicillin and cefotaxime-sodium antibiotics. We investigated the rate of tolerance of embryogenic calli of 'Chardonnay' and 'Richter 110' for a selectable marker conferring resistance to kanamycin on MSE medium. In addition to quantify the most reasonable concentration of kanamycin for genetic transformation, we tried to determine it by the GUS+ regenerated transgenic plants. To decrease damage and cell death in transformated tissue we investigated water insoluble polyvinylpyrrolidon (Polyclar AT) antioxidant and phenol binding dithioerythritol (DTE). Afterwards we attempted the regeneration of transformated cells on hormon free medium, transfered monthly to fresh medium.

Results

We studied somatic embryogenesis and direct organogenesis from different parts of grapevine to establish and develope plant regeneration systems in order to extend the range of genotypes for genetic transformation. In the first process a lot of efforts were made to induce somatic embryos from various tissues of grapevine. We obtained calli on leaves, petioles and stems of tested grape genotypes but took a long time until showing. After observing the formation of calli, they did not proved embryogenic and appeared basically softer and lighter than normally. When we initiated callus from pistils, the production didn't prove embryogenic as well. Despite application of 1.1 mg/l 2,4-D and 0.2 mg/l BAP hormons, the method did not show any results in this case, as the use of combination of 1.1 mg/l 2,4-D and 0.05 mg/l TDZ hormons neither.

Plant regeneration via organogenesis experiments from leaves, petioles and stem segments of 'Chardonnay', 'Korai bibor', 'Orpheus' closed without considerable regenerations. We attempted to estimate the organogenetic potencial of the cells of the base of buds, the different genotypes in what range to be able to generate shoots from meristematic tissue. According to datas, 0.1 mg/l BAP concentration was the

Table 2 Overview of *Agrobacterium*-treatment experiments

treatment	the way of inoculation		wash-out and	co-cultivation	elimination		drying	maintenance
	way	time (min.)	drying	(hour)	shake (min.)	antibiotic		
1	shake	120	–	48	120	100 mg/l cn	–	100 mg/l cn
2	shake	60	–	48	60	100 mg/l cn	–	100 mg/l cn
3	shake	30	–	48	30	100 mg/l cn	–	100 mg/l cn
4	shake	30	–	24	30	100 mg/l cn	–	100 mg/l cn
5	shake	30	MSE	48*	30	200 mg/l cn	+	200 mg/l cn
6	drop	–	–	48	–	–	–	200 mg/l cn
7	drop	–	–	48	–	500 mg/l cn	–	200 mg/l cn
8	drop	–	–	48	–	1000 mg/l cn	–	200 mg/l cn
9	drop	–	-	48	–	1000 mg/l cn	+	200 mg/l cn
10	drop	–	–	48	–	–	–	200 mg/l cn 300 mg/l ce
11	drop	–	–	48	–	–	+	200 mg/l cn 300 mg/l ce

cn= carbenicillin, ce= cefotaxime-sodium, + treatment – no treatment

most appropiate to regenerate plant (16%) from the base of buds at 'Chardonnay', 0.25mg/l TDZ proved the most efficient (55%) at 'Korai bíbor' (*Table 3*). To get regenerated 'Richter 110' rootstock via organogenesis we adopted the most suitable concentrations of these two hormons together; 0.1 mg/l BAP and 0.25mg/l TDZ. We obtained a few regenerated plants with very low efficiency (*Figure 1*).

Table 3 Results of shoot regeneration from the cells of the base of buds at different hormon concentrations

genotype	hormon	conc.(mg/l)	average*	standard deviation
Chardonnay	–	–	4	2
Chardonnay	TDZ	0,25	8,3	1,5
Chardonnay	BAP	0,1	16	4
Chardonnay	BAP	0,5	2,7	1,5
Chardonnay	BAP	1,0	7	2,6
Korai bibor	–	–	0,3	0,6
Korai bibor	TDZ	0,25	55,3	5,7
Korai bibor	BAP	0,1	46,7	2,9
Korai bibor	BAP	0,5	2	2
Korai bibor	BAP	1,0	10	2,6

*(shoot regenerated amount of cuttings/ amount of cuttings)×100

Figure 1 *In vitro* regenerated 'Richter 110' rootstock from the cells of the base of bud

The further aim of our survey was to optimize the conditions of genetic transformation The sensibility to carbenicillin of EHA101(pTd33) strain was tested on half strenght of MS medium with different concentrations of the antibiotic. The bacteria couldn't be noticed at 200 mg/l carbenicillin as a result, exemption from the bacterium can be maintained at this concentration. After using different concentrations of carbenicillin we didn't experience any effect to 'Chardonnay' calli, forwards we adopted a combination with cefotaxime-sodium. The application of 200 mg/l carbenicillin and 300 mg/l cefotaxime-sodium proved successfully to eliminate bacteria. To improve the conditions of genetic transformation we inspected the various methods. We experienced that, the techniques of dipping into bacterial suspension and shaking with bacteria caused extensive necrosis symptoms on plant material. We managed to slightly moderate necrosis of embryogenic cultures with declining the time of dipping and co-cultivation. In the case of removing the dipping treatment the death symptoms were canceled. In practice we verified that, the co-cultivation with *Agrobacterium* for short time reduces the frequency of transformation. Accordingly, the most efficient method was the transfer of a small quantity (40–50 μl) of bacterium suspension to the surface of embryogenic callus on solid medium, followed 2 days co-cultivation. By this method it is possible to cut the symptoms of death and to avoide the drawbacks of dipping technique. The most appropiate somatic embryos to transformate were in below of 1 mm, in early globular stage and in the same age. We experienced if they were bigger than 1 mm, the browning symptoms were very strong. To eliminate bacteria after 2 days co-cultivation, they were transfered to solid hormonfree medium containing these two different antibiotics. For the selection of transformed cells, 20 mg/l kanamycin appeared to be sufficient quantity because we obtained GUS+ transformated plants only at this kanamycin concentration. In none of the cases we could regenerate plants without using antioxidant and phenol-binding (*Table 4*). Accordingly the selection medium always includes 4 g/l water insoluble Polyclar AT and 0.1 g/l DTE antioxidants to assist the regeneration systems. Application of them gives sufficient protection to the cells against toxic phytometabolites. By this method we successfully regenerated genetically transgenic 'Richter 110', 'St George' and 'Chardonnay' grapevines in our research with model genes. The transformated plants were tested by PCR analysis (data not shown).

Table 4. Obtained GUS+ transgenic plants by optimising the factors

	plant material	antioxidant and phenol binding*	selection agent	concentration (mg/l)	GUS+ plants
1.	embryogen callus	–	kanamycin	20	–
2.	embryogen callus	–	kanamycin	50	–
3.	embryogen callus	–	kanamycin	100	–
4.	embryogen callus	+	kanamycin	20	+
5.	embryogen callus	+	kanamycin	50	–
6.	embryogen callus	+	kanamycin	100	–
7.	embryos	–	kanamycin	20	–
8.	embryos	–	kanamycin	50	–
9.	embryos	–	kanamycin	100	–
10.	embryos	+	kanamycin	20	+
11.	embryos	+	kanamycin	50	–
	embryos	+	kanamycin	100	–

*- no treatment, + treatment

Conclusion

To facilitate the development of transgenic grapevines, we did efforts to establish a rapid system for regenerating on different grape rootstock and scion varieties via somatic embryogenesis, organogenesis, and to develope a more efficient gene transformation system. In spite of many reports on successful transgenic plant production in grape, routine transformation remains difficult. Mainly, this is due to highly genotype-dependent regeneration ability, often with low embryogenesis and organogenesis induction, moreover low embryo conversion rates. Further, conditions of *Agrobacterium* co-cultivation need to be carefully manipulated, the avoidance of oxidative stress being one important point. Minimum handling may be equally important, also the choice and the used quantity of selectable markers and antioxidants. Consequently, conditions of transformation and selection are needed carefully consideration for each genotype.

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