TRANSFORMATION OF SOMATIC EMBRYOS OF *VITIS* SP. (GRAPEVINE) WITH DIFFERENT CONSTRUCTS CONTAINING NUCLEOTIDE SEQUENCES FROM NEPOVIRUS COAT PROTEIN GENES

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Breeding of grapevines, one of the world's most widely-grown fruit crop, as in the case with many woody crops, is hampered by genetic heterozygozity and long generation time. Transformation offers new alternatives in the genetic improvement of grapevine. Nepoviruses are the grapevine viruses of greatest economic importance because of their worldwide occurrence and the severe damages they cause. An *Agrobacterium*-mediated gene transfer system relying on repetitive embryogenesis has been used to regenerate transgenic grapevines. Embryogenic cultures of *Vitis vinifera* (Russalka - selfpollinated) and 110 Richter (*Vitis rupestris x Vitis berlandieri*) were transformed with *Agrobacterium tumefaciens* strain LBA 4404 containing various binary plasmids, pBinGUSint, carrying the marker gene β-glucuronidase (GUS), and seven different constructs containing chimeric coat protein (CP) genes of grapevine fanleaf virus (GFLV), including nontranslatable and truncated forms of the CP gene, and arabis mosaic virus (ArMV), respectively. Putative transformed embryos were selected by continued proliferation on kanamycin containing medium. Embryos transformed with the plasmid pBinGUSint were shown to express the GUS gene by histochemical analyses.

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O melhoramento de videiras, uma das plantas mais cultivadas no mundo, tal como muitas outras plantas lenhosas, é dificultado devido à sua elevada heterozigocidade genética e longo ciclo vegetativo. Assim a engenharia genética torna-se um instrumento importante num programa de melhoramento de videiras. Os nepovirus são os vírus da videira com maior importância económica, devido à sua distribuição pelo mundo inteiro e aos graves danos que provocam. Um sistema de transformação genética através de *Agrobacterium* baseado na embriogénese somática repetitiva, foi utilizado para regenerar vinhas transgénicas. Culturas embriogénicas de *Vitis vinifera* (Russalka - auto-polinizada) e Richter 110 (*Vitis rupestris* x *Vitis berlandieri*) foram transformadas com *Agrobacterium tumefaciens* estirpe LBA 4404, contendo vários plasmídeos binários, pBinGUSint,

transportando diferentes marcadores genéticos, β-glucuronidase (GUS), e sete construções diferentes contendo quimeras do gene da proteína da cápsula do vírus do urticado da videira (GFLV) e do vírus mosaico-arábico (ArMV), incluindo formas não transcríveis e incompletas, respectivamente. Os embriões transformados foram selecionados através da sua contínua proliferação em meios contendo kanamicina. A expressão do gene marcador foi demonstrada através de análises histoquímicas do produto do gene GUS.

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INTRODUCTION

Grapevine fanleaf nepovirus (GFLV), the most widespread soil-borne grapevine virus, together with arabis mosaic virus (ArMV) and other nepoviruses is responsible for grapevine fanleaf disease. Control of nematode vectors by soil fumigation is largely inefficient. Furthermore the use of nematicides is being restricted because of the detrimental effects on the soil inhabiting fauna and pollution of surface waters. Therefore new approaches for introducing virus resistance to grapevine would be desirable.

It has been shown that the expression of the viral coat protein (CP) even in altered forms can protect plants efficiently against virus infection (BEACHY et al. 1990; LINDBO & DOUGHERTY 1992). Given the economic importance of nepoviruses, several laboratories are working on the introduction of CP genes into plants in order to induce virus resistance (STEINKELLNER et al. 1991; BARDONNET et al. 1994; KRASTANOVA et al. 1995; MAURO et al. 1995).

Somatic embryogenesis offers an interesting opportunity for genetic transformation by the repetitive process of proliferation of embryos in culture (TSOLOVA et al. 1995). These new secondary embryos are formed from single epidermal cells of primary embryos (POLITO et al. 1989; MACHADO et al. 1995a), ensuring the genetic uniformity of the resulting transformants. From what has been reported so far, not only the regeneration of transgenic plants from transformed cells, but also the efficiency of the selection procedure is a major problem in the transformation of grapevines due to the extreme sensitivity of *Vitis* tissue to kanamycin (COLBY & MEREDITH 1990).

We present the principal steps of an *Agrobacterium*-mediated gene transfer system based on repetitive embryogenesis. Somatic embryos were obtained from immature zygotic embryos of seedless *V. vinifera* (TSOLOVA & ATANASSOV 1994) and from anthers of 110 Richter (LE GALL et al. 1994).

These explants were transformed with 6 different constructs of the CP gene of GFLV, carrying either the full-length CP, 3 truncated forms of the CP and 2 untranslatable forms of the CP gene either in antisenes (AS) or sense (S) orientation (GÖLLES 1994), and the ArMV CP gene.

The β-glucuronidase (GUS) gene (VANCANNEYT et al. 1990) was used to gain information about transformation, behaviour of the tissue during the cocultivation with *Agrobacterium tumefaciens*, and the selection procedures that followed. The obtained data served as guidelines for the handling of presumptive transformants with different forms of the GFLV or ArMV CP gene.

MATERIAL AND METHODS

PLASMID CONSTRUCTION

For the construction of plant transformation vectors containing different forms of the GFLV CP gene the full-length CP gene was first cloned into the vector pSP73 (Promega). The resulting plasmid pGFLV-CP (GÖLLES 1994) was used for the amplification of the various forms of the GFLV CP gene by PCR with specific primers. The particular genes were inserted into the BglII site of the plant expression vector pGA643 (AN et al. 1988). The obtained plasmids are the following: pGA-CP+ contains the full-length CP gene of GFLV with an introduced start codon. pGA-CP differs from the former by a deletion of 15 bp within the CP gene corresponding to the nt 238 - 252 of the CP gene of GFLV strain F13 (SERGHINI et al. 1990). pGA-AS carries the same gene construct as pGA-CP+ but in antisense orientation, resulting in an untranslatable form of the gene. In the plasmid pGA-S two stop codons were introduced after the inserted start codon by PCR. The sequence GGATTAGCTGGTAGA was changed to GGATAAGCTGGTTGA resulting in an untranslatable CP gene in sense orientation. pGA5'TR contains a CP cDNA which is shortened by 138 bp at the 5'-end. Plasmid pGA3'TR carries a CP gene with a truncation of 168 bp at the 3'-end of the gene.

The binary plasmid pROK-ArMV contains the ArMV CP gene downstream of the enhanced 35S promotor and the TMV omega leader sequence and before the NOS terminator sequence (BERTIOLI et al. 1991).

PLANT MATERIAL

Two repetitively embryogenic *Vitis*-lines were used in our transformation experiments: Line 7-3/2E1 (selfpollinated Russalka 3) was established by inducing embryogenesis from immature ovules of this stenospermocarpic *Vitis vinifera* cultivar (TSOLOVA & ATANASSOV, 1994). The second embryogenic line was a long-term somatic embryogenic callus culture obtained from the vegetative tissues of anthers of the grapevine rootstock 110 Richter (*Vitis rupestris x Vitis berlandieri*) (LE GALL et al. 1994).

Culture conditions were as previously described (MACHADO et al. 1995b; LE GALL et al. 1994).

KANAMYCIN SENSITIVITY

The influence of different concentrations of kanamycin on the root development and the adventitious embryogenesis of seedless grapes (line 7-3/2E1, Russalka - selfpollinated) was determined by culturing somatic embryos on modified NN69 medium (TSOLOVA & ATANASSOV 1994) with 12.5, 25, 50, and 100 mg/l kanamycin.

PLANT TRANSFORMATION AND SELECTION

An *Agrobacterium tumefaciens* LBA4404 suspension, containing the wanted plasmid, was inoculated from an overnight culture and grown to a density of OD600=0.6. For cocultivation, the suspension was diluted 1:50 in 3 % sucrose solution and white embryogenic clusters were dropped into the *Agrobacterium* suspension. After incubation at room temperature (5 - 10 min) for inoculation, the embryos were placed onto solid modified NN69 medium without charcoal.

After a 48 h cocultivation with the bacterium embryos were transferred to plates containing modified NN69 medium with 300 mg/l cefotaxime and activated charcoal in order to inhibit further agrobacterial growth.

Kanamycin was added as a selective agent after 2 months of proliferation without selection pressure. We started either directly with 100 mg/l kanamycin or increased the concentration stepwise during successive monthly transfers (12.5; 25; 50; 75; 100 mg/l).

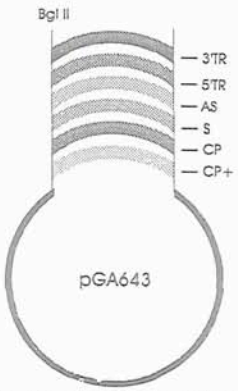
Analysis of the GUS Activity

Expression of the introduced GUS gene in the new generation of somatic embryos was analysed by the x-Glu (5-bromo-4-chloro-3-indolyl-glucuronide) histochemical assay according to Jefferson et al. (1987). After 14 days and subsequently in intervals of 2 months, the embryos were removed from putatively transformed clusters, exposed to x-Glu for 24 h at 37°C, and observed by light microscopy.

RESULTS

Plasmids

Plant transformation vectors containing various forms of the GFLV CP gene are shown in Fig. 1. All constructs were tested for their expression in *Nicotiana benthamiana* as a herbaceous model plant. Expression was determined on a transcriptional level by RT-PCR and, in the case of the full-length CP gene construct, also by ELISA. With all of these gene constructs plant lines expressing the gene in a correct manner were obtained (Gölles et al. pers. commn.). The transformation of *N. tabacum cv. White Burley* with pROK-ArMV has been reported previously (Steinkellner et al. 1991).



3'TR ATG GGA TTA GCT GGT AGA .... GAC GGA ACT ACA TAG 168 nt
5'TR 138nt ATG CCT ACT TTC AAG ATA .... AGT TTC CCA GTC TAG
AS CTA GAC TGG GAA ACT TGG .... TCA AGC TTA TCC CAT
S ATG GGA TAA GCT GGT TGA ..... AGT TTC CCA GTC TAG -15 nt
CP ATG GGA TTA GCT GGT AGA ..... AGT TTC CCA GTC TAG -15 nt
CP+ ATG GGA TTA GCT GGT AGA ..... AGT TTC CCA GTC TAG

Fig. 1. Schematic presentation of the 6 different vectors and primers used for their amplification used for transformation. CP+ contains the entire coat protein sequence; CP contains the coat protein gene with an internal deletion of 15 nucleotides; S contains the same sequence in sense orientation, but without start codon, so that no protein synthesis is to be expected; AS contains the CP+ sequence in antisense orientation, therefore also yielding no protein, and; 5'TR and 3'TR are truncated sequences either at the 3' or 5' end of the CP+ sequence shortened 168 and 138 nucleotides respectively.

Kanamycin Sensitivity

To optimize the level of kanamycin during selection we observed a kanamycin response curve after 2 months exposure to the antibiotic (Fig. 2) with special attention to the root development of grape somatic embryos. Only high concentrations (more than 100 mg/l) were lethal. Up to 50 mg/l kanamycin secondary embryogenesis was observed in some of the

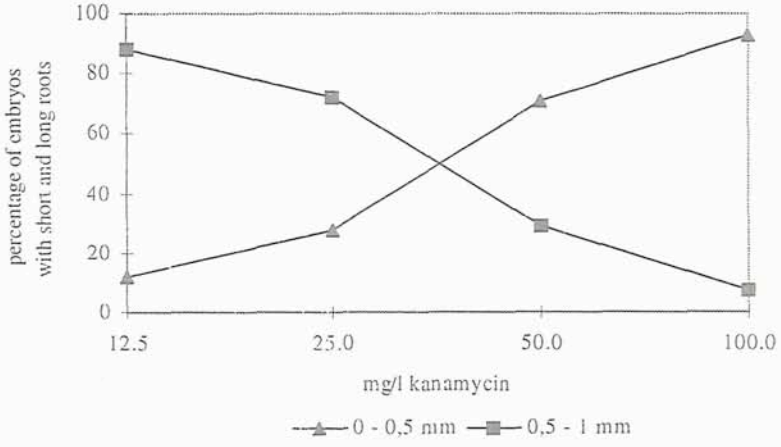


Fig. 2. Influence of different concentrations of kanamycin on the percentage of embryos of *Vitis* embryogenic line 7-3/2E1 with short and long roots (N = 120).

treated explants. Thus a kanamycin concentration of ≥75 mg/l is needed in order to inhibit the formation of non-transformed secondary embryos.

Transformation and Selection

Perl et al. (1994) reported tissue necrosis and subsequent cell death after very short exposures of grape embryogenic tissue to diluted cultures of *Agrobacterium*. Therefore, to define the optimal incubation time for the explants we inoculated white embryogenic culture for 5 - 10 min at room temperature with bacterial suspension. Local tissue necrosis could be observed in some of the explants, but during the following subcultures a high amount of embryos grew on the tissue surface and the embryogenic competence was maintained.

Parallel testing of selection procedures with 100 mg/l kanamycin and stepwise increasing the concentration during successive subcultures (12.5; 25; 50; 75; 100 mg/l) showed that an initial concentration of 100 mg/l kanamycin caused a strong reduction of growth and a strong inhibition of the embryogenic process. Because of this inhibitory effect we decreased the kanamycin concentration stepwise to 25 mg/l in part of these embryogenic cultures. Recovery of proliferation of embryogenic cultures could be obtained and a stepwise increase of the selective pressure could be applied again. However, also those cultures which were maintained at 100 mg/l kanamycin during the successive subcultures recovered and proliferated, although to a much reduced extent. We found that the strategy of proliferation without kanamycin for 2 months and a stepwise increase of the kanamycin concentration to a final concentration of 75 mg/l is the best suitable way to regenerate and select transformed embryos.

Somatic embryos transformed with the GUS gene and selected for kanamycin resistance for 5 and 10 months respectively, showed a strong GUS-activity in histochemical assays (Fig. 3), confirming that the developed transformation system is suitable to transform grapevine. Different media to induce germination are under investigation.

All six constructs of the GFLV CP gene and the plasmid pROK-ArMV were used to transform somatic embryos from line 7-3/2E1. Embryogenic callus from 110 Richter was transformed with the plasmids pGA-CP+, pGA-CP, pGA-AS, pGA-S, and pGA5'TR by now. Currently the selection of somatic embryos transformed with the different forms of the GFLV or ArMV CP genes has almost been completed (Fig. 4) and regeneration of transgenic plants will be carried out following the optimized procedures.

DISCUSSION

Conditions for gene transfer in grapevine were elaborated, investigating the influence of kanamycin on somatic embryos and evaluating the efficiency of the transfomation method by GUS visualization. Obviously somatic embryos and adventitious somatic embryogenesis as a regeneration process are more tolerant towards the toxic and inhibitory effects of kanamycin than adventitious grape shoots obtained through organogenesis, as was reported by Colby & Meredith (1990) and Mullins et al. (1990). Similar results were also described by Martinelli & Mandolino (1994).

Six plasmids containing different forms of the GFLV CP gene were constructed and together with the ArMV-CP gene, used for grapevine transformation.

After germination of the embryos we plan to analyze the transgenic plants both at the transcriptional and, in case of the full-length CP, translational level. Plant lines, which show the highest expression level will be used for challenge infection experiments in order to determine the protection against homologous and heterologous nepoviruses.

As indicated by results of coat protein-mediated protection obtained with *N. benthamiana* (Bardonnet et al. 1994) the presented strategy of pathogen-derived resistance is an important contribution to resistance breeding in grapevine.

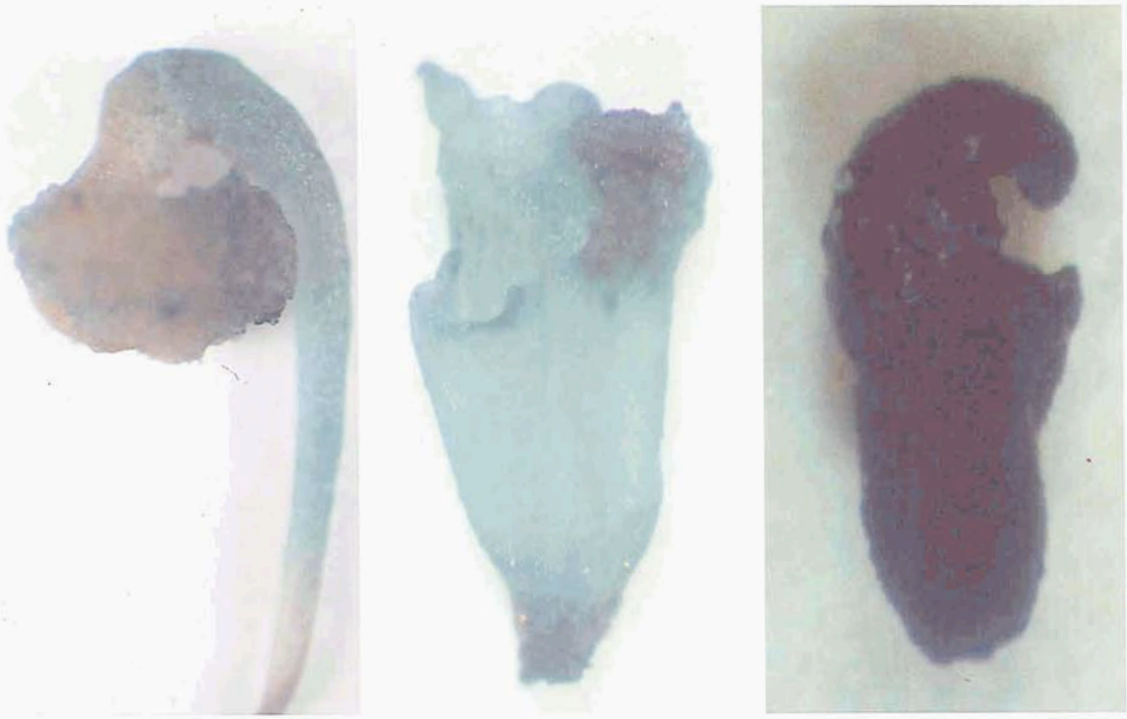


Fig. 3. GUS-activity in grape somatic embryos (embryogenic line 7-3/2E$_1$): a- 14 days after cocultivation the blue colour is localized mainly in root and cotyledonary tissue: b- after 10 months in culture with selection pressure GUS-positive reaction over the whole embryo; c- after 12 months in culture with selection pressure deeply blue coloured embryo.

Fig. 4. Transgenic embryos of line 7-3/2E$_1$ after several months of selection (75 mg/l kanamycin).

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