Genetically Engineered Grapevines Carrying GFLV Coat Protein and Antifreeze Genes

Georgy P. GUTORANOV
Ivan J. TSVETKOV
Violeta M. COLOVA-TSOLOVA
Atanas I. ATANASSOV

SUMMARY

Biotic and abiotic stress has a negative effect on both the quality and quantity of grape production. Like many woody crops, grape has been relatively recalcitrant to *in vitro* manipulations. The crucial point in the process of genetic transformation is to have cells that are able to both regenerate and be transformed. A regeneration system seems to be a major problem in the transformation process. Somatic embryogenesis is the favoured regenerative protocol in genetic transformations of grapes. Comparison of an embryogenic and organogenic system in grape demonstrated that organogenesis frequently leads to chemical transformation of tissues. In this respect we started to develop and apply procedures suitable for the genetic transformation of grapevine. Two sources of explants were used for embryo induction. In the first case, immature zygotic ovules of *Vitis vinifera* seedless genotypes were used. In the second case *in vivo* leaf tissues from rootstocks *Vitis rupestris* cv. Rupestris du Lot and 110 Richter (*Vitis berlandieri* x *Vitis rupestris*). Continual transfer to fresh medium maintained embryogenic cultures. *Agrobacterium tumefaciens* mediated transformation of enbryogenic cultures of seedless grapes (*Vitis vinifera* L.) with constructs containing the gene encoding the coat protein of Grape Fanleaf Virus (GFLV) and with four constructs containing genes encoding for an antifreeze protein. An embryogenic culture of rootstock *Vitis rupestris* cv. Rupestris du Lot was transformed with a construct carrying the β-glucoronidase (GUS) gene. The first transformed plantlets have been regenerated from somatic embryos and the presence of the NPTII gene was verified by PCR and Southern blot analyses.

KEY WORDS

grape, genetic transformation, somatic embryogenesis, freeze resistance, virus resistance, transgenic plants

Institute of Genetic Engineering
2232 Kostinbrod-2, Bulgaria

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INTRODUCTION

Apart from the biological limitations (long generation time, genetic heterozygosity, large plant size), the tradition and economic attitude of the region growing a particular variety further hampers the genetic improvement of grapevine. The application of genetic engineering techniques may make it possible to transfer a single trait into a grape variety while leaving the distinctive characteristic of the variety unchanged. The incorporation of desirable genes into the plant cell by molecular, biological and genetic engineering techniques promises great potential for the genetic improvement of grapevine. Much progress has been made in the last few years in the genetic manipulation of grapes, as reviewed by Gray and Meredith (1992) and Torregrosa (1995). The objective of the present research was the development of an *Agrobacterium tumefaciens* mediated genetic transformation system based on grapevine repetitive embryogenesis and the introduction of genes that confer cold and virus resistance to grapevines.

MATERIALS AND METHODS

Plant material

Embryogenic cultures consisting of embryos arrested in a more advanced stage of development and multiplied by secondary embryogenesis were established from immature zygotic embryos of seedless grape genotypes (Russalka 3 - selfpolinated 7-3/2 E1 and Russalka 3 x Corinth white - B) and also from leaf tissue of grapevine rootstock *Vitis rupestris* cv. Rupestris du Lot. and 110 Richter (*Vitis berlandieri* x *Vitis rupestris*). Culture conditions were previously described (Tsolova and Atanassov, 1994, 1996).

Kanamycin sensitivity

The influence of different concentrations of kanamycin on the secondary embryogenesis of rootstocks (*V. rupestris* and 110 Richter) and seedless grapes (line B and line 7-3/2 E1) were determined. Embryogenic clusters were placed on modified NN 69 medium supplemented with 25, 50, 75, 100 and 125 mg/l kanamycin.

Constructs

(1)pBIN GUS int.–binary vector pBIN19 (Bevan, 1984) containing the glucoronidase (GUS) and neomycin phosphotransferase (*NPTII*) reporter genes under control of 35S CaMV promotor and introduced into *A. tumefaciens* strain LBA 4404.

(2)pGA-CP+(GFLV) (Golles, 1994, 1996) *A. tumefaciens* – strain LBA 4404 containing the full length coat protein gene of GFLV inserted into the *Bgl*II site of the plant expression vector pGA 643 (An *et al*., 1988) with an introduced start codon.

(3)pAtf 11, pAtf 62, pAtf 78, pB5 – four constructs containing genes encoding different antifreeze proteins in pPCV 91 plasmids under the control of 35S CaMV promotor in *A. tumefaciens* strain GVE 3101 (Dr. C. Kontz, personal communication).

Genetic transformation and selection

An overnight culture of *Agrobacterium tumefaciens* suspensions containing the different constructs, were diluted in YEB liquid medium to OD_{600}=0.8. Embryogenic clusters were incubated in this suspension for 15-20 minutes at room temperature under vacuum. Somatic embryos were cultured on NN 69 hormone- and charcoal-free medium. After 48 h cocultivation, the embryogenic clusters were transferred to selective medium with 500 mg/l cefotaxime (Duchefa). Two type of selection were used: (1) late permanent - the selective agent (100 mg/l kanamycin) was included in the regeneration medium after two months of free proliferation of the transformed embryos; and (2) early stepwise - the selective agent was added at a lower concentration (25 mg/l kanamycin) to the regeneration medium immediately after co-cultivation with *A. tumefaciens*, and increased during the successive monthly transfer of the culture.

Analysis of transformants

Expression of the introduced GUS gene in the somatic embryos was analysed by the X-Gluc (5-bromo-4-chloro-3-indolyl-glucoronide) histochemical assay as described by Jefferson *et al.* (1987). After 14 days (and subsequently in intervals of two months) the embryos were exposed to X-Gluc for 24 h at 37°C and observed by light microscopy. DNA from leaves was isolated according to the protocol of Fulton *et al.* (1995). Stable incorporation of the *NPT*II gene was assayed by DNA amplification using the Polymerase Chain Reaction (PCR) (Saiki *et al*., 1990), and Southern blot (Southern, 1975). DNA was blotted and probed with a DIG-labeled heterologous *NPT*II DNA probes according to the manufacturer's protocols for the gene screen plus membranes (Du Pont) and the non-radioactive labeling and detection of nucleic acid. Amino acid analyses were done using dry plant material according to the manufacturer's protocol of the amino analysator.

RESULTS

Initiation of embryogenic culture

Possibilities for induction of embryogenesis were available due to in fact there be proembryogenic groups of cells in the initial explants (Fig.1). Chilling and the concentration of growth regulators in the culture medium were used as induction factors. Newly formed embryogenic clusters typically contained embryos in all developmental stages (ranging from globular, heart-shaped, torpedo and

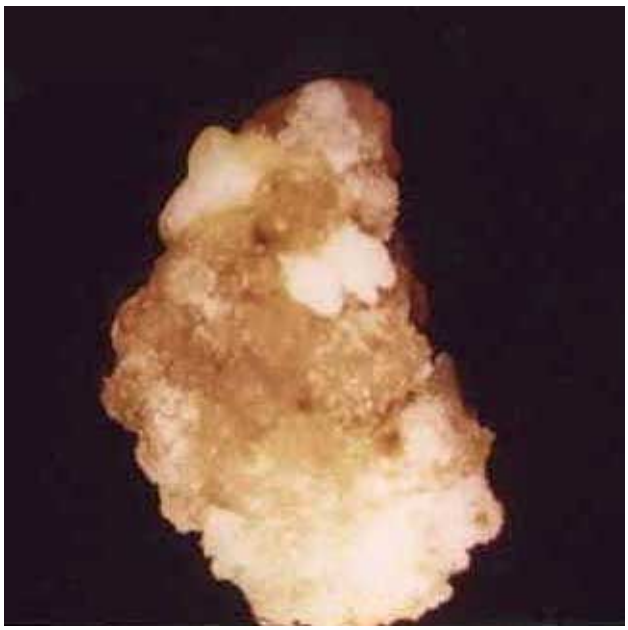


FIGURE 1. Primary embryogenic structure of *Vitis rupestris* cv. Rupestris du Lot

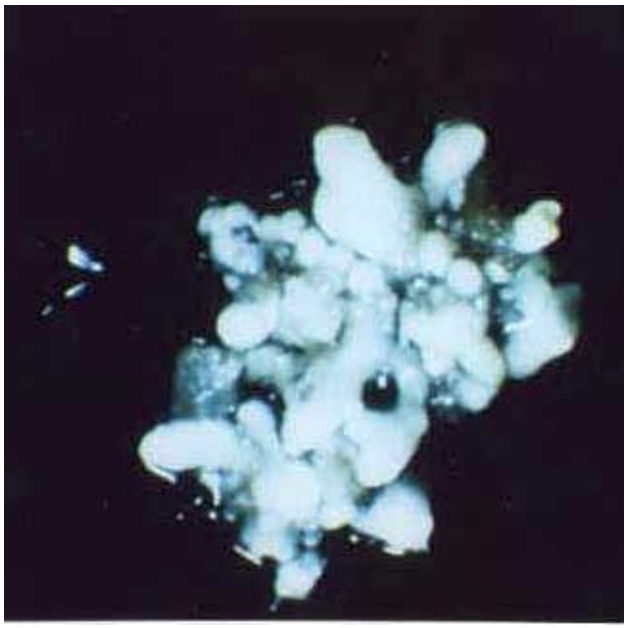


FIGURE 2. Embryogenic cluster of seedless genotype B

cotyledonary) (Fig.2, 3). Secondary embryos developed on the surface of primary embryos in all these stages. A selective subculture of embryos in heart versus torpedo stage allowed the isolation of highly embryogenic lines suitable for transformation.

Kanamycin sensitivity

To optimize the level of selection, a kanamycin response curve was plotted after 40 days of exposure to the antibiotic for each of the embryogenic genotypes. We specifically tested the effect of kanamycin on the root development of grape somatic embryos. Only high concentrations of the antibiotic

FIGURE 3. Regeneration of seedless genotype 7-3/2E1 via direct somatic embryogenesis

(more than 100 mg/l kanamycin) proved lethal to somatic embryos. Secondary embryogenesis was observed in some of the treated explants at concentrations of up to 50 mg/l kanamycin. Under the toxic and inhibitory effects of kanamycin, it is clear that somatic embryos and adventitious somatic embryogenesis are more stable during regeneration than adventitious shoots.

Genetic transformation and plantlets regeneration

The four antifreeze constructs were transformed into the embryogenic line B (Rusalka 3 x Corinth white). The CP GFLV construct was transformed into line 7-3/2 E1 (self-pollinated Russalka 3). Embryogenic cultures of *V. rupestris* cv. Rupestris du Lot were transformed with the GUS reporter gene. Tissue necrosis and subsequent cell death after short exposure of grape somatic embryos to kanamycin has been reported by Perl *et al.* (1994). In our protocol embryogenic clusters were inoculated and incubated for 15-20 min at room temperature under vacuum. Local tissue necrosis was observed in some of the explants, but during the subsequent sub-cultures large amounts of embryos overcame this process and retained it's embryogenic competence. After 13 months under selection in tissue culture, and 5 months plantlet regeneration on hor-

mone-free medium, 15 plants of line 7-3/2 E1 (transformed with CP gene of GFLV) and 12 plants of line B (transformed with Afp 78 gene), have been regenerated.

Analyses of transgenic plants

The GUS histochemical assay was analyzed after 14 days of co-cultivation with *A. tumefaciens*. GUS expression was exhibited mainly in the roots and cotyledons of inoculated cultures. Blue colored cells were recognized inside of the explants by light microscopy of cross sections of the same embryos according to results reported by Da Camara Machado *et al.* (1995). The GUS activity of the new generation of culture after two months free proliferation (without selective pressure) was similar. The whole positive embryogenic culture was recognized after 10 months in culture under selective conditions (Fig.4). This result corresponds to the efficiency of the regeneration capacity of repetitive embryogenesis in our cultures. After plantlet regeneration, 15 clones of seedless genotype 7-3/2 E1 transformed with CP gene of GFLV; and 12 clones of seedless genotype B transformed with Atf 78 gene were analyzed by PCR and Southern blot (Figures 5 and 6). Two plants from genotype 7-3/2 E1 (self-pollinated Russalka 3), three plants from genotype B (Russalka 3 x Corinth white), one from *Vitis rupestris* cv. Rupestris du Lot. and one from 110 Richter, had successfully integrated the *NPT*II reporter gene. Amino acid analyses were made with one clone of transformed genotype B, two clones of genotype 7-3/2 E1 and their respective control plants (Table 1). The quantity of practically all the amino acids in the free dry material of genotype B was lower than the control plant. In contrast to the general tendency, the level of alanine in the transgenic plants was approximately 14% higher than the control plants, which could be due to the expression of the recombinant alanine-rich antifreeze protein in the transgenics.

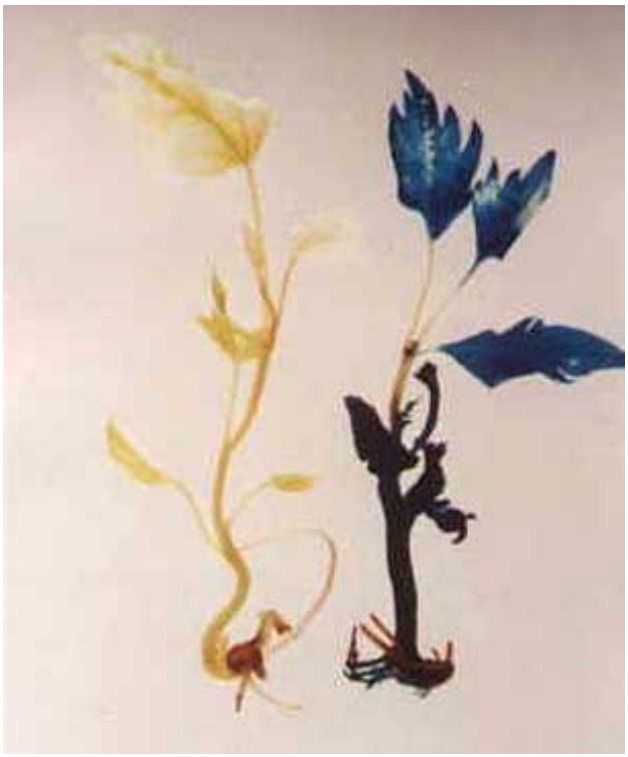
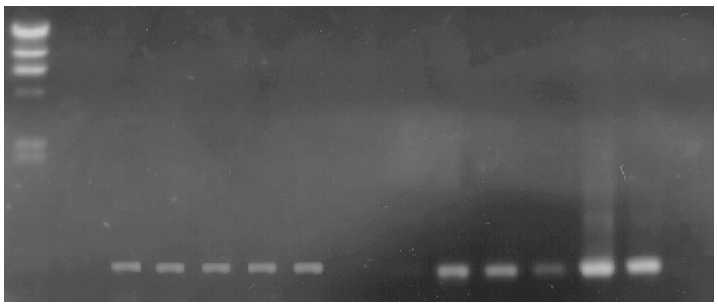


FIGURE 4. Transformed *Vitis rupestris* cv. Rupestris du Lot with a strong positive GUS reaction

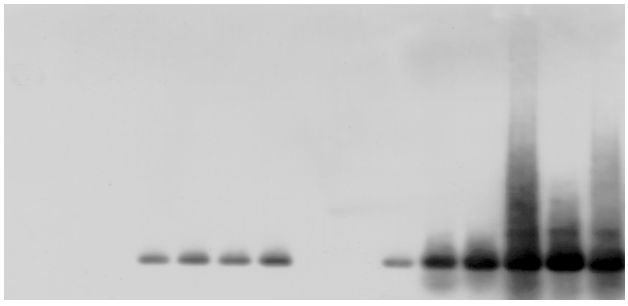
TABLE 1. Results of Aminoacid analyses

Amino acid	AA in absolutely dry material %				
	B control plant	B pAtf 78 - clone 1	7-3/2 E1 - clone 1	7-3/2 E1 - clone 2	7-3/2 E1 - control pl.
1. Lysine	0.35	0.31	0.29	0.26	0.25
2. Hystidine	0.16	0.12	0.14	0.12	0.09
3. Arginine	1.43	0.95	1.36	1.13	1.08
4. Aspargine	0.60	0.54	0.42	0.42	0.47
5. Threonine	0.25	0.25	0.17	0.17	0.17
6. Serine	0.29	0.29	0.18	0.18	0.18
7. Glutamine	0.94	0.81	0.80	0.85	0.73
8. Proline	0.30	0.34	0.28	0.23	0.30
9. Glycine	0.28	0.31	0.20	0.18	0.19
10. Alanine	0.28	0.32	0.21	0.20	0.21
11. Cysteine	0.02	0.01	0.01	0.01	0.01
12 Valine	0.24	0.26	0.18	0.17	0.18
13. Methionine	0.02	0.02	0.02	0.01	0.02
14. Isoleucine	0.19	0.18	0.13	0.13	0.13
15. Leucine	0.39	0.38	0.25	0.24	0.26
16. Tyrosine	0.14	0.13	0.07	0.06	0.06
17. Phenylalanine	0.24	0.24	0.18	0.17	0.17



Lanes:
1 Hind III molecular marker;
2 and 15 untransformed controls (7-3/2 E1 and B);
3-4 transformed plants (CP GFLV 7-3/2 E1);
5-6-7 transformed plants (pAtf 78 B);
8-9 negative transformed plants (pAtf 78 B);
10 transformed plant (CP GFLV *Vitis rupestris* cv. Rupestris du Lot);
11 transformed plant (CP GFLV 110 Richter);
12-14 plasmid controls

FIGURE 5. Detection of the NPT II gene by PCR in DNA of transgenic somatic embryo-derived regenerants.



Lanes:
1 empty
2-3 untransformed controls (7-3/2 E1 and B);
4-5 transformed plants (CP GFLV 7-3/2 E1);
6-7 and 10 transformed plants (pAtf 78 B);
8-9 negative transformed plants (pAtf 78 B);
11 transformed plant (CP GFLV *Vitis rupestris* cv. Rupestris du Lot);
12 transformed plant (CP GFLV 110 Richter);
13-15 plasmid controls.

FIGURE 6. Detection of the NPT II gene by southern blot in DNA of transgenic somatic embryo-derived regenerants.

DISCUSSIONS

The crucial factor in grapevine transformation is the ability of a cell to regenerate and as well as be transformed. For this reason embryogenic cells are good candidates for genetic transformation. Similar findings were also described by Martinelli *et al.* (1994), Krastanova *et al.* (1995), Perl *et al.* (1996), Kikkert *et al.* (1996). Genetic transformation is an alternative to conventional breeding. It is less-time consuming and the preciseness of this approach holds much promise for the genetic improvement of grapevine. Many issues in term of the quality of the vine and the dessert grape need to be addressed since these factors are largely determined by secondary metabolites present in the berry, and the regulation of these substances on gene level are only know being unraveled.

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