DEVELOPMENT AND OPTIMIZATION OF GENETIC TRANSFORMATION SYSTEM FOR GRAPE

VÝVOJ A OPTIMALIZACE SYSTÉMU GENETICKÉ TRANSFORMACE U REVY VINNÉ

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

E-mail: edina.novak@uni-corvinus.hu

ABSTRACT

We have established a successful *Agrobacterium*-mediated transformation system for several important grapevine cultivars. Genetic transformation is based on the existence of an effective regeneration method. Different types of explants have been tested for their ability to produce somatic embryos on inducing medium. To optimize the conditions of the transformation system we examined the effectiveness of different *Agrobacterium*-treatments, the use of antioxidants and phenol-bindings, and the adequate amount of kanamycin for the purpose of selection. The developed transformation system allowed the recovery of germinating transgenic embryos and regenerated plantlets.

Key words: *Agrobacterium tumefaciens*, somatic embryogenesis, plant grow regulator, transgenic plantlet

Acknowledgements: This work was supported by grants National Scientific Research Found (OTKA) Grant No. T049438/2005. The authors thank Veronika Tóth for helpful technical assistance.

INTRODUCTION

Transgenic approach to genetic improvement of traditional wine grape cultivars would allow modification of traits such as disease and pest resistance, product quality, and production efficiency without altering the essential characteristics of the cultivar. In addition, genetic transformation would facilitate identification and isolation of novel genes utilizing reverse genetics. Successful application of gene technology requires an efficient genetic transformation and reliable regeneration system. Embryogenic cultures have been established for some grape species, despite somatic embryogenesis remains genotype dependent (Maillot et al., 2006). Somatic embryos are often used as targets for transformation studies. Different parts of grapevine have been tested for their ability to produce somatic embryos under induction conditions, such as anthers (Vidal et al., 2003; Perrin et al., 2004), leaves (Li et al., 2001) and ovaries (Kikkert et al., 2005; Gambino et al., 2007). When the total regeneration process is considered, the development of transgenic shoots from somatic embryos frequently represents a limiting step (Iocco et al., 2001). The researchers were able to induce the calli to regenerate embryos and intact transgenic plants. The successful transformation of grapevine has been reported using the *Agrobacterium*-mediated system (Yamamoto et al., 2000; Iocco et al., 2001; Das et al., 2002). Generally antibiotic resistance has been applied for the selection of transformed cells, and neomycin phosphotransferase II (*nptII*) has been used as the selectable marker gene (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, developing somatic embryogenesis, optimizing the conditions of transformation, as well as the different factors influencing selection and the efficiancy of regeneration.

MATERIALS AND METHODS

We examined the regeneration via organogenesis from young leaves, petioles and stem segments cultured on MS solid medium supplemented with 10g/l saccharose and 3 mg/l 6-benzylaminopurine (BAP). In vitro cultures were incubated under long period of darkness at 28 °C. Shoot regeneration was also attempted from the base of buds. Somatic embryogenesis was induced on different tissues; anther filaments, leaves, stem segments and pistils for several different grape genotypes. Embryogenic callus was initiated from anther filaments most of the cases. Anthers were collected from inflorescences of different cultivars e. g.: 'Richter 110' rootstock, Vitis vinifera cv. 'Chardonnay' in the bud stage. Anthers were disinfected with the use of sodium hypochloride solution (Clorox 10%) keeping in it for 15 minutes then rinsed three times in sterile distilled water. Excised anthers were incubated at 28°C in darkness on MSE induction medium (Mozsár et al., 1994) consisted of Murashige and Skoog basal salts and vitamins, supplemented with 20 g/l saccharose, 70 mg/l FeEDTA, 1.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/l 6-benzylaminopurine (BAP) and were solidified with 7 g/l Oxoid agar. The pH was adjusted to 5.8. We also used the same induction medium supplemented with 0.05 mg/l TDZ for some grape cultivars. Resulting

callus culture were transfered monthly to fresh medium, somatic embryos were maintained on hormone-free medium. In order to determine the optimal conditions of effective transformation system, embryogenic callus of ‘Chardonnay’, ‘Richter 110’, were co-cultivated with different Agrobacterium strains. All vectors contained nptII coding region conferring kanamycin resistance for selection of transformed plant cells. These experiments were essentially differed from transferring bacteria to calli. In some cases embryogenic calli were co-cultivated with Agrobacteria in 20 ml sterilized MSE liquid medium with or without hormons for 24 or 48 hours. The bacteria were removed by rinsing the calli in liquid culture medium or steril water containing 100-300 mg/l carbenicillin for different time (30, 60, 120 minutes), then they were collected by filtering. In the other cases small amount of bacterial suspension (20-30 μl) were applied onto the surface of callus on solid medium in 5-10 mm diameter, co-cultivated for 48 hours. After treatment the plant material was maintained on selective medium contained 100-1000 mg/l concentrations of carbenicillin or supplemented with 300 mg/l cefotaxime-sodium antibiotic to eliminate Agrobacteria. We carried out some experiments to controll the tolerance of embryogenic callus for kanamycin selection agent on MSE medium. In addition to quantify the most reasonable amount of kanamycin for selection from 20 mg/l, 50 mg/l and 100 mg/l concentrations, we tried to determine the quantity of it by the existence of viable regenerated transgenic plants. To reduce damages caused by stress, save the cells through transformation process, we tested the effect of 4 g/l water insoluble polyvinylpyrrolidon (Polyclar AT) antioxidant and 0.1 g/l phenol -binding dithioerythritol (DTE).

RESULTS

We studied direct organogenesis from different parts of grapevine but this process did not prove enough successful on the contrary we could effectively obtain somatic embryos from anther tissues. Various plant growth regulator (PGR) combinations were applied to induce somatic embryogenesis from different grape genotypes. Besides BAP and 2,4-D, the suitability of thidiazuron (TDZ) was verified, according to the possession of citokinin and auxin effect, is a good initiator of callus development. Successfully we obtained a rapid method for the induction of somatic embryogenesis and effective plant regeneration derived from anther tissue.

To improve the conditions of genetic transformation we developed a new method which is based on minimum-handling. We experienced, the techniques of submerging in bacterial suspension caused extensive necrosis symptoms on plant material. We were able to slightly moderate symptoms of 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. 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, which was followed 48 hours co-cultivation with *Agrobacterium*.

By this method it is possible to cut down the symptoms of cell death and to avoide the drawbacks of dipping technique. The most appropiate somatic embryos to transformate were about 1 mm, in early globular stage and approximately at the same age (Figure 1).

Figure1. Somatic embryos in globular stage

Figure 2. Transformed embryos under selective conditions

The application of 200 mg/l carbenicillin and 300 mg/l cefotaxime-sodium in the selection medium proved efficient to eliminate bacteria therefore we adopted this combination of antibiotics. For the selection of transformed embryos (Figure 2), 20 mg/l kanamycin appeared to be sufficient quantity because we obtained regenerated transgenic plants only at this kanamycin concentration. In none of the cases could we regenerate grape plants without using antioxidant and phenol-binding so these chemicals seemed to be absolutely necessary for the effective transformation system as the application of them gives sufficient protection to the cells against toxic phytometabolites. Accordingly the selection medium always includes 4 g/l water insoluble Polyclar AT antioxidant and 0.1 g/l DTE phenol-binding. By this newly developed technics we successfully transformed with useful genes and regenerated ‘Richter 110’ and ‘Chardonnay’ grapevines in our research (Figure 3, 4). The transgenic status of plants was confirmed by PCR analysis (data not shown).

Figure 3. In vitro newly formed transgenic plantlet

Figure 4. Transgenic plant

DISCUSSION

In spite of many reports on successful transgenic plant production in grape, routine transformation remains difficult. Mainly, this is due to a highly genotype-dependent regeneration ability, often with low embryogenesis induction, moreover low embryo conversion rates. Further, the avoidance of oxidative stress being one important point after *Agrobacterium* co-cultivation. Minimum handling may be equally important, also the used quantity of selectable markers and the necessary of antioxidants and phenol-bindings. Consequently conditions of transformation and regeneration have need to be carefully considerated for each genotype.

This study has described the development of a transformation system which can be applied to grapevine cultivars of importance to the wine industry.

LITERATURE

Das, D., Reddy, M., Upadhyaya, S., Sopory, S. (2002): An efficient leaf disc culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera* L.). Plant Cell Rep. 20:999-1005.

Gambino, G., Ruffa, P., Vallania, R., Gribaudo, I. (2007): Somatic embryogenesis from whole flowers, anthers and ovaries of grapevine (*Vitis* spp.). Plant Cell Tiss. Organ Cult. 90:79-83.

Iocco, P., Franks, T., Thomas, M. R. (2001): Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. Transgen. Res. 10:105-112.

Kikkert, J. R., Striem, M. J., Vidal, J. R., Wallace, P. G., Barnard, J., Reisch, B. I. (2005): Long-term study of somatic embryogenesis from anthers and ovaries of twelve grapevine (*Vitis* sp.) genotypes. In Vitro Cell Dev. Biol. - Plant 41: 232-239.

Li, Z., Jayasankar, S., Gray, D. J. (2001): Expression of a bifunctional green fluorescent protein (GFP) fusion marker under the control of thre constitutive promoters and enhanced derivatives in transgenic grape (*Vitis vinifera*). Plant Sci. 160:877-887.

Maillot, P., Kieffer, F., Walter, B. (2006): Somatic embryogenesis from stem nodal sections of grapevine. Vitis 45:185-189.

Mozsar, J., Sule, S. (1994): A rapid method for induction of somatic embryogenesis and plant regeneration from cultured anthers of *Vitis riparia*. Vitis 33:245-246.

Perl, A., Lotan, O., Abu-Abied, M., Holland, D. (1996): Establishment of an *Agrobacterium* mediated transformation system for grape (*Vitis vinifera* L.): the role of antioxidants during grape-*Agrobacterium* interactions. Nat. Biotechnol. 14:624-628.

Perrin, M., Gertz, C., Masson, E. J. (2004): High efficiency initation of regenerable emryogenic callus from anther filaments of 19- grapevine genotypes grown worldwide. Plant Sci. 167:1343-1349.

Torregrosa, L., Péros, J. P., Lopez, G., Bouquet, A. (2000): Effect of hygromycin, kanamycin and phosphinothricin on the embryogenic callus development and axillary micropropagation of *Vitis vinifera* L. Acta Hort. 528:403-406.

Vidal, J. R., Kikkert J. K., Wallace P. G., Reisch B. (2003): High efficiency biolistic co-transformation and regeneration of ‘Chardonnay’ (*Vitis vinifera* L.) containing npt-II and antimicrobial peptide genes. Plant Cell Rep. 22:252-260.

Yamamoto T., Iketani H., Ieki H., Nishizawa Y., Notsuka K., Hibi T., Hayashi T., Matsuta N. (2000): transgenic grapevine plants expressing a rice chitinase with enhanced resistance to fungal pathogens. Plant cell Rep. 19:639-646.