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L. Martinelli · G. Mandolino

Genetic transformation and regeneration of transgenic plants in grapevine (*Vitis rupestris* S.)

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Abstract Isolated somatic embryos from petiole-derived callus cultures of *Vitis rupestris* Scheele have been employed in experiments on genetic transformation. Co-cultivation of somatic embryos during embryogenesis induction with *Agrobacterium tumefaciens* strain LBA4404, which contains the plasmid pBI121 carrying the neomycin-phosphotranspherase and the β-glucuronidase genes, produced transformed cellular lines capable of recurrent somatic embryogenesis. Precocious selection for high levels of kanamycin (100 mgl^{-1}) was an important part of our transformation protocol. Transformed lines still have strong β-glucuronidase expression as well as stable insertion of the marker genes after 3 years of in-vitro culture, during which they have maintained their capacity to organize secondary embryos and to regenerate transgenic plants with an agreeable efficiency (13%).

Key words *Agrobacterium tumefaciens* · Genetic transformation · GUS · Somatic embryos · *Vitis rupestris*

Introduction

Species of the genus *Vitis* are particularly difficult to transform: stable transgenic grapevine genotypes, for example, have not yet been reported.

The potentiality of biolistic transformation on grapevine cell cultures is currently under investigation (Hébert et al. 1993). Regeneration from transformed *Agrobacterium rhizogenes* root explants has not been described so far (Hemstad and Reisch 1985; Gribaudo and Schubert 1990; Guellac et al. 1990). *Agrobacterium tumefaciens* seems a more amenable vector, since plant regeneration has already been reported for several genotypes starting from tissues adopted for transformation (Hirabayashi et al. 1976; Mullins and Srinivasan 1976; Krul and Worley 1977; Barlass and Skene 1979, 1980; Rajasekaran and Mullins 1979; Bouquet et al. 1982; Mauro et al. 1986; Stamp and Meredith 1988 a, 1988 b; Cheng and Reisch 1989; Clog et al. 1990; Monnier et al. 1990; Stamp et al. 1990; Martinelli et al. 1993).

Protocols for transgenic grapevine production by *Agrobacterium*-mediated transformation have already been proposed for different *Vitis* species. They are based on cell cultures (Baribault et al. 1989), leaf, petiole or stem explants (Mullins et al. 1990; Colby et al. 1991; Berres et al. 1992), fragmented shoot apex cultures (Baribault et al. 1990) and somatic embryo sections (Mullins et al. 1990) utilized as tissues to be transformed. In spite of this, suitable and reproducible protocols are not available, and several unsolved problems lower the efficiency of the process. Apparently there is no barrier to *Agrobacterium* infectivity (Baribault et al. 1990; Mullins et al. 1990), but the choice of the regenerating model remains the crucial aspect.

Somatic embryogenesis derived from single cells (Krul and Worley 1977) has been judged unsuitable for transformation in commercial *Vitis* genotypes (Mullins et al. 1990). On the other hand, regenerating systems where groups of cells take part in the process are also poorly adapted, since transformation followed by shoot regeneration leads to chimeric plants (Baribault et al. 1990; Colby et al. 1991; Berres et al. 1992).

Additionally, the choice of the gene which allows an accurate selection of the transformed cells appears to be a decisive issue. The neomycin phosphotransferase gene is a good marker, but *Vitis* tissues are extremely sensitive to the antibiotic kanamycin (Baribault et al. 1989, 1990; Colby and Meredith 1990; Guellac et al. 1990; Mullins et al. 1990; Colby et al. 1991; Hébert et al. 1993). An effective and continuous selection pressure for kanamycin resistance cannot in fact be applied because of the inhibitory effect on regeneration of this antibiotic when used at relatively high concentrations (Mullins et al. 1990).

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L. Martinelli (✉)
Istituto Agrario, via Mach 1, 38010 San Michele all'Adige, Trento, Italy

G. Mandolino
Istituto Sperimentale per le Colture Industriali, Bologna, Italy

In the present paper we present an attempt to improve the efficiency of *Agrobacterium*-mediated genetic transformation of species of the genus *Vitis*, avoiding both the production of chimeric tissues and the loss of the inserted genes. This study has been carried out on a well-established culture of petiole-derived *Vitis rupestris* somatic embryos able to generate recurrent secondary somatic embryogenesis (Martinelli et al. 1993), and to regenerate transgenic plants with an agreeable efficiency.

Materials and methods

Induction of somatic embryogenesis

The embryos employed in the present work differentiated from petiole-derived calli of *V. rupestris* Scheele following the protocol of Martinelli et al. (1993). A first cycle of secondary somatic embryogenesis was started from 196 somatic embryos isolated from liquid cultures and placed horizontally on an induction medium. The induction of somatic embryogenesis and subcultures of the embryogenic callus were carried out on two solid media, alternatively half strength MS- or full strength NN-based media [Murashige and Skoog (MS) 1962; Nitsch and Nitsch (NN) 1969], both containing 0.8% Difco Bacto-agar and supplemented with 1 mgl^{-1} of Indole-3-acetic acid (IAA) or 0.1 mgl^{-1} of Indole-3-butyric acid (IBA), respectively. The cultures were incubated at 25 °C in dim light (15 µmol $m^{-2}s^{-1}$). The medium was replaced monthly.

Embryo culture

For the elongation, multiplication and separation of embryos, clusters of small somatic embryos (length 1–2 mm) were separated from the embryogenic callus and placed in a 125-ml flask containing 50 ml of liquid NN-based medium with 0.1 mgl^{-1} of IBA. Flasks were closed with a cottonwool plug and covered with aluminum foil. Cultures were incubated at 25 °C in dim light (15 µmol $m^{-2} s^{-1}$), shaken continuously at 90 rpm, and the medium was renewed every 3 weeks.

Kanamycin sensitivity

To optimize the level of kanamycin for the selection of the transformed tissues, a kanamycin response curve was evaluated during a 1-month selection. Based on this, somatic embryos during embryogenesis induction on solid media, as well as maturation in liquid cultures and germination, were cultured with 15, 25, 50, 100 and 150 mg l^{-1} of the antibiotic added to the media after autoclaving. To verify the kanamycin effect on the meristems, buds were tested at the same concentrations.

Transformation

Bacteria were grown in a solution of 2.85 mM K_2HPO_4, 0.82 mM $MgSO_4$, 0.01% NaCl, 0.04% yeast extract, 1% mannitol and 100 mgl^{-1} of kanamycin. Infection was accomplished by a 5-min submersion of selected somatic embryos into an overnight culture of *A. tumefaciens* strain LBA4404 carrying the plasmid pBI121 (Jefferson et al. 1987). This plasmid is a derivative of pBin19 (Bevan 1984) and contains both the neomycin-phosphotranspherase (NPT) gene under the control of the nopaline-synthase (NOS) promoter and terminator, and the *E. coli* β-glucuronidase gene fused to the Cauliflower Mosaic virus (CaMV) 35*s* promoter.

Embryos were blotted on filter paper and then placed horizontally on solid induction medium, with or without scratching with a needle, to prepare them for co-culture. The cultures were incubated at 28 °C in the dark. After 3 days the embryos were moved to fresh medium containing 300 mgl^{-1} of cefotaxime and incubated at 26 °C in the dark.

Culture maintenance on selective medium

Twenty-day-old cultures were moved to fresh medium containing 100 mgl^{-1} of kanamycin and 300 mgl^{-1} of cefotaxime, and incubated at 25 °C in dim light (15 µmol $m^{-2}s^{-1}$). The selection was carried out over a period of 9 months during which only white and embryogenic tissues were kept in culture. After 1 year the concentrations of kanamycin and cefotaxime were reduced to 50 and 100 mgl^{-1}, respectively. Four months later, these levels were scaled down to 50 mgl^{-1} for both chemicals, and after a further 4 months kanamycin was reduced to 25 mgl^{-1}. During callus subcultures, clusters of small embryos were periodically sampled to induce multiplication and elongation of isolated embryos in liquid cultures. Such clusters were isolated for the first time after a 7-month subculture of the embryogenic callus. They were cultured on a liquid NN-based medium containing 0.1 mgl^{-1} of IBA, 50 mgl^{-1} of kanamycin and 100 mgl^{-1} of cefotaxime. Two months later the levels of kanamycin and cefotaxime were reduced to 25 and 50 mgl^{-1} and four months later to 15 and 50 mgl^{-1}, respectively.

The overall process was repeated starting with 496 somatic embryos selected from liquid cultures and induced to produce secondary embryos on 50 mgl^{-1} of kanamycin and 100 mgl^{-1} of cefotaxime. During the subsequent liquid culture phase, 25 mgl^{-1} of kanamycin and 50 mgl^{-1} of cefotaxime were used.

Plant regeneration and culture

Single somatic embryos were isolated from liquid cultures and placed with the radicals downward on NN-based medium containing 1.5% sucrose, gelled with 0.8% Difco Bacto-agar, and supplemented with BA (1 mgl^{-1}) and IBA (0.1 mgl^{-1}). The medium was renewed monthly.

A sample of 52 somatic embryos was inseminated on this medium containing 50 mgl^{-1} of kanamycin and 100 mgl^{-1} of cefotaxime, and subjected to a chilling treatment (15 days in the dark at 4 °C). Additionally, 73 somatic embryos were tested in the absence of kanamycin but in the presence of chilling. Finally, a population of 285 somatic embryos in four distinct replications were induced to regenerate plants in the absence of both kanamycin and chilling.

The embryo-derived plantlets were separated from the embryo tissues and cultured on growth-regulator-free NN-based medium containing 1.5% sucrose and 0.8% Difco Bacto-agar, in order to promote rooting and elongation.

The same medium was employed for the micropropagation of grown plants.

Both regenerating embryos and plant cultures were incubated in a climate room at 25 °C with a 16 h photoperiod (70 mol $m^{-2}s^{-1}$ cool white light).

Media preparation and culture vessels

The pH of the media was adjusted with NaOH to 5.7 before autoclaving for 20 min at 121°C and 1 atmosphere When needed, filter-sterilized IAA was added after autoclaving.

For solid cultures of both somatic embryos and regenerating embryos, 30 ml of medium were dispensed on plastic Petri dishes of 9-cm diameter. For plant cultures, 70 ml of medium was dispensed in magenta boxes.

Marker-gene evaluation

Periodically, embryos were randomly sampled from liquid cultures to screen for evidence of the insertion of the marker genes. Assays have also been performed at the end of a complete cycle of recurrent somatic embryogenesis after 12, 18 and 36 months of selection on kanamycin. GUS expression was analyzed according to Jefferson (1987). Longitudinal and horizontal sections were incubated overnight at 37 °C with a 1 mM solution of 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). The same technique was used for checking the tissues during plant regeneration and for assaying regenerated plantlets. Leaf tissues needed to be scraped with a needle to promote X-Gluc infiltration. Green tissues were incubated with 75% lactic acid and autoclaved for 15 min at 121 °C and 1 atmosphere. Coloured sections were observed under a stereo microscope.

For the molecular assays, DNA was extracted from about 1 g of well-developed putatively-transformed fresh somatic embryos sampled after 12, 18 and 36 months of selection, respectively.

Genomic DNA was prepared from transformed and untransformed *V. rupestris* embryos by the CTAB method (Rogers and Bendich 1985). Additionally, whole regenerated plantlets, randomly chosen, were used to check the insertion of the GUS gene into the plant genome. The plantlets were ground under liquid nitrogen, and genomic DNA prepared following the same method used for embryo cultures. DNA samples (about 12 μg) were digested either with *Eco*RI (40 units) or *Hin*dIII (40 units each; both enzymes from Boehringer, Mannheim). The digestion mixture was further purified, precipitated, and electrophoresed on a 1% TAE (Tris-acetate 40 mM, EDTA 1 mM, pH 8.0) agarose gel at 1 V/cm. Gels were blotted onto nylon membranes (Hybond-N, Amersham) that were subsequently prehybridized overnight at 65 °C in hybridization solution (6×SSC, 5×Denhardt's, 0.5% SDS, salmon sperm DNA 50 μg ml^{-1}). After prehybridation, the labelled probe was added to the same solution, and the hybridation carried out overnight at 65 °C. The filters were then washed three times in 3×SSC/0.1% SDS and subjected to autoradiography.

The plasmid pBI221, carrying the GUS gene, was extracted from an overnight culture of *Escherichia coli* (strain XL1 Blue, Stratagene), and digested with *Eco*RI and *Hin*dIII. The 3.1-kb fragment containing the β-glucuronidase (GUS) gene, the CaMV promoter and the NOS terminator was gel-purified and radioactively labelled for 4 h at room temperature in the presence of 10 μCi of ^{32}P-dCTP and five units of the Klenow fragment of DNA polymerase I (Promega).

Observation with the scanning electron microscope

Embryos were fixed for 12 h in 3% gluteraldehyde in 0.1 M phosphate buffer at pH 7.0. Fixation was followed by dehydration through a graded alcohol series. The specimens were critical-point dried, fixed on holders with silver adhesive, coated with gold in a vacuum spatter coater, and analyzed with a Hitachi S-2300 scanning electron microscope using a tungsten filament at 25 kV.

Results and discussion

Production of stably-transgenic somatic embryos

In the genus *Vitis*, in-vitro systems of recurrent embryogenesis have been frequently described (Krul and Worley 1977; Gray 1989; Matsuta and Hirabayashi 1989; Vilaplana and Mullins 1989; Martinelli et al. 1993). This same process, has proved to represent a relevant part of a suitable strategy for achieving genetic transformation in *V. rupestris* (Mullins et al. 1990).

In our protocol (Martinelli et al. 1993) somatic embryogenesis develops in recurrent cycles. The first cycle consists of two distinct steps occurring on solid and in liquid cultures, respectively (Fig. 1). In the first step, embryogenesis is induced from isolated somatic embryos, starting with the production of embryogenic callus and ending with the regeneration of clusters of small somatic embryos. The second step accomplishes elongation, maturation, and separation of the embryos. This recurrent production of embryos can be repeated over and over, since new cycles can be started as soon as maturation of individual embryos occurs. Alternatively, isolated somatic embryos can be induced at this point to regenerate plants.

The co-culture of plant tissues with *A. tumefaciens* during the induction of embryogenesis promotes the delivery of foreign genes to embryo-derived cellular clones (Lippincott and Lippincott 1969). In our case the tissues of the embryo entered into a phase of active cell division and within a few days the embryo surface was covered by numerous developing structures. The attachment of the bacteria to the cell surface was seen through the scanning electron microscope by observing how the bacterial clumps are tightly anchored to plant tissues by synthesized fibrils (Fig. 2) (Mooney and Goodwin 1991). Wounding of embryo tissue proved unnecessary for achieving DNA delivery and insertion, since both scratched and intact embryos gave rise to transformed somatic embryos.

Vitis tissues have been described as extremely sensitive to kanamycin (Baribault at al. 1989; Colby and Meredith 1990). Based on this, the selection of transformed tissues from different explants has been carried out in the presence of low levels of the antibiotic (Baribault et al. 1990; Guellec et al. 1990; Mullins et al. 1990; Colby et al. 1991; Hébert et al. 1993). We believe, however, that rigorous selection is necessary in order to avoid the establishment of chimeric tissues (Baribault et al. 1990; Colby et al. 1991; Berres et al. 1992). In our experience, at all concentrations tested, kanamycin proved to be seriously toxic, since tissue browning, growth inhibition and organ structure degeneration were commonly observed in treated explants. However, during embryogenic induction on solid medium (150 mgl^{-1}) and maturation in liquid phase (100 mgl^{-1}), only high concentrations of the antibiotic proved clearly lethal to somatic embryos. In our cultures, embryogenic competence appeared within a 2-month culture, but at least 7 months were necessary to obtain groups of polarized somatic embryos. Under selective conditions, kanamycin-sensitive tissues deteriorated while small white spots of resistant cells arose: only these were selected. During the following subcultures a large amount of white kanamycin-resistant embryogenic callus was produced. Histochemical assays confirmed that these white tissues exhibited GUS activity (Fig. 3 b), while dark non-kanamycin-resistant tissues never turned blue when properly assayed. Because of the adoption of precocious selection at high levels of kanamycin, it is reasonable to conclude that only a few resistant transformed cells took part in the embryogenic process; this was also supported by the fact that the *E. coli* β-glucuronidase gene showed a homogeneous expression in the transformed tissues: all cells of secondary somatic embryos turned blue after a few hours of incubation with X-Gluc (Fig. 3 c, d). No endogenous GUS activity was detected in control tissues (Fig. 3 a).

In a 3-year selection, two independent cycles of secondary embryogenesis have been completed. In the first, 196 somatic embryos were employed in co-cultivation experiments, and in the second, 496. The number of somatic embryos, maintained in culture because of their capacity to produce small clumps of white cells putatively resistant to kanamycin, reduced substantially for the first and the second cycle respectively to 3.1% and 3.4%. All selected cell clumps originated embryogenic calli which differentiated clusters of somatic embryos (58% and 33% during the first and the second cycle, respectively). The selective condi-

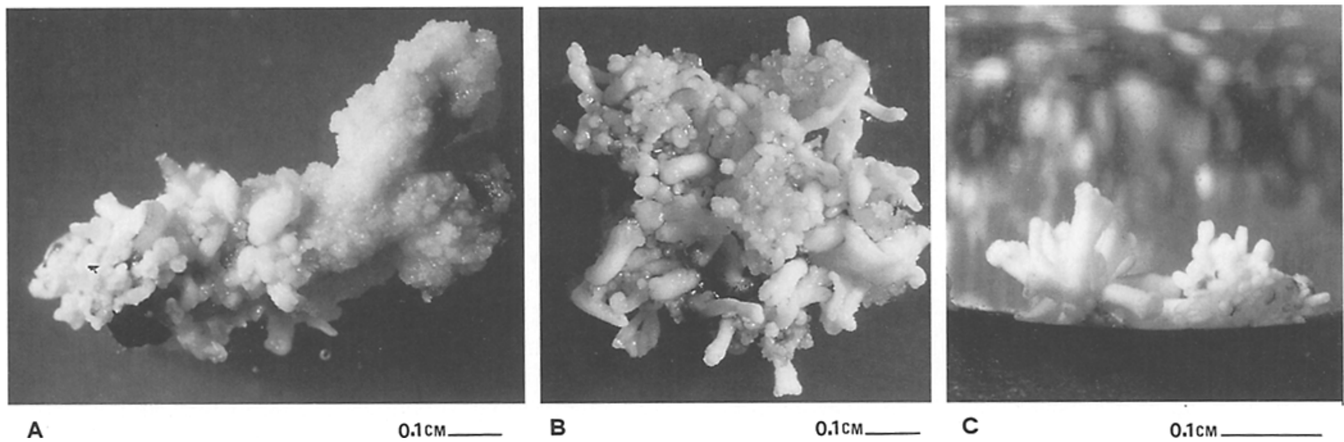


Fig. 1A – C Secondary embryogenesis. **A** Isolated somatic embryo grown on solid medium and actively producing callus-like tissues on its surface. **B** Embryogenic callus with clumps of polarized somatic embryos. **C** Clusters of young somatic embryos in liquid culture

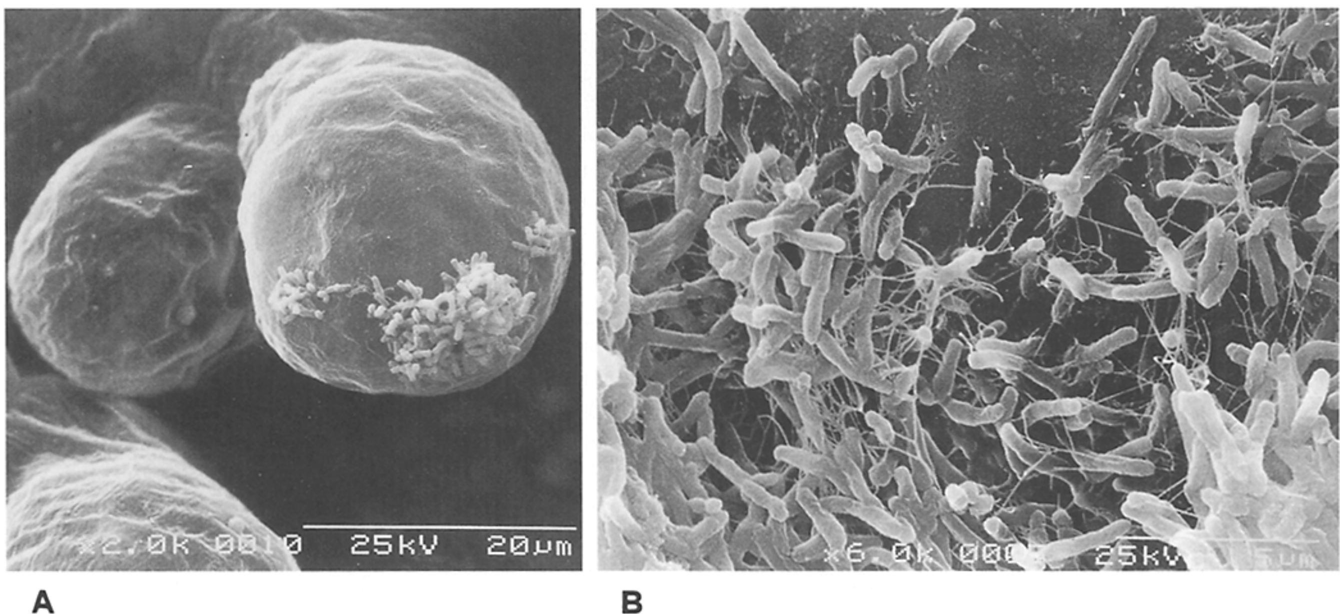


Fig. 2A, B Somatic embryo co-cultured with *Agrobacterium tumefaciens* during secondary embryogenesis induction as seen with the scanning electron microscope. **A** A secondary embryogenetic structure infected by a bacterial clump. **B** Particulars of a bacterial clump: fibrils allow strong contact of the bacteria with the embryo surface

tions were modified during the subculture progress, as described under Materials and Methods. Nevertheless, the decrease in antibiotic concentration did not alter the level of expression of the GUS gene.

In agreement with the high level of GUS activity showed by transformed embryos (Fig. 3 c, d), the integration of the GUS gene within the DNA of *V. rupestris* was evident when analyzed at the molecular level. In three assays carried out 12, 18 and 36 months after the transformation, a sharp 3.1-kb band appeared consistently in Southern blottings of *Eco*RI-*Hin*dIII-digested DNA when hybridized to the GUS fragment of pBI221 (Fig. 4, lanes 1, 3 and 5); in *Eco*RI-digested samples this band is not present but is substituted by two heavier bands (Fig. 4, lanes 2, 4 and 6). In untransformed embryos, by contrast, no hybridization occurred at all (Fig. 4, lanes 7 and 8).

Comparable results were observed when regenerated plantlets were tested. In Fig. 6, in fact, the same pattern of hybridization occurred (lanes 2 and 3). Additionally, when undigested genomic DNA was blotted, the lower-molecular-weight bands are substituted by a unique band corresponding to the undigested DNA (lane 1).

These data, along with histochemical assays and stable antibiotic resistance, showed that a highly-stable integration of foreign genes in *V. rupestris* somatic embryos was achieved.

Only 4 weeks were required for inducing maturation and multiplication of somatic embryos in liquid culture. The abundant embryo production at this stage compensated for the low efficiency of the initial process.

Fig. 3A – D Untransformed and transformed somatic embryos. **A** Longitudinal section of a non-transformed somatic embryo showing no endogenous GUS activity. **B** A somatic embryo 1 month after infection and induction: kanamycin-resistant tissues exhibit GUS activity while kanamycin-sensitive cells appear dark brown. **C** A cluster of polarized somatic embryos with high GUS activity. **D** Transverse section of a transformed somatic embryo: the GUS activity is expressed homogeneously in all cells of the embryo

Transgenic plant regeneration

Regenerative capacity has been described as a major problem for transgenic grapevines (Colby et al. 1991). In spite of this drawback, we obtained plant regeneration with a relevant efficiency from distinct transformation events: in fact, in the absence of kanamycin, both chilled and unchilled embryos regenerated plants at good levels (respectively 10% and 13%) within a 7-month culture. As a result, respectively 11 and 36 different clones were obtained

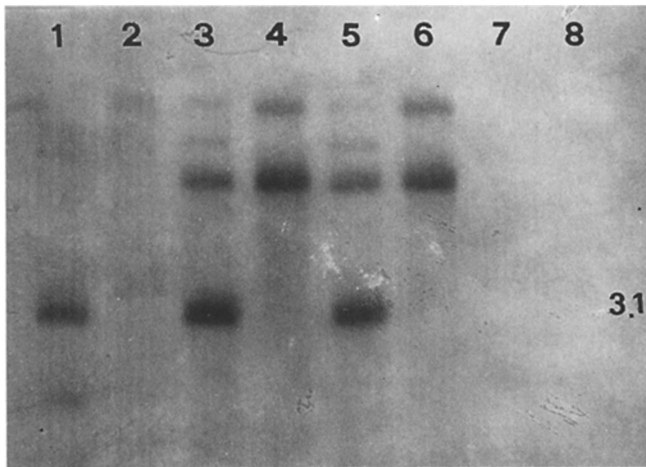


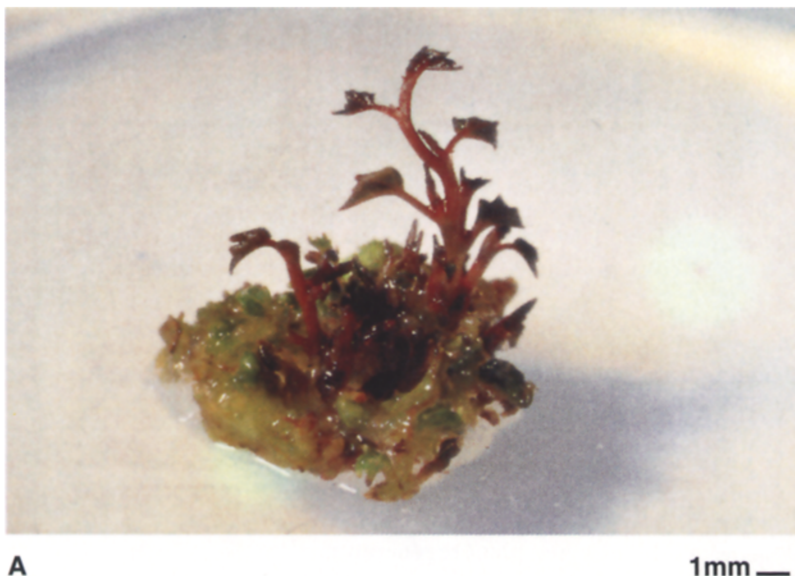
Fig. 4 Autoradiograph of a Southern blot of DNA extracted from embryo cultures transformed at different times. *Lane* 1, 12-month-old transformed culture, *Eco*RI and *Hin*dIII; *lane* 2, 12-month-old, *Eco*RI; *lane* 3, 18-month-old, *Eco*RI and *Hin*dIII; *lane* 4, 18-month-old, *Eco*RI; *lane* 5, 36-month-old, *Eco*RI and *Hin*dIII; *lane* 6, 36-month-old, *Eco*RI; *lane* 7, untransformed, *Eco*RI and *Hin*dIII; *lane* 8, untransformed, *Eco*RI

from distinct transformation events. Since similar values were obtained for untransformed *V. rupestris* somatic embryos during germination induction (Martinelli et al. 1993), we assume that the presence of foreign DNA in the genome does not affect the regeneration capability. The embryos produced a green disorganised callusing tissue and from this tissue plant regeneration occurred via organogenesis, with many individual shoots regenerated from each embryo (Fig. 5 a). Shoots were dissected from the embryogenic tissues for growing, rooting, and micropropagating.

Histochemical assays showed a strong GUS activity in the deformed embryogenic tissues and in the regenerating shoots, as well as in the grown plantlets (Fig. 5 b) and in the roots (data not shown). Because of the presence of the epicuticular wax, leaf wounding is necessary for a proper incubation with X-Gluc. In contrast, undamaged root tissues are very sensitive to this substrate since a strong blue coloration appears after a few hours of incubation.

The molecular analysis of both transformed embryo cultures (Fig. 4) and regenerated plantlets (Fig. 6) fully demonstrates the stable insertion of the exogenous gene into the plant genome. Consequently, no loss of the chimeric GUS gene occurs following both the long-term embryo cultures and the plant regeneration events.

These results are a corollary of our transformation strategy. In other systems, by contrast, difficult regeneration of whole plants and chimerical production and/or expression of the inserted genes have been reported as a disadvantage of the techniques adopted (Baribault et al. 1989, 1990; Mullins et al. 1990; Colby et al. 1991). Nevertheless, here we have a system leading to good genome stability of the inserted genes and to a low level of somaclonal variation. The effect is a suitable overlap between transgenic and regenerating cells.



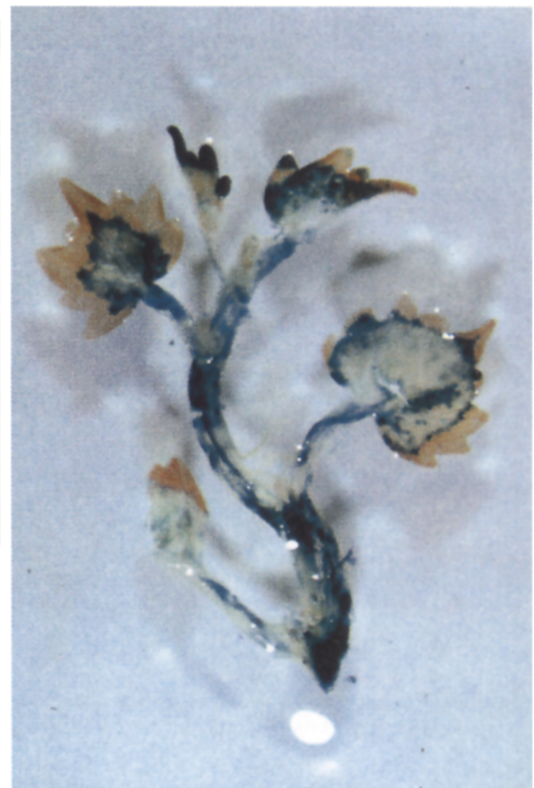


Fig. 5A, B Plant regeneration from a transgenic somatic embryo. **A** Regeneration occurs via organogenesis and many individual shoots are often produced from each embryo. **B** GUS-positive regenerated plantlet separated from the embryogenic tissues

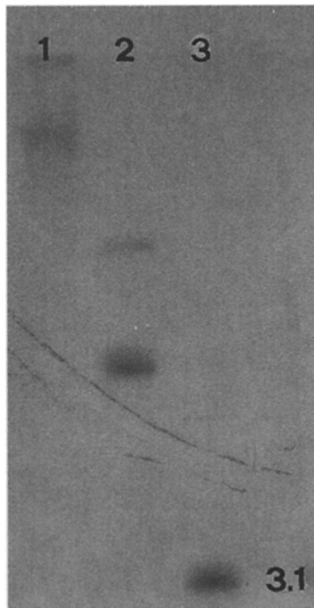


Fig. 6 Autoradiograph of a Southern blot of genomic DNA extracted from a transgenic plantlet. *Lane* 1, undigested DNA; *lane* 2, *Eco*RI-digested DNA; *lane* 3, *Eco*RI and *Hind*III digested-DNA

In contrast, when kanamycin was present in the medium, embryos failed either to turn green or to evolve any morphogenic structures. However, when transferred on kanamycin-free medium, embryos gradually developed greenish deformed structures. The inhibitory effect of kanamycin on the organogenesis of grapevine has already been described (Baribault et al. 1990; Colby and Meredith 1990; Mullins et al. 1990). However, our results prove the non-requirement for selection on kanamycin during the plant regeneration stage.

Conclusions

The present work is a contribution to the success of genetic transformation in grapevine. Our transformation strategy highlights some of the potential advantages of using somatic embryos for inserting foreign genes. In fact, from a single transformed somatic embryo (or a part of a single embryo) it is possible, through secondary embryogenesis, to propagate transformed tissues which give rise to a population of stably-transformed secondary somatic embryos. This population would be a clone originating from a single transformation event, and the data provided show that these transformed clones remain stable for 3 years. Most remarkable, they are competent to regenerate transgenic plants with a suitable efficiency.

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