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FINAL REPORT

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BREAU FOR ECONOMIC GROWTH, AGRICULTURE AND TRADE

TITLE OF PROJECT:
TRANSGENIC SEEDLESSNESS IN GRAPES BY USING GENETIC ABLATION OF SEEDS

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Executive Summary

From the point of view of the consumer, seeds are an unattractive aspect of these fruits. Many high yielding, fine tasting grape cultivars have already been produced by conventional breeding, yet, some of these cultivars are often seedy, and their market value is low. Seedless cultivars are sold in much higher prices in the export markets as compared to seeded cultivars. A seedless grape cultivar may be sold in a price as high a 5 times as the seeded cultivar on the same export markets. It has been demonstrated that selected genes can be introduced into grapes by molecular transformation, and plants were successfully regenerated. This proposal aims to develop new grapes cultivars which produce seedless fruits. This purpose will be achieved by introducing heterologous lethal genes from other origins which are specifically expressed in developing seeds leading to the destruction of seeds as they develop inside the fruit. The overall aim of this proposal is to examine the feasibility of utilizing such genetic ablation methodologies in order to transform seeded table grapes into seedless, via one gene insertion. The introduction of such new modified cultivars into the market will be relatively easy because the overall properties of the fruit, except its seed bearing, will not be altered. Moreover, no special concern with respect to growth conditions, plant protection and agrotechnical treatment will be required. The main findings of this project are:

1. Improvement of regeneration abilities, transformation efficiencies and the long term maintenance of embryogenic cultures.
2. Transformation of several seeded cultivar of grapes and first analysis of seeded versus seedless fruits of these transformed plants.
3. Cryopreservation techniques and their role in improving transformation efficiencies.

Research Objectives:

The overall objectives of this project were to assist the South African growers and research institution with an up to date technology aiming to improve the quality of a major crop of South Africa. Grape export from South Africa exceed yearly up to 300,000 tons and provide a major income to the industry. The seeded cultivars consisted till now up to 50% of the total cultivars exported. Consumers preference is changing in the recent years to only seedless cultivars and the price for seeded cultivars on the export markets is dropping significantly. On the other hand those seeded cultivars are well know on the markets as well as the horticulture protocols to grow them are well established in South Africa. The general idea of this project is to test the feasibility of using genetic engineering technology to induce seed ablation in the same old and familiar cultivars. It will enable to re-introduce them to the markets as seedless cultivars thus maintaining all their previously well accepted characters.

During the last 10 years several protocols were developed for grape transformation. Although several research group conduct those studies worldwide, grape is till considered a recalcitrant cultivar for transformation (see: Perl and Eshdat 1998; Perl et al 2004) for reviews.

The specific research objectives of this proposal are the following:

1. Improve the efficiency of plant regeneration, grape transformation and the long term maintenance of embryogenic cultures.
2. Produce embryogenic culture of additional commercially important grape cultivars.
3. Study the use of cryopreservation of embryogenic cultures and their effect on transformation efficiency.
4. Construct plant expression vectors that upon transformation into plants will express lethal genes (e.g. *RNase T1; Strepavidin*), under the control of the seed and embryo specific promoter, in the developing seed.
5. Produce transgenic plants that will express the specific lethal genes, only in the developing seeds.
6. Analyze the transgenic plants with respect to their berry and seed development during fruit maturation.

Methods and Results:

Topic 1: Cryopreservation of grapevine (*Vitis vinifera* L.) embryogenic cell suspensions by encapsulation-dehydration and subsequent plant regeneration

Plant materials

Embryogenic cell-lines were developed from anthers of *V. vinifera* L. cv. Red Globe. Methods for anther culture, induction of somatic embryogenesis and the long-term maintenance of the embryogenic calli were previously described. Briefly, embryogenic calli were maintained on MGN medium composed of NN medium supplemented with 18 g/l maltose, 1 g/l casein enzymatic hydrolysate, 4.6 g/l glycerol and 1 mg/l NOA. The pH was adjusted to 6.0 and medium was solidified with 0.26% Gelrite prior to autoclaving at 121 °C and 1.1 kg/cm for 15 min. Embryogenic cell suspensions were established by transferring calli into MGN liquid medium and maintained on a gyratory shaker (90 rpm) at 25 °C in the dark. The cells were weekly subcultured to fresh MGN liquid medium and utilized throughout this study.

Preculture and cryopreservation

Embryogenic cell suspensions were first-step precultured for 2 days in MGN liquid medium supplemented with increasing concentrations of 0.25, 0.5, 0.75 and 1 M sucrose, with 12 h for each concentration. The precultured cells were then suspended in a mixture of 2% (w/v) Na-alginate, 1 M sucrose and 2 M glycerol. The mixture was dropped using a sterile pipette into 0.1 M $CaCl_2$ solution containing 1 M sucrose and 2 M glycerol at room temperature and left for 30 min, to form beads (about 4 mm in diameter). Each bead contained 25% cells. The beads were second-step precultured for 3 days in MGN liquid medium containing 1 M sucrose. The beads were rapidly surface dried by blotting with cellulose tissue, placed on sterilized filter paper in 9-cm Petri dish and dehydrated by air in a laminar-flow chamber at room temperature for up to 10 h. The water content of the beads was measured on a fresh weight basis by drying them in an oven at 80 °C for 48 h. Following dehydration, ten precultured beads were transferred into a 10-ml cryotube and immersed directly in liquid nitrogen for 1 h.

Viability

Cryotubes removed from liquid nitrogen were rapidly thawed in a water bath at 40 °C for 3 min. The beads were post-cultured at 25 °C in the dark in a Petri dish containing 40 ml solidified (0.26% Gelrite) MGN medium supplemented with 2.5 g/l AC. Viability was determined using the triphenyltetrazolium chloride reduction assay (TTC test). Five beads were incubated in 3 ml of 0.6% TTC solution in 0.05 M potassium phosphate buffer (pH 7.5) at 28 °C for 24 h and further treated according to Steponkus and Lanphear. Viability was measured 3 days after post-culture and expressed as the percentage of reduction in TTC activity over control (immediately after encapsulation without any preculture and dehydration).

Regrowth and re-establishment of liquid cultures

Regrowth was determined by measuring fresh weight increase per gram of beads over 30 days of post-culture on MGN solid medium at 25 °C in the dark. Embryogenic cell suspensions were re-established by suspending the beads in MGN liquid medium maintained on a gyratory shaker (90 rpm) at 25 °C in the dark. Subculture was done weekly.

Plant regeneration

Ten beads were placed in a Petri dish containing 40 ml of solidified MG medium, lacking NOA. Cultures were maintained at 25 °C under a 16-h photoperiod with a light intensity of 45 μmol/s per m^2 provided by cool white fluorescent tubes. Within 3 weeks embryos developed and covered the outside surface of the beads. The total number of embryos in 1 g fresh weight of cells (for each treatment) was counted after 4 weeks of post-culture. Embryos were further divided into three different stages of development as described . The percentage of embryo at either globular, heart or torpedo syages was estimated by reference to the total number of embryos counted in each treatment. Single embryos at the torpedo stage, approximately 3–4 mm long, were placed in Magenta GA7 vessels containing 50 ml of solidified (0.26% Gelrite) WPM medium supplemented with 30 g/l sucrose, 0.2 mg/l NAA and 2.5 g/l AC. The culture conditions were the same as for embryo maturation. Subculture was done

every 4 weeks until each plantlet developed a profuse root system and grape-like shoot morphology.

In all experiments, at least ten beads were used in two replicates. Each experiment was repeated twice. Data from two independent experiments were pooled and expressed as the mean±S.E.

Results

Effect of dehydration on viability

Changes in the water content of beads were measured hourly during a 10 h period of dehydration (Fig. 1). The initial water content was 67.7% on a fresh weight basis; it rapidly decreased to 20.6% after 6 h and then gradually dropped to 16.2% after 10 h. The viability of both dehydrated and cryopreserved cells was greatly influenced by the water content of the precultured beads following dehydration periods ranging from 0 to 10 h (Fig. 2). The viability of dehydrated cells was 96% at the beginning of dehydration (0 h); it slowly decreased to 84% as the water content fell from 67.7 to 20.6% and then declined sharply to 42% at a water content of 16.2%, after 10 h of dehydration. The viability of cryopreserved cells increased as the water content of the beads decreased. The highest viability (78%) was recorded with 20.6% water content after 6 h of dehydration, any further dehydration reduced viability.

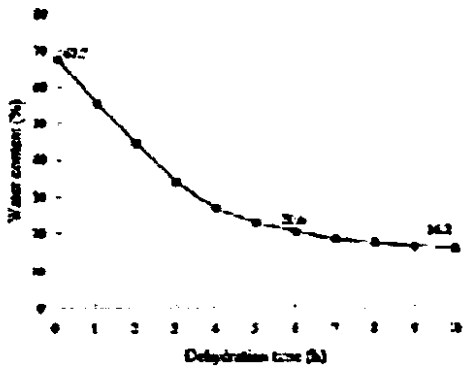


Fig. 1. Changes of the water content of encapsulated cells after 3 days of second-step preculture with 1 M sucrose during dehydration by air drying in a laminar flow chamber.

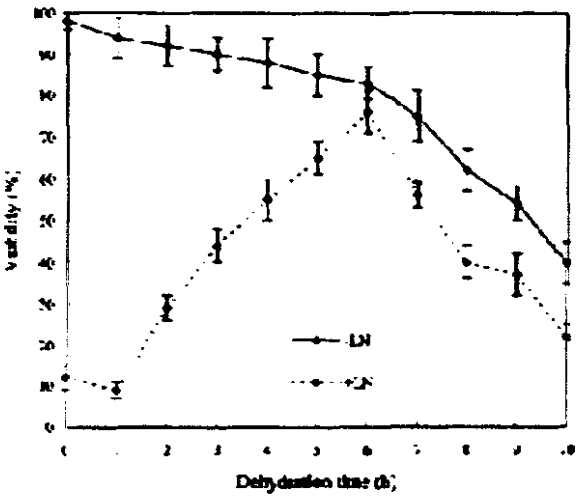


Fig. 2. Effect of dehydration on the viability of encapsulated cells following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Embryogenic cell suspensions were first-step precultured for 2 days on increasing concentrations of 0.25, 0.5, 0.75 and 1 M sucrose, with 12 h for each concentration. Precultured cells were encapsulated and second-step precultured on 1 M sucrose for 3 days. Viability was measured by the TTC test 3 days after post-culture and expressed as the percentage reduction in TTC activity over control (immediately after encapsulation, without preculture and dehydration). Bars represent S.E.

3.2. Effect of preculture on viability

Preculture had a significant effect on the viability of dehydrated and cryopreserved cells (Fig. 3). At the end of the first-step preculture on increasing sucrose concentrations, viability of dehydrated and cryopreserved cells was only 38 and 20%, respectively. An increase in the duration of the second-step preculture promoted the viability of dehydrated and cryopreserved cells. The optimal viability of dehydrated cells was obtained with 2–5 days of preculture, while 2–4 days of preculture gave the best viability of cryopreserved cells.

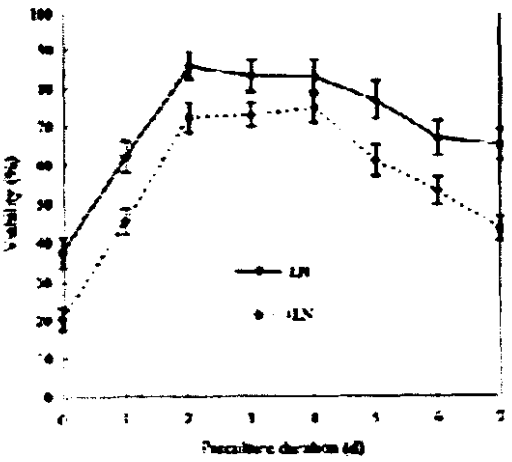


Fig. 3. Effect of preculture duration on the viability of encapsulated cells following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Following the second-step preculture on 1 M sucrose for 0–7 days, encapsulated cells were dehydrated for 6 h. Viability was measured by the TTC test 3 days after post-culture and expressed as the percentage reduction in

TTC activity over control (immediately after encapsulation, without preculture and dehydration). Bars represent S.E.

Effect of post-culture medium on viability

The effect of the post-culture medium composition on the viability of dehydrated and cryopreserved cells was studied in four different media. All liquid media tested greatly reduced viability compared with solid media (Fig. 4). The addition of AC to the solid post-culture medium promoted the viability of cryopreserved cells. AC was also found to promote the viability of dehydrated cells but this effect was less significant compared to solid medium devoid of AC.

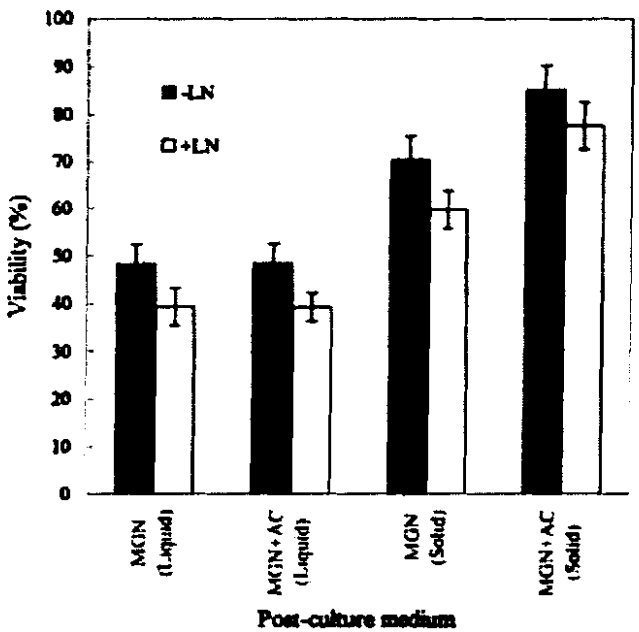


Fig. 4. Effect of post-culture medium on the viability of encapsulated cells following dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Following the second-step preculture on 1 M sucrose for 3 days, encapsulated cells were dehydrated for 6 h. Viability was measured by the TTC test 3 days after post-culture and expressed as the percentage reduction in TTC activity over control (immediately after encapsulation, without preculture and dehydration). Bars represent S.E.

Regrowth and re-establishment of liquid cultures

Regrowth of dehydrated and control cells began much earlier and proceeded faster than the cryopreserved cells (Fig. 5); a 5-day lag phase was found in regrowth of cryopreserved cells. The increase in the fresh weight of cryopreserved cells was only half compared to control cells 30 days after regrowth. Following regrowth, cell suspensions were re-established by transferring the cells to MGN liquid medium. The

growth pattern of cell suspensions obtained from cryopreserved cells was similar to control cells after two subcultures (data not shown).

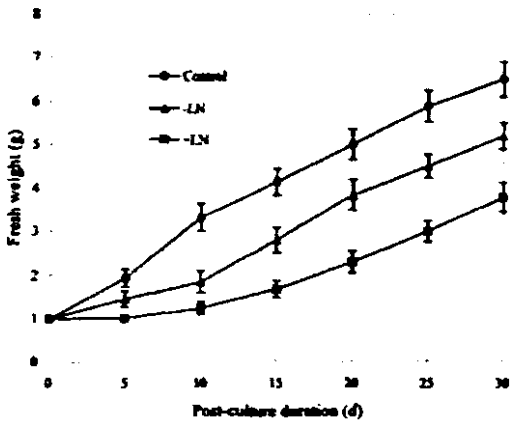


Fig. 5. Comparison of regrowth of encapsulated cells following encapsulation (control), dehydration alone (-LN) and freezing in liquid nitrogen (+LN). Regrowth was determined by measuring the increase in fresh weight per gram of beads over 30 days of post-culture on MGN solid medium. Bars represent S.E.

Plant regeneration

The embryogenic potential of cryopreserved cells was studied by culturing them on MG solid medium. Cryopreserved cells were found to have an improved regeneration potential compared to dehydrated and control cells (Fig. 6). Cryopreserved cells regenerated embryos much earlier and produced many more embryos at the various developmental stages than dehydrated and control cells (Fig. 6, Table 1). Dehydration treatment was also found to promote embryo regeneration compared to control but with a lower efficiency than cryopreservation. The number of embryos developed following the dehydration treatment exceeded the control by more than 4-fold. Cryopreservation enabled a 7-fold increase in the number of regenerated embryos compared with control. While no embryos at the torpedo stage were developed from control cell, 16 and 29% of embryos at this stage were developed from dehydrated and cryopreserved cells respectively (Table 1). Embryos at torpedo stage (4 mm) that were transferred to plant regeneration medium started to root within 4 weeks. A profuse root system and normal grape-like shoot morphology were visible 8–10 weeks later. No visible differences in leaf morphology could be observed between plants regenerated from cryopreserved cells and those from control cells (data not shown).

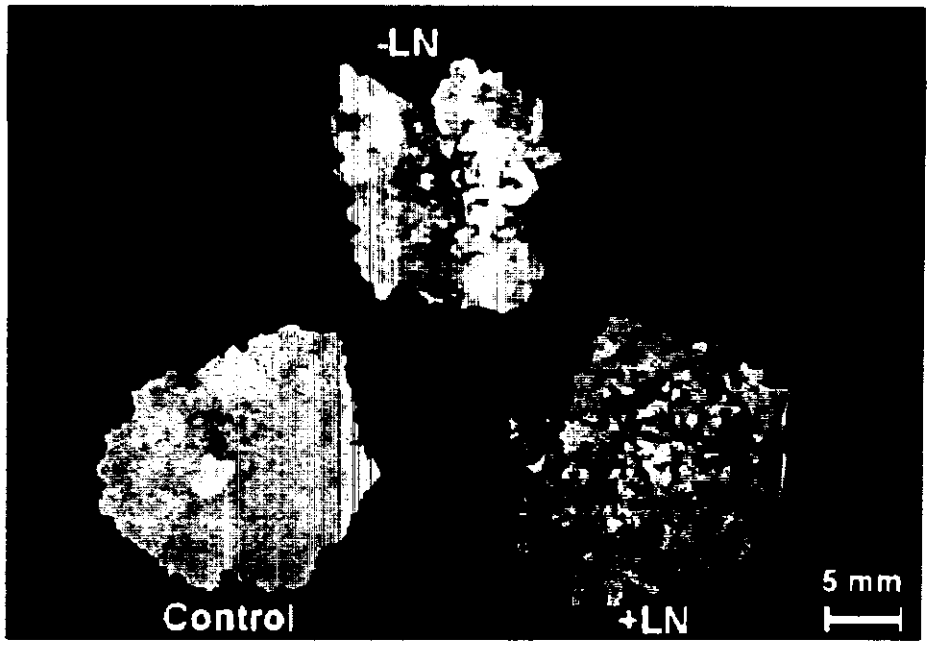


Fig. 6. Embryo development of encapsulated cells following encapsulation (control), dehydration alone (-LN) and freezing in liquid nitrogen (+LN) 4 weeks after post-culture on MG solid medium.

Table 1. Proportion of different embyogenic stages produced by encapsulated cells following encapsulation (control), dehydration alone (-LN) and freezing in liquid nitrogen (+LN)

Treatment	Stages (%)			Total no. embryos
	Globular	Heart	Torpedo	
Control	88 ± 7	12 ± 3	0	106
-LN	62 ± 8	22 ± 4	16 ± 2	428
+LN	40 ± 6	31 ± 5	29 ± 4	785

The total number of embryos in 1 g fresh weight of cells for each treatment was counted 4 weeks after post-culture on MG solid medium. Proportion of different embryogenic stages was presented as the mean±S.E.

Topic 2: Cryopreservation of grapevine (*Vitis* spp.) embryogenic cell suspensions by encapsulation-vitrification

Plant materials

The rootstock 110 Ritcher (*V. berlandieri x V. rupestris*) was used for optimization of the process parameters during encapsulation-vitrification procedure. Another rootstock (41B, *V. vinifera x V. berlandieri*) and four cultivars of *V. vinifera* (Red globe, Sugarone, Gammy, Chardonay) were also used in these experiments. A long-

term somatic embryogenic callus culture was developed from the vegetative tissues of anthers of *Vitis* spp. Methods for anther culture, induction of somatic embryogensis and maintenance of embryogenic cultures were described previously. Briefly, embryogenic calli were maintained on MGN medium, which comprised NN medium supplemented with 18 g l^{-1} maltose, 1 g l^{-1} casein enzymatic hydrolysate, 4.6 g l^{-1} glycerol and 1mg l^{-1} NOA. The pH was adjusted to 6.0 and media were solidified with 2.6 g l^{-1} Gelrite prior to autoclaving at 121°C and 1.1 kg cm^{-2} for 15 min. Embryogenic cell suspensions were established by transferring the calli to MGN liquid medium and maintained on a gyratory shaker (90 rpm) at 25 °C in the dark. Cells were subcultured weekly on fresh MGN medium. Cell suspensions at this stage were used throughout this study.

Encapsulation-vitrification and cryopreservation

Unless stated otherwsie, cell suspensions were precultured for 3 days in MGN liquid medium supplemented with increasing concentrations of 0.25, 0.5, and 0.75 M sucrose, with 1 day for each concentration.

In order to determine the effect of different final sucrose concentrations in preculture medium on tolerance of cell suspensions to dehytration and subsequent freezing, four sucrose concentrations (0.25, 0.5, 0.75 and 1 M) were tested, with each being the final concentration. The cell suspensions were precultured with an initial sucrose concentration of 0.25 M, which was raised in 1 day step of 0.25 M until the designated final sucrose concentration was reached. In each concentration treatment, preculture was continued at the final concentration, to a total preculturing time of 4 days. Thus, the cells treated with a final sucrose concentration of 0.25 M were exposed to 0.25 M sucrose for 4 days, while those designated for 0.5 M spent 1 day at 0.25 M and 3 days at 0.5 M, and so on. Cell suspensions precultured without any sucrose (0 M) were used as controls.

Following preculture, the cells were suspended in MGN medium containing 2.5% (w/v) Na-alginate, and 0.4 M sucrose. The mixture was dripped with a sterile pipette into 0.1 M $CaCl_2$ solution containing 0.4 M sucrose at room temperature and left for 20 min, to form beads (about 4 mm in diameter), each bead containing 25% cells. The beads were then rapidly surface dried by blotting with cellulose tissue and dehydrated with PVS2 solution (Sakai et al. 1990) at 0 °C for 270 min prior to

direct immersion in LN for 1 h. PVS2 solution contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO in 0.4 M sucrose solution (pH 5.8). After dehydration, the beads were rapidly surface-dried by blotting with cellulose tissue and then 10 beads were transferred into a 10-ml cryotube and immersed directly in LN for 1 h.

Viability

On removal from LN, the cryotubes were rapidly thawed for 3 min in a water bath at 40 °C. Ten beads were then post-cultured at 25 °C in the dark in each 9-cm Petri dish containing 40 ml of MGN solid medium supplemented with 2.5 gl^{-1}AC for viability assessment. Viability was estimated by means of a triphenyltetrazolium chloride reduction assay (TTC test). Five beads were incubated in 3 ml of 0.6% TTC solution in 0.05 M potassium phosphate buffer (pH 7.5) at 28 °C for 24 h and were then further treated according to Steponkus and Lanphear (1967). Viability was measured 3 days after post-culture and expressed as the percentage reduction in TTC activity over control (immediately after encapsulation without any preculture and dehydration).

Regrowth

After viability assessment, ten beads were transferred to each 9-cm Petri dish containing 40 ml of MGN solid medium and kept at 25 °C in the dark for regrowth, which was determined by measuring the fresh weight increase per gram of beads over 30 days of post-culture (Wang et al. 2002). Embryogenic cell suspensions were re-established by suspending the beads in MGN liquid medium maintained on a gyratory shaker (90 rpm) at 25 °C in the dark. Subculture was done weekly.

Plant regeneration

Surviving cells were transferred to a 9-cm Petri dish containing 40 ml of solidified MG medium., without NOA for embryo development (Wang et al. 2002). Cultures were maintained at 25 °C under a 16-h photoperiod with a light intensity of 45 □mol s^{-1} m^{-2} provided by cool white fluorescent tubes. About 4 weeks later, individual embryos at torpedo stage, approximately 3-4 mm long, were placed in Magenta GA7 vessels containing 50 ml of solidified (2.6 gl^{-1} Gelrite) WPM medium supplemented with 30 gl^{-1} sucrose, 0.2 mgl^{-1} NAA and 2.5 gl^{-1} AC. The

culture conditions were the same as for embryo maturation. Subculture was done every 4 weeks.

Biochemical analysis

At the end of preculture and before encapsulation, cell suspensions precultured with the various final sucrose concentration were collected for analysis of total soluble sugar and total soluble protein contents. Precultured cells were quickly rinsed three times in a solution of 0.8 M polyethlene glycol (PEG) 4000 (pH 5.8) and drained on filter paper (Jitsuyama et al. 2002). Total soluble sugar was extracted and measured. The drained cells were dried in an oven at 60 °C for 48 h, and then ground to a fine powder with a pestle and mortar. The dried powder (100 mg) of each sample was extracted with 10 ml of 80% ethanol in a water bath at 70 °C for 1 h. The ethanol extracts were collected in a plastic tube and the tissues were extracted twice more with 5 ml of 80% ethanol in a water bath at 70 °C for 45 min each time. The ethanol extracts were combined and evaporated dryness at 60 °C, and then 3 ml of double-distilled water was added to the percipitate in each tube, followed by centrifugation at 10,000 rpm for 10 min. The supernatants were filtered through a 0.45-□m HPLC certified syringe filters for measurements of the soluble sugars in an HPLC 360 Autosampler.

Total soluble protein was determined according to Bradford. Drained cells (300 mg FW) were ground with a pestle and mortar and extracted with 3 ml of 50 mM phosphate buffer (pH 7.0), followed by centrifugation at 10,000 rpm for 10 min. The supernatants were collected for determination of protein content at 595 nm in a Cary 1E spectrophotometer with bovine serum albumin (BSA) as a standard.

In all experiments, at least 10 beads were used in each of three replicates. Each experiment was repeated twice. Data from two independent experiments were pooled and expressed as the mean ± standard error.

Results

Effect of loading solution on viability

The effect of a loading solution containing 2 M glycerol and 0.4 M sucrose on osmotolerance of precultured cells to PVS2 was tested. Encapsulated cells were osmoprotected with a loading solution at 24 °C for perods ranging from 0 to 120

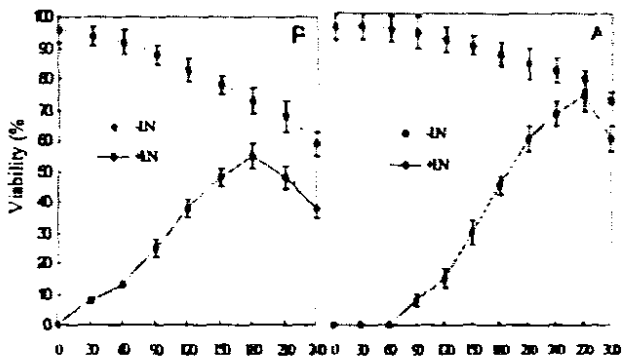
min, prior to dehydration by PVS2 for 270 min at 0 °C. The loading solution treatment did not influence viability, which was about 85 or 76%, respectively, for cells that were dehydrated alone (-LN) or cryopreserved (+LN), regardless of the loading treatment time. Therefore, direct dehydration of encapsulated cells with PVS2 was used in the present study.

In vitrification-based cryopreservation procedures, cells have to be dehydrated by exposure to a highly concentrated solution such as PVS2, prior to immersion in LN. However, the direct exposure of tissues to PVS2 without osmoprotection caused harmful effects, because of osmotic stress or chemical toxicity, which has been described as a mojar factor in determining the success of cryopreservation of cells by vitrification and a loading treatment was deemed essential.

Effect of dehydration on viability

We studied the effects of the duration of dehydration with PVS2 at both 0 and 24 °C on viability of cryopreserved cells. Dehydration time and exposure temperature significantly affected viability. At 24 °C, the viability of encapsulated cells (-LN) increased sharply with increasing dehydration time (Fig. 1A). The highest viability (58%) of cryopreserved cells (+LN) was achieved following 180 min of dehydration. At 0 °C, the viability of dehydrated cells (-LN) decreased gradually with increasing dehydration time; survival of cryopreserved cells (+LN) started to increase after 60 min of dehydration and reached a maxium (76%) after 270 min of dehydration.

The incubation time and temperature of the vitification solution are the two important factors affecting the survival of cryopreserved cells. It was previously shown that over- exposure to the vitrification solution can cause chemical toxicity and excessive osmotic stress. The optimal time of exposure to PVS2 varies among plant species, and also depends on the temperature during exposure. In the present study, the optimal exposure time to PVS2 at 24 °C was 180 min. compared with that at 25 °C, dehydration at 0 °C usually led to higher viability, and also the incubation time was largely extended, which gave much more flexibility for handling a large number of samples at the same time. The penetration of the vitrification solution to cells was reported to be faster at 24 °C than at 0 °C, and the fast penetration may damage the cell membrane, causing leakage and resulting in the loss of vital components and subsequent poor viability.

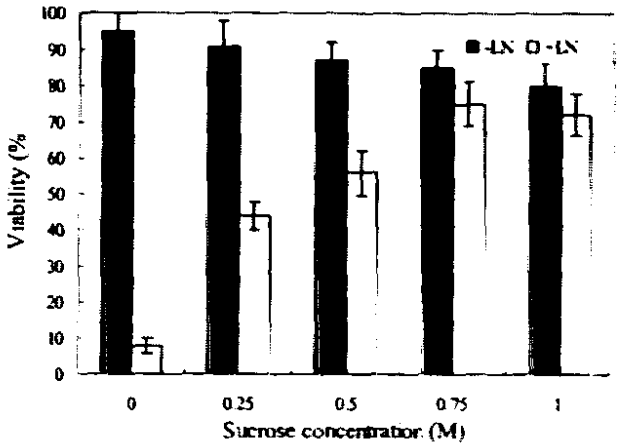


Effect of sucrose concentration in preculture medium on viability

The concentration of sucrose in the preculture medium exerted a strong effect on the viability of cells that were dehydrated (-LN) and cryopreserved (+LN) (Fig. 2). The viability of dehydrated cells (-LN) decreased with increasing sucrose concentration in the preculture medium, whereas that of cryopreserved cells (+LN) increased as the sucrose concentration increased with an optimal viability (76%) achieved at 0.75 M sucrose.

In the preliminary studies, we further investigated the effect of the duration (ranging from from 1 to 7 days) of preculture with 0.75 M sucrose on the viability of cryopreserved cells. The results showed that the viability of cryopreserved cells remained fairly constant (at about 76%) as the preculture time increased from 1 to 4 days, and then started to decreased. Therefore, a preculture treatment with 0.75 M

sucrose for a total time of 3 days used in all the following experiments. The level of total soluble protein of precultured cells increased as the sucrose concentrations in the preculture medium increased from 0 to 1 M, but there was no significant difference between 0.75 and 1 M (Fig. 3). A prerequisite for successful cryopreservation is induction of a high level of tolerance to dehydration and subsequent freezing. Recently, sucrose has been widely reported to be the most frequently used sugar in cryopreservation techniques to establish this tolerance. The intracellular sucrose and fructose contents of precultured cells significantly increased as the sucrose concentration in the preculture medium increased, up to 1 M (Fig. 4A and B). The glucose level remained unchanged, regardless of the sucrose concentrations in the preculture medium (Fig. 4C). Cold acclimaton has widely been used to enhance survival of cryopreserved shoot tips and cell. We hypothesize that sugar incorporation is not the only factor involved in enhancing the freezing tolerance of cell suspensions, as was previously suggested.



Regrowth

Regrowth of control cells began much earlier and proceeded faster than that of cryopreserved cells; a 5-day lag phase was found in regrowth of cryopreserved cells. This is consistent with previous findings that cryopreserved cells were slower in their regrowth, generally having 3- to 5-days lag time. The general morphology of cells recovered from cryopreservation were similar to that of control cells (Fig. 6). The increase in the fresh weight of cryopreserved cells (+LN) was about 52% of that of control cells 30 days after regrowth. Following regrowth, cell suspensions were re-established by transferring the cells to MGN liquid medium. The growth pattern of cell suspensions obtained from cryopreserved cells was the same as that of control cells after two subcultures (data not shown). The results reported here were consistant with those of Wang et al. (2002) for *Vitis vinifera* L. cv. Red Globe.

Plant regeneration

About 2 weeks after post-cultured on solid MG medium, cryopreserved cells started to develop embryos. Within 3 weeks the embryos covered the outside surfaces of the beads (data not shown). Embryos at torpedo stage (3-4 mm) that were transferred to plant regeneration medium started to root in about 2 weeks. A profuse root system and normal grape-like shoot structure formed 8 weeks later. Leaf morphologies of plants regenerated from cryopreserved cells were similar to those of plants from control cells (data not shown). The pattern of plant regeneration reported from the present study was similar to that reported for *Vitis vinifera* cv. Red Globe (Wang et al. (2002).

Cryopreservation of Vitis rootstocks and cultivars

The protocol developed during the present study was applied to cryopreservation of another rootstock B41 (*V. vinifera x V. Berlandieri*)) and four cultivars (*Vitis vinifera* L.) (Table 1). All of them survived freezing in LN with the highest (82%) and lowest viability (42%) achieved for cv. Red Globe and rootstock B41, respectively. Although they differed in their survival rates, cryopreserved cells of all the rootstocks and cultivars tested were able to resume regrowth, albeit with different regrowth indexes, and to be re-established in cell suspension cultures.

To the best of our knowledge, this is the first report of successful cryopreservation of cell suspensions of grapevine by encapsulation-vitrification. With the optimized parameters reported here, 76% survival of cryopreserved cell suspensions (Ritcher 110) was achieved. The cryopreserved cells retained their potential for regrowth and for embryo regeneration and development.

In conclusion, we have developed a general, simple and highly efficient method for cryopreservation of cell suspensions of a genetically wide range of commercial grapevine rootstocks and scion cultivars. The protocol reported in the present study may pave the way for a routine, highly efficient and synchronous method for cryopreservation of cell suspensions of *Vitis* spp.

Table 1. Cryopreservation of cell suspensions of several *Vitis* cultivars and rootstocks by encapsulation-vitrification. Embryogenic cell suspensions were precultured for 3 days on increasing concentration of 0.25, 0.5 and 0.75 M sucrose, with 1 day for each concentration. Precultured cells were encapsulated and dehydrated with PVS2 at 0 °C for

270 min prior to direct immerison in liquid nitrogen. Ten samples were used in each of three replicates. Each experiment was repeated twice. Data from two independent experiments were pooled and expressed as means ± standard error.

Cultivar	Viability [1] (%)	Regrowth index[2]
V. berlandieri x *V. rupestris*		
110 Ritcher	76 ± 7	0.52 ± 0.06
V. vinifera		
Red Globe	82 ± 6	0.60 ± 0.08
Superior Seedless	66 ± 5	0.48 ± 0.05
Chardonay	53 ± 6	0.44 ± 0.05
Gammy	46 ± 5	0.40 ± 0.04
V. vinifera x *V. Berlandieri*		
B41	42 ± 5	0.32 ± 0.04

[1]Viability was measured by the TTC test and expressed as the percentage reduction in TTC activity over control (immediately encapsulation, without preculture and dehydration).

[2]Regrowth index was calculated as:

$$\frac{\text{fresh weight of cryopreserved cells at day 30} - \text{fresh weight of cryopreserved cells at day 0}}{\text{fresh weight of control cells at day 30} - \text{fresh weight of control cells at day 0}}$$

Topic 3:

***Agrobacterium*-mediated transformation of *Vitis vinifera* L. cell suspensions: improvement of transformation efficiency and transgenic plant regeneration**

Materials and methods

Initiation and maintenance of embryogenic cell suspensions

Embryogenic cultures were developed from anthers of *Vitis vinifera* L. cv. Red Globe. Methods for anther culture, induction of somatic embryogensis and maintenance of embryogenic cultures were described previously.Briefly, embryogenic calli were maintained on MGN medium composed of NN medium supplemented with 18 g/l maltose, 1 g/l casein enzymatic hydrolysate, 4.6 g/l glycerol and 1mg/l 2-naphthoxyacetic acid (NOA). The pH was adjusted to 6.0 and media were solidified with 0.26% Gelrite prior to autoclaving at 121°C and 1.1 kg cm^{-2} for 15 min. Embryogenic cell suspensions were established by transferring the calli to MGN liquid medium and maintained on a gyratory shaker (90 rpm) at 25°C in the dark. Cell suspensions were weekly subcultured on fresh MGN medium.

Cryopreservation of embryogenic cell suspensions

Embryogenic cell suspensions were cryopreserved by encapsulation-dehydration procedure, as decribed in detail by Wang et al. (2002). Cell suspensions were first-step precultured for 2 days in MGN liquid medium supplemented with increasing concentrations of 0.25, 0.5, 0.75 and 1 M sucrose, with 12 h for each concentration. The precultured cells were encapsulated and were then second-step precultured for 3 days in MGN liquid medium containing 1 M sucrose. Following preculture, the beads were rapidly surface-dried and dehydrated by air drying in a laminar-flow chamber at room temperature and humidity for 6 h prior to direct immersion in liquid nitrogen for 1 h. Beads removed from liquid nitrogen were rapidly thawed in a water bath at 40°C for 3 min and were then post-cultured at 27°C in the dark in a Petri dish containing 40 ml of MGN solid medium supplemented with 2.5g/l activated charcoal (AC) for survival and regorwth. After 30 days of post-culture, embryogenic cell suspensions were re-established by suspending the beads in MGN liquid medium maintained on a gyratory shaker (90 rpm) at 25°C in the dark. Subculture was carried out weekly. After 2-3 times of subculture, cryopreserved cell suspensions showed the same growth pattern as that of control cells and used for transformation experiments.

Transformation and plant regeneration

The pCAMBIA 2301 binary vector carrying the reporter b-glucuronidase (GUS) gene and the neomycin phosphotransferase II (*npt* II) gene Both under the control of the 35S promoter was introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation and used for transformation experiments

Agrobacterium cultures were grown overnight on a gyratory shaker (100 rpm) at 28°C in the dark in 50 ml of YEP medium supplemented with 50 mg/l kanamycin and 15 mg/l rifampicin, pH=7.2. The cultures were centrifuged at 5000 rpm for 10 min and resuspended in 50 ml of induction medium composed of YEP supplemented with 100 uM acetosyringone, pH=5.2. After additional 2 h of incubation at 28° C (100) rpm, bacteria were centrifuged at 5000 rpm for 10 min and resuspended to OD_{630} = 0.6 with NN medium. Embryogenic calli (1 g fresh weight) were inoculated with 20 ml of bacteria for 10 min at 25° C. Calli were dry-blotted and transferred onto solid NN

medium containing 100□M acetosyringone for co-cultivation at 25° C in the dark for 48 h. Calli were washed 3 times in 50 ml of NN medium containing 300 □g/ml cefotaxim to remove bacteria. Calli were transferred to liquid selection medium composed of MG medium supplemented with 300 mg/l cefotaxim and kanamycin or paromomycin. In case paromomycin was used, it was added stepwise to the cultures from 5, 10, 15 to a final concentration of 20 mg/l, with 2 days for each of the first three concentrations, and then maintained at 20 mg/l paromomycin for 5 weeks (Mauro et al., 1995), to select antibiotic-resistant transformed cells . Non-transformed cells were grown in the same medium with or without paromomycin as a control to verify the efficiency of the antibiotic selection. Calli during the selection stage were maintained at 25° C in the dark with shaking at 100 rpm. Subculture was performed every 4 days.

Survived calli and somatic embryos, which developed on calli cultured on the selection medium, were transferred separately to solid germination medium composed of MG supplemented with 300 mg/l cefotaxim and 50 mg/l paromomycin. Following 5-8 weeks of culture on the germination medium, green embryos at torpedo stage (about 4 mm) were cultured on rooting medium composed of woody plant medium supplemented with 30 g/l sucrose, 0.1 mg/l naphthalene acetic acid (NAA), 2.5 g/l activated charcoal (AC) and 300 mg/l cefotaxim. The plant during germination and rooting stages were kept at 25° C under a 16-h photoperiod with a light intensity of 45 □mol s^{-1} m^{-2} provided by cool white fluorescent tubes. Subculture was performed every two weeks. Normal-looking grape plantlets with profuse root system formed 8 weeks after culturing on the rooting medium.

Testing of transgenic plants

Transformation efficiency was confirmed by histochemical GUS assay. Tissues were vacuum infiltrated with a 5-bromo-4-chloro-3-glucuronic acid buffer for 10 min and then incubated at 37° C overnight. After staining, tissues were cleared in 70% ethanol and stored in 90% ethanol. Transformation efficiency was assessed by recording GUS expression in calli and embryos at the end of selection stage. The results are presented as the percentage of GUS-positive tissues per 100 mg cells.

After rooting and transfer to WMP without cefotaxin for micropropagation, the presence of the *npt*II gene in plantlets was detected by Southern blots. DNA was extracted using DNAzol ES (Molecular Research Center, Inc., Cincinnati, OH) from the putatively transformed and nontransformed plantlets.

Experimental design

To determine the toxic effect of kanamycin and paromomycin concentrations on cell suspensions, 4 different concentrations of kanamycin (40, 60, 80 and 100 mg/l) and paromomycin (10, 15, 20 and 25 mg/l) were added to cell suspension cultures. The growth rate of cell suspensions was measured for each antibiotic.

From the first experiment, it was found that paromomycin at 10 mg/l was effective in killing cells within a few days of culture. Therefore, an experiment was conducted to further test the effect of different paromomycin concentrations (10, 15, 20 and 25) on selection of antibiotic resistant cells and embryo formation. Selection was started by adding 5 mg/l paromomycin to the selectine medium and then the concentation increased at 5 mg/l every 2 days until a desired concentration was reached. The period of the selective stage lasted for 5 weeks

The effects of kanamycin and paromomycin on tranformation efficiency were compared. In this experiment, 80 mg/l kanamycin were consistantly used during the whole selective stage (for 5 weeks), When paromomycin was used conce as described in Transformation and plant regeneration above. The period of the selective stage was 5 weeks.

Finally, cryopreserved cell suspensions were used as the target tissues for transformation. In this experiment, non-cryopreserved cells were used as the control. Paromomycin at 20 mg/l was used as the selective agent, as described in the stable transformation.

Results

Toxic effects of kanamycin and paromomycin concentrations on embryogenic cell suspensions

Fig. 1 shows the sensitivity of untransformed embryogenic cell suspensions of *Vitis vinifera* L. cv. Red Globe to kanamycin and paromomycin concentrations. Cell suspensions treated with 60, 80 and 100 mg/l kanamycin stopped growing after 6 days of culture and turned totally browning after 8 to 10 days of culture. Therefore, a kanamycin concentration of 80 mg/l was chosen in our transformation experiment. Compared with kanamycin, killing effect of paromomycin on cell suspensions was much faster. With a paromomycin concentration as low as 10 mg/l, cell suspensions stoped growing after 3 days of culture and totally died after only 5 to 6 days of culture. Based on the above data, effect of paromomycin concentrations in a range of 10 to 25 mg/l on transformation efficiency was further studied in the following experiment.

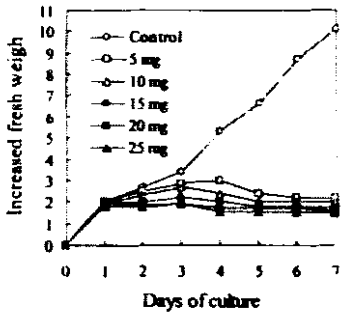


Fig. 1. Effect of paromomycin concentrations on growth of cell suspensions of *Vitis vinifera* L. cv. Red Globe.

Effect of paromomycin concentrations on transformation efficiency

Effect of paromomycin concentrations in the range of 10 to 25 mg/l on transfromation efficiency of cell suspensions of *Vitis vinifera* L. cv. Red Globe was documented in Table 1. The number of cell clusters survived on selection medium decreased, while the number of embryos developed on selection medium increased, as paromomycin concentration increased from 10 to 20 mg/l. A further increase in this antibiotic concentration did not influence these two parameters. Transformation efficiency, determined by percentage of GUS-positive cell clusters and embryos, was markedly increased as paromomycin concentration increased with a maxium, 62 and 70% for cell clusters and embryos obtained, respectively, when 20 mg/l paromomycin was used for selection.

Table 1. Effect of paromomycin concentrations on transformation efficiency of embryogenic cells of *Vitis vinifera* L. cv. Red Globe.

Paromomycin concentrations (mg/l)	Number of cell clusters survived on selection medium/100 mg cells	% of GUS-positive cell clusters	Number of embryos developed on selection medium/100 mg cells	% of GUS-positive embroys
5	123	17	5	30
10	115	26	8	45
15	103	42	18	61
20	90	62	25	70
25	85	60	28	68

A comparison of kanamycin and paromomycin in transformation efficiency

In this experiment, 80 mg/l kanamycin and 20 mg/l paromomycin were compared (Table 2). Although the number of cell clusters survived in kanamycin-containing medium were nearly twice of those selected by paromomycin, the number of embryos and transformation efficiency, determined by percentage of GUS-positive cell clusters and embryos, were significantly higher with paromomycin than with kanamycin as the selective agent.

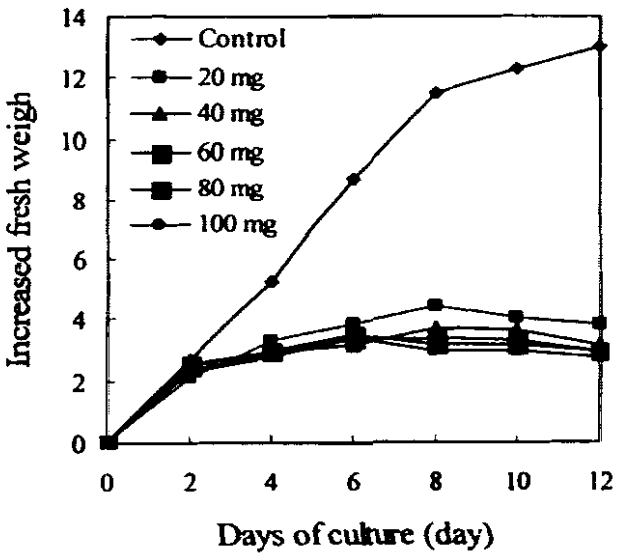


Fig. 2. Effect of kanamycin concentrations on growth of cell suspensions of Vitis vinifera L. cv. Red Globe.

Table 2. A comparison of kanamycin and paromomycin on transformation efficiency of embryogenic cells of *Vitis vinifera* L. cv. Red Globe.

Antibiotics	Number of cell clusters survived on selection medium/100 mg cells	% of GUS-positive cell clusters	Number of embryos developed on selection medium/100 mg cells	% of GUS-positive embryos
Kanamycin (80 mg/l)	155	42	8	45
Paromomycin (20 mg/l)	85	61	27	71

Effect of transformation of cryopreserved cell suspensions on transformation efficiency

Table 3 summarized the effect of transformation of cryopreserved cell suspensions on tranformation efficiency. There were no differences in the number of cell clusters survived on the selection medium and transformation efficiency when cryopreserved cell suspensions were used for transformation, compared with non-cryopreserved cells (control). However, the number of embryos developed from cryopreserved cell suspensions on selection medium were nearly three times of those from non-cryopreserved ones.

Table 3. Effect of cryopreserved embryogenic cells on transformation efficiency of *Vitis vinifera* L. cv. Red Globe.

Cell types	Number of cell clusters survived on selection medium/100 mg cells	% of GUS-positive cell clusters	Number of embryos developed on selection medium/100 mg cells	% of GUS-positve embryos
Non-cryopreserved cells (control)	85	58	30	75
Cryopreserved cells	83	55	85	80

Effect of transformation of cryopreserved cell suspensions on their conversions to embryos and plantlets

The number of embryos developed from transformed cryopreserved cells on germination medium were significantly higher than those from non-cryopreserved cells (Table 4). Also, the embryos produced from transformed cryopreserved cells retained their much greater ability to convert to plantlets than those from non-cryopreserved cells, when transferred on rooting medium.

Table 4. Effect of transformation of cryopreserved embryogenic cells of *Vitis vinifera* L. cv. Red Globe on their conversions to embryos and plantlets.

Cell types	Number of embryos developed on germination medium/100 mg cells	Number of plantlets regenerated on rooting medium/100 embryos
Non-cryopreserved cells (control)	85	25
Cryopreserved cells	150	40

Analysis of the putative transformants

At the end of selection in the presence of 20 mg/l paromomycin, calli and embryos, which developed from calli under selection, were use for GUS staining. A strong uniform blue color reaction was produced in tissues developed from paromomycin resistant embryogenic calli. Tissues from non-transformed embryogenic cultures stained negatively.

Analysis of the leaves of GUS-positive transgenic plantlets by PCR revealed amplified fragments coinciding with the GVA CP gene, while no band was detected in non-transformed plantlets.

Topic 4:

Induced seedlessness in two commercial seeded grape cultivars

To produce transgenic grapes that express a lethal gene under the control of a seed specific promoter. The toxic gene product will interfere with the normal development of the seed, leading consequently to seed abortion and seedllesness.

Material and methods

Engineering of expression vectors for *Agrobacterium* - mediated transformation of grape

1. Promoters:

Our genes were placed under the control of two different promoters, assumed to confer seed specific expression,

- A seed coat specific promoter, obtained from Dr. Brain Miki and used in the first year of the project.

- An ovary specific promoter. We have further used the ovary specific promoter DefH9.

2. Suicide genes

a. The vector containing the *RNase* gene, as a lethal gene, under the control of a seed coat specific promoter. We have further cloned this gene also under the control of an ovary specific promoter DefH9.

b. We have used the avidin gene as a new potential lethal gene. Avidin is known to bind biotin, an important vitamin required for normal cell metabolism. When trying to overexpress avidin using organ specific promoter, tissue ablation was observed. As an alternative approach for seedlessness, we assumed that expression of avidin under the previously mentioned seed specific promoter may lead to seed ablation and seedlessness.

The engineered cassettes were then removed from the modified pUC derived vector and cloned into a PZP binary vector. The latter vector was then mobilized into the EHA 105 *Agrobacterium* strain.

Plant material

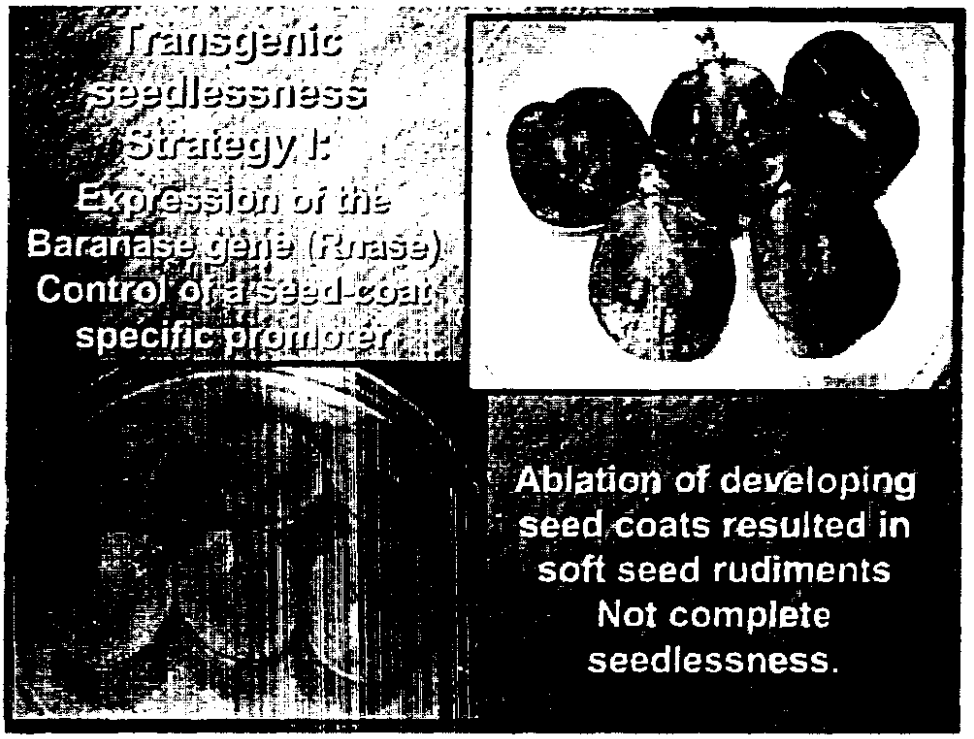
Transformation studies were carried out in Israel using the important grape cultivar "Red Globe" and 529 – a local seeded Muscat selection. Embryogenic cultures of Red Globe were produced in our laboratory.

Transformation of tomato

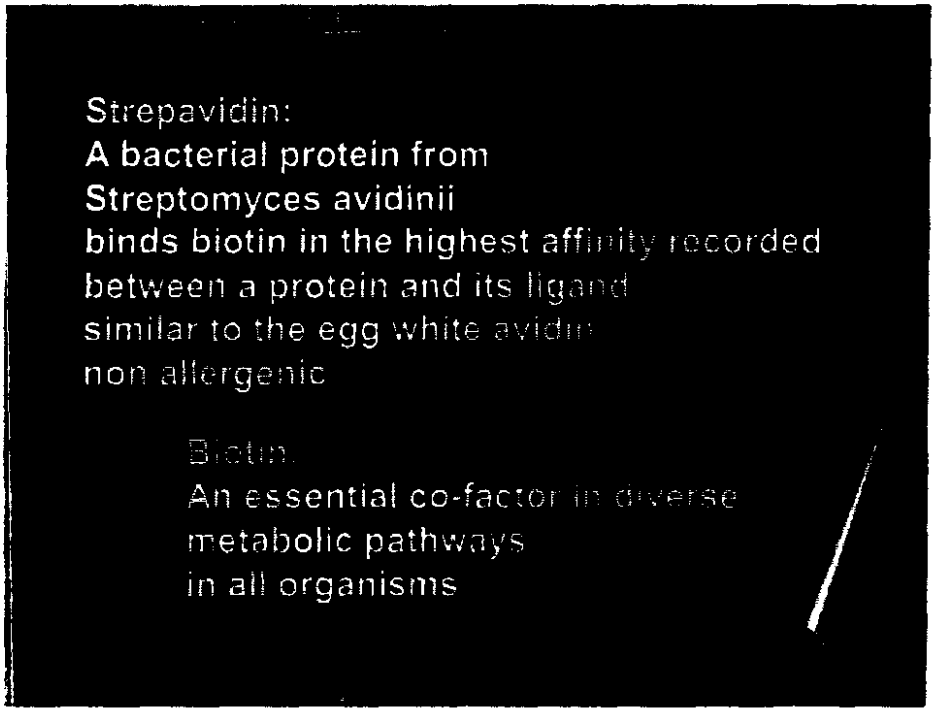
Since we assumed that the evaluation of grape berries will take place beyond the scope of this project, we tested the feasibility of our hypothesis as well as our recently prepared construct - in tomatoes. Tomatoes were transformed using the Seed-Coat - RNase construct. Transgenic plants were transferred to the greenhouse and set fruits. Mature fruits were cut and in order to study seed ablation. Out of 12 independent transgenic plants, 9 were totally seedless while 3 had few seeds within the fruits. These promising data supported our hypothesis that a functional *RNase* gene under the control of a seed specific promoter may induce transgenic seedlessness.

Transformation of Grape

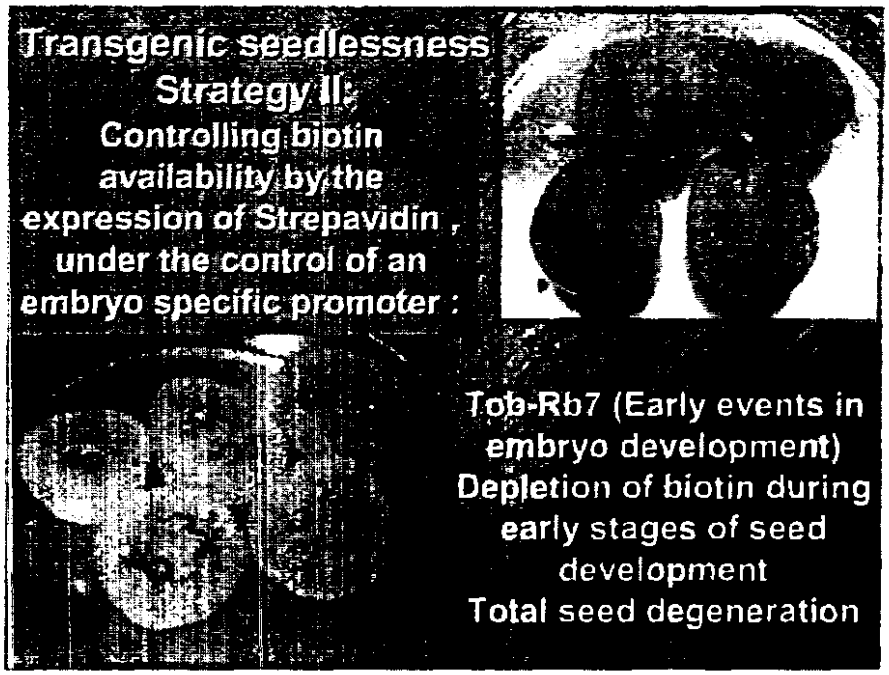
We have transformed the cultivar Red-Globe; a red seeded worldwide known cultivar. Transgenic plants were produced and evaluated in the field till fruit set. These plants carried the Rnase gene under the control of the seed coat specific promoter. Upon fruit set we have analyzed fruit set in 5 different transgenic lines of Red Globe. One plant had normally looking seeded berries. It may indicate that it was not transformed or gene expression was not able to confer seedlessness. On the other hand, the 4 additional plants had completely normal looking berries. We were happy to find out that all of them were seedless and contained only very small seed rudiments. Control untransformed plants had fully developed seeds (Fig. 1).



We have transformed the cultivar 529; a white seeded Muscat cultivar. Transgenic plants were produced and evaluated in the field till fruit set. These plants carried the Strepavidin gene under the control of the Tob-Rb7 seed specific promoter. The advantages of Strepavidin in blocking biotin and inducing cell death is exemplified in Fig. 2.



Upon fruit set we have analyzed fruit set in 8 different transgenic lines of 529. We were happy to find out that all of them were seedless and contained no seed rudiments. Control untransformed plants had fully developed seeds (Fig. 3).



c). Scientific impact of collaboration and technology transfer:

Travel: Both PI's met 3 times during this project, twice in South Africa and once in Israel. An additional meeting took place during August 2002 in Hungary as both PI's were attending the International Symposium for Grape Breeding and Genetics and presented talks describing the progress made.

Transfer of know-how: Constructs were transferred to the South African group for transformation experiments. Working protocols for transformation were transferred to the South African group for experiments. Embryogenic cultures were transfer to the South African group for transformation experiments.

Description of project impact: The recent results of seedless grape obtained by transformation was actually the first proof of concept that biotechnology can induce seedless fruits in trees. The results of the project are not used yet, but it is anticipated that they will pave the way for the development of seedless grape and other crops as well via genetic engineering.

Publication:

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2. Wang, Q., Gafny, R., Sahar, N., Mawassi, M., Tanne. E. and Perl, A. (2002). Cryopreservation of grapevine (*Vitis vinifera* L.) embryogenic cell suspensions and subsequent plant regeneration by encapsulation-dehydration. Plant Sci. 162:551-558.
3. Perl, A., Sahar, N., Farchi, S., Colova –Tsolova, V. , Holland, D. and Gollop, R. 2000. Conventional and biotechnological breeding of seedless table grapes in Israel.

Proceeding of VI International Symposium on Grapevine Physiology and Biotechnology, Heraklion, Greece, 91-92.

4. Wang, Q., Mawassi, M., Sahar, N., Li, P., Colova-Tsolova V., Gafny, R., Sela, I., Tanne, E. and Perl, A. (2004). Cryopreservation of grapevine *(Vitis* spp.) embryogenic cell suspensions by encapsulation-vitrification. Plant Cell Tissue. & Organ Culture (in Press).
5. Ginzberg, I., Perl, A., Genser, M., Wininger, S., Nemas,C. and Kapulnik Y. (2004) Expression of streptavidin in tomato resulted in abnormal plant development that could be restored by biotin application.
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6. Qiaochun Wang, Ping Li, Nachman Sahar, Munir Mawassis, Ron Gafny, Ilan Sela, Ednna Tanne and Avihai Perl

Agrobacterium-mediated transformation of *Vitis vinifera* L. cell suspensions: improvement of transformation efficiency and transgenic plant regeneration (submitted)

7. Perl, A., Colova-Tsolova, V. and Eshdat, Y. Grape Transformation: A practical manual. In: Curtis, I. (ed), Transgenic Crops of the World, Kluwer Academic Publishers (in press).

PROJECT GRANT NUMBER CDR TA-MOU-98-C14-177
FINAL REPORT

1 February 2001 to 30 April 2004

Submitted to the U.S. Agency for International Development; Bureau for Global Programs, Field Support and Research; Center for Economic Growth

TRANSGENIC SEEDLESSNESS IN GRAPES BY USING GENETIC ABLATION OF SEEDS

Principal Investigator : Dr Avi Perl

Grantee Institution : Volcani

Collaborator : Miss P Burger

Institution : ARC Infruitec-Nietvoorbij

Project Number :

Grant Number : CDR TA-MOU-98-C14-177

Grant Project Officer :

Project Duration : Three years

Executive summary

Although putatively transformed plantlets were obtained in this project it was very difficult to replicate and the procedures cannot be regarded as routine techniques. After initially experimenting with several different media compositions to initiate somatic embryogenesis, three media were selected for further use. Explants were cultured on a basal solid Nitsch & Nitsch medium (1969) with three different concentration combinations of BA (N^6-Benzyladenine) and 2,4D (2,4-Dichlorophenoxyacetic acid). Initially, both leaflets and petioles derived from *in vitro* plants were used as explant source, but as petioles gave better results, only petioles were used towards the end of the project. Different systems of co-cultivation of *Agrobacterium* and embryogenic plant material were investigated to transfer the gene of interest into plant material. Initially solid medium were used for all the transformation steps (co-cultivation, killing of *Agrobacterium* and selection of transformants). However, since serious problems with *Agrobacterium* overgrowth was experienced, the system used in Dr Perl's laboratory was investigated. This make use of liquid culture for transformation and selection. Although liquid culture gave good control of and prevented *Agrobacterium* overgrowth, problems were experienced with the browning of embryogenic material. Finally, a system was developed that incorporated the good qualities of both the solid and the liquid systems. Co-cultivation was done on solid medium. This was followed by "washing" embryogenic material in liquid medium supplemented with antibiotics to kill *Agrobacterium*. Afterwards, embryos were plated onto solid medium containing antibiotics to prevent *Agrobacterium* overgrowth and Kanamycin to select putative transformants. Various approaches were followed to germinate putative transformed embryos. These included media without plant growth regulators, media with BA and GA and media on either petridishes or in glass jars. However, it proved very difficult and mostly unsuccessful and plants were only obtained on basal Nitsch medium without growth regulators in 100 ml glass jars or with the same medium supplemented by 1,5 mg/l BA. The plants that did develop were screened by PCR techniques. However, although it seemed that some of the plants might have been transformed, the bands were faint and it could not be established beyond doubt that these plants were transformed.

Research objective

At the ARC Infruitec-Nietvoorbij seedless grapes are developed by conventional cross-breeding and embryo rescue techniques, involving the use of seedless parents. Since crosses are made, progeny characteristics are unpredictable and differ from that of both parents. This implicates that a single characteristic of a certain cultivar cannot be changed or improved by using cross-breeding. However, by implementing certain specific biotechnological techniques it is possible to alter a certain characteristic of a known cultivar (for example, to introduce powdery mildew resistance into a susceptible cultivar or to change the seeded characteristic to seedlessness). The objective of this project was the development of genetic transformation techniques for seeded cultivars important in the South African industry and the development of seedless variants from these by using genetic transformation techniques and a seed ablation gene to alter the seeded characteristic to one of seedlessness.

Although scientists have started to experiment with genetic transformation of grapevine about 15 years ago, most scientists will agree that it is still not a routine technique and that germination of embryos remain an obstacle, making it an impediment for use in molecular biology. Furthermore, different cultivars have different cultural requirements (personal communication with Drr Colova, Reustle, Martinelli and Gribaudo). Gentic transformation was carried out successfully by amongst others, Mauro, Kikkert & Reisch, Perl, Krastanova, Martinelli, Mullins *et al* and Toregrossa

Materials and Methods

Abbreviations used:

MS = Murashige and Skoog medium (1962)
N = Nitsch and Nitsch medium (1969)
BD = Bouquet and Davis medium (1989)
BA = N^6-Benzyladenine, NOA = (2-Naphtoxy)acetic acid, DTT = Dithiothreitol, IAA = Indole-3-acetic acid, TDZ = 1-Phenyl-3-(1,2,3-Thiadiazol-5-YL)Urea, CPPU = N-(2-Chloro-4-pyridyl)-N'-phenylurea, IASP = Indole-3-acetyl-L-aspartic acid, 2,4-D = 2,4-Dichlorophenoxyacetic acid

Initiation of embryogenic material

The plant material into which the gene(s) of interest are introduced is somatic embryos and it therefore was logic to start-off the project by initiating somatic embryos. The chosen explant material for this purpose were *in vitro* leaflets and petioles, because these material are readily available throughout the year. The influence of the explant source and different media compositions on the initiation of embryogenic callus and somatic embryos were compared.

Throughout the project explants of the following cultivars were cultivated to supply enough material for transformations : Bonheur, La Rochelle, Dauphine, Majestic and Regal. Somatic embryos of Redglobe were received from Dr Perl at the Volcani institute in Israel. Regal is a seedless cultivar, but has a noticeable large seed trace. Somatic embryos, when initiated were multiplied every six to eight weeks on a maintenance medium.

The composition of the media used at the start of the project to initiate somatic embryos are given in Table 1.

Table 1. Media used to initiate somatic embryos from *in vitro* leaflets and petioles of the cultivars Dauphine, Regal, Bonheur and La Rochelle.

1 N + 1 µM BA + 5 µM 2,4-D + 10µM ABA
2 N + 1 µM BA + 5 µM 2,4-D + 10 µM ABA + 3 g/l Gelrite (instead of agar)
3 N + 3 g/l Gelrite + 20 µM 2,4 D + 9 µM BA
4 N + 3 g/l Gelrite + 10 µM 2,4 D + 4,5 µM BA
5 Half MS + 0,8 g casein acid hydrolysate + 60g/l sucrose + 0,1 g/l inositol + 5 mg/l IASP + 2 mg/l NOA + 0,2mg/ BA + 2g/l activated charcoal
6 N + B&Davis amino acids and 1 g/l caseien acid hydrolysate + 1 µM BA + 5 µM 2,4-D
7 N + 266 mg/l $Ca(NO_3)_2$ instead of $CaCl_2$ + 3 g/l Gelrite + 20 µM NOA + 4 µM TDZ (no PHE)
8 N macro + MS micro + N vit. + 60 g sucrose + 3 g/l Gelrite + 2,5 µM 2,4 D + 2,5 µM NOA + 5 µM CPPU
9 N + 1 µM BA + 5 µM 2,4-D (Control)

B&D = Bouquet & Davis medium (1989)
N = Nitsch & Nitsch medium (1969)
MS = Murashige & Skoog medium (1962)

Table 2. Media used for initiation of somatic embryos of the cultivar Majestic.

1. N + 10 g/l agar + 1 µM BA + 5 µM 2,4-D (Control)
2. N + 3 g/l Gelrite (instead of agar) + 1 µM BA + 5 µM 2,4-D
3. N + 3 g/l Gelrite + 20 M 2,4 D + 9 µM BA
4. N + 3 g/l Gelrite + 10 µM 2,4 D + 4,5 µM BA
5. MS + 0,2 g/l glutamine + 5 mg/l $AgNO_3$ + 2 mg/l 2,4 D + 0,2 mg/l BA + 2,5 g/l activated charcoal
6. N + B&D amino acids + 1 g/l instead of 0,1 g/l casein hydrolysate + 1 µM BA + 5 µM 2,4-D
7. N + 266 mg/l $Ca(NO_3)_2$ instead of $CaCl_2$ + 0,413 g/l phenylalanine + 3 g/l Gelrite (instead of agar) + 20 µM NOA + 4 µM TDZ
8. N + 3g/l Gelrite + 5 uM 2,4 D + 5 uM NOA + 5 uM TDZ

Young *in vitro* leaflets and petioles (five of each per petridish) were cultivated on each of these different media and sixteen dishes were used per medium. Leaflets and petioles were cultured in dark at 25 ± 2°C. Based on the results obtained from the above experiments only petoles were used in further attempts to initiate embryogenic material and also based on the results obtained with these various media, only three were used in further attempts at embryo initiation. Embryogenic material were initiated throughout the project to supply in the need for sufficient material for transformation.

The media used till the end of the project are the folowing :

Medium 1 : N + 3 g/l Gelrite + 5 µM 2,4 D+ 1 µM BA

Medium 2 : N+ 3 g/l Gelrite + 20 µM 2,4 D + 9 µM BA

Medium 3 : N + 3 g/l Gelrite + 10 µM 2,4 D + 4,5 µM BA

Maintenance of somatic embryos

Embryogenic material were transferred to maintenance medium for multiplication in order to have had a supply of material for transformations throughout the project.

Initially a growth regulator free medium based on MS medium, but with lower nitrogen levels was used for maintenance of somatic embryos. However, after a few months this medium failed to give satisfactory results with Bonheur and Majestic and embryos ceased multiplication and eventually turned brown. This lead to the loss of about half of the material. Amendments were made by replacing agar with 2 g/l Gelrite and adding 5 uM NOA. Although this improved the multiplication of somatic embryos, it was still not satisfactorily and the medium was adapted further. Good results were obtained with the following medium and it was used till the end of the project : Nitsch macro elements, MS micro elements, Nitsch vitamins, Bouquet & Davis amino acids, 2 g/l Gelrite, 0,2 g/l casein, 60 g/l sucrose, 0,1 g/l inositol, 2 g/l activated charcoal, 10 µM NOA, 1 µM BA and 20 µM IAA.

Transformation of somatic embryos

Initially all steps in the transformation procedure were done on solid medium in petridishes - a protocol that was previously followed successfully in our laboratory with indicator genes (this work was done by a former colleague under another project). However, overgrowth of *Agrobacterium* occurred more frequently when the seed ablation genes were transferred as compared to the experience with indicator genes. This caused serious problems and sometimes all the plates were overgrown by *Agrobacterium*. In an attempt to get rid of these problems the system used by Dr Perl for killing *Agrobacterium* and selection of transformants were used instead. Dr Perl's procedures make use of liquid culture and it was reasoned that *Agrobacterium* would be more readily exposed to antibiotics in the liquid system as compared to solid medium in petridishes. Somatic embryos were transferred from petridishes to a liquid system two weeks before the transformation steps were done. Transformation and selection were done in liquid culture according to the method and media of Dr Perl. Claforan at 400 mg/l was added to medium to kill *Agrobacterium* and embryos were washed in this medium by replacing it every hour for three times. For selection of transformants Kanamycin was added to this Claforan containing medium. Kanamycin concentration was gradually increased from 50 mg/l to 100 mg/l over a one month period. Embryos were plated on solid maintenance medium with Kanamycin (100 mg/l) after a further month in liquid culture.

Initially this system seemed to be working well, but after a number of attempts at transformation the results with this method were not fully satisfying. Embryos turned brown after a few days in liquid culture (even before co-cultivation with *Agrobacterium*) and a protocol involving both solid and liquid culture was finally established. Co-cultivation of embryogenic material and *Agrobacterium* was done

on solid maintenance medium for 48h. The liquid phase (maintenance medium supplemented with 500 mg/l Claforan and 4.6 g/l glycerol and no gelling agent) was used to wash transformed embryos and kill off *Agrobacterium* after co-cultivation. The medium was replaced every hour for three times. Afterwards embryos were plated on a solid maintenance medium supplemented with 400 mg/l Claforan. Towards the end of the project the medium was supplemented with 250 mg/l Timentin (Ticarcillin: Clavulanic acid; 15:1) instead of Claforan to prevent *Agrobacterium* overgrowth as we obtained better results with this antibiotic in the control of *Agrobacterium*. For selection this medium was supplemented with Kanamycin (increasing the concentration from 50 mg/l to 100 mg/l over a one month period). In selection medium agar was used instead of Gelrite.

Germination of putative transformants

Various approaches were followed for the germination of transformants that were selected on kanamycin medium.

Initially transformants that were selected on kanamycin medium were plated on a germination medium (Basal medium of Bouquet and Davis, 1989, containing no growth regulators and 15 g/l sucrose) in petri dishes. Previously this medium was used successfully to germinate untransformed somatic embryos. In addition, putative transformants were cultured for two days in liquid medium supplemented with either 2mg/l BA or 2 mg/l GA_3 before it was transferred to solid Nitsch medium with no growth regulators in 100 ml glass jars. In another attempt at germination putative transformants were plated on solid shoot elongation medium with 1 mg/l BA in petri dishes as well as in 100ml glass jars following selection.

However, results were not satisfactorily as no plants developed and the method of Perrin *et al* (2001) was followed in an effort to develop embryos into plants. Embryos were transferred from a long-term culture medium onto a pre-conditioning medium. After 4 weeks on this medium embryos were transferred onto a torpedo initiation and development medium. Torpedo stage embryos were transferred onto a medium for conversion into plants.

Finally, embryos were plated on basal Nitsch medium with either no plant regulators or with 1.5 mg/l BA in 100 ml glass jars.

PCR studies of plantlets

Putatively transformed plantlets were multiplied *in vitro* by one-bud cuttings to supply enough material for PCR studies and plant material for future further studies (greenhouse and vineyard). PCR studies were done by Dr Yolanda Peterson at the ARC Infruitec-Nietvoorbij to verify whether plantlets were genetically transformed. DNA was extracted from the plantlets using the CTAB method. The primers used to test for the success of transformation were the *npt*II primers.

Results and discussion

Initiation of embryogenic material

In the earliest initiation experiments it was established that a higher percentage of embryos developed from petioles and therefore only petioles were used in further experiments. Only the results obtained with Majestic are represented (Table 3) as results from the other cultivars were similar, but fewer explants developed embryogenic material.

TABLE 3. The development of somatic embryos from leaves and petioles of the cultivar Majestic.

Medium	Number of explants with embryos	
	Leaves	Petioles
1 (control)	-	1
2	-	3
3	1	-
4	2	2
5	-	-
6	-	1
7	-	1
8	-	-
Total	3	8

The effect of the three different media compositions on initiation of somatic embryogenesis is illustrated for the cultivars La Rochelle and Bonheur in Table 4 (This is for one culture date only, data for other dates and cultivars are not illustrated).

TABLE 4. Development of somatic embryos in two seeded cultivars.

Medium	Number of explants	Number of explants callusing	Number of explants developing embryos	Percentage of explants developing callus clumps	Percentage of callus clumps developing embryos	Percentage of explants developing embryos
LA ROCHELLE						
N + 5µM 2,4D + 1µM BA	230	97	1	42,2	1,03	0,43
N + 20µM 2,4D + 9µMBA	230	25	3	10,9	12,0	1,30
N + 10µM2,4D + 4,5µMBA	240	42	5	17,5	11,9	2,08
TOTAL	700	164	9	23,4	5,49	1,48
BONHEUR						
N + 5µM 2,4D + 1µM BA	210	137	1	65,2	0,73	0,48
N + 20µM 2,4D + 9µM BA	200	88	-	44,0	-	-
N+ 10µM2,4D + 4,5µM BA	250	199	-	79,6	-	-
TOTAL	660	424	1	64,2	0,15	0,24

An interesting observation was made that callus developing on the same medium varied in texture depending on the cultivar. La Rochelle callus were much smaller in volume, while those developing in Bonheur where voluminous, but watery (Fig 1). A higher number of the Bonheur explants also developed callus as compared to La Rochelle. It was also clear that the watery callus is non-embryogenic. Although fewer explants developed callus in La Rochelle a higher percentage of these were embryogenic. The difference in appearance of watery and embryogenic callus is illustrated in figure 1 and the development of embryos from embryogenic callus in figure 2.

Another interesting observation was that explants did not always gave the same results on the same medium when cultured at different dates. For example, on a later culture date, embryos developed only on medium containing 10µM 2,4D and 4,5µM BA for the cultivar La Rochelle and not on either of the other two media.

Transformations

The initial problems experienced with *Agrobacterium* overgrowth on solid medium and browning of embryos in liquid culture were solved by using a procedure that combined both procedures. Co-cultivation on solid medium, followed by washing of embryos in liquid culture and plating of embryos on solid medium gave satisfactorily results and was followed up to the end of the project. The inclusion of the antibiotic Timentin in addition to Claforan also helped to decrease *Agrobacterium* overgrowth. The final procedure is as follows :

1). Put a volume of an *A. tumefaciens* culture, with the "seed ablation" gene, in 20 ml liquid maintenance medium (with 4,6g glycerol added and no activated charcoal or agar).
2). Transfer young somatic embryos to this, shake lightly and let stand for 10 minutes
3). Decant liquid and plate embryos on solid maintenance medium in petridishes. Leave for 48 hours in a dark growth chamber at 25 ± 2 °C.
4). Transfer embryos to 50 ml liquid maintenance medium with 20g/l sucrose, 100 mg/l DTT and 300 mg/l Claforan (cefotaxime) or 250 mg/l Timentin in an Erlenmeyer flask. Put on a shaker (80 – 100 rpm) for 1 hour. Replace the medium every hour for three times.
5). Transfer embryos to solid maintenance medium (60 g/l sucrose, 6 g/l agar) with 25 mg/l Kanamycin and 400 mg/l Claforan or 250 mg/l Timentin. Cultivate in dark at 25 ± 2 °C, replacing medium every two weeks and gradually increase the Kanamycin concentration to 100 mg/l.
6). After two to three months putative transformed embryos are selected and transferred to a solid germination medium (Nitsch, 1969 medium with 1 mg/l BA or without growth regulators) in 100 ml glass jars.

Germination of putative transformed embryos

We found it very difficult to germinate putative transformed embryos and had no success with the method of Perrin *et al.* (2001). The only embryos that germinated and developed into plantlets were those that were cultured in 100 ml glass jars on either basal Nitsch medium or on this medium

supplemented by 1,5 mg/l BA after selection on maintenance medium in petri dishes. However, this also could not readily be replicated and sometimes no germination occurred on these media. Plantlets with normal leaflets developed within two and a half to three months after embryos were transferred to glass jars (Fig 3 and 4).

PCR Analysis

Analysis of transformed grapevine plantlets

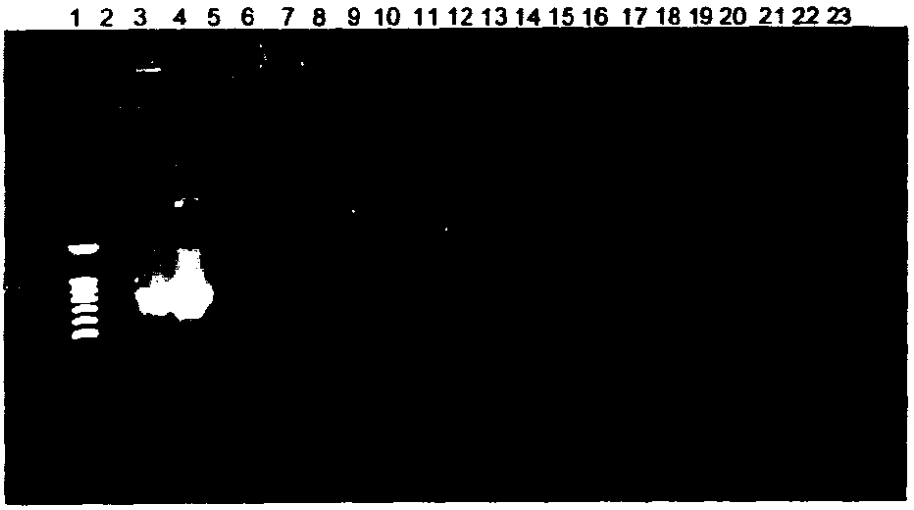
DNA was extracted from the 19 samples using the CTAB method.

The primers used to test for the success of transformation were the *npt*II gene primers. Two different DNA concentrations and two different primer annealing temperatures were tested to verify the results.

Summary of results
The samples 9, 11, 12, 13, 16, 18 and 19 may be considered putatively positive for the presence of the *npt*II gene. Because of the faintness of the bands I would recommend redoing the PCR with new DNA preparations, since DNA quality may have influenced the results.

Gel 1

100 ng of DNA and an annealing temperature of 55°C were used in the PCR

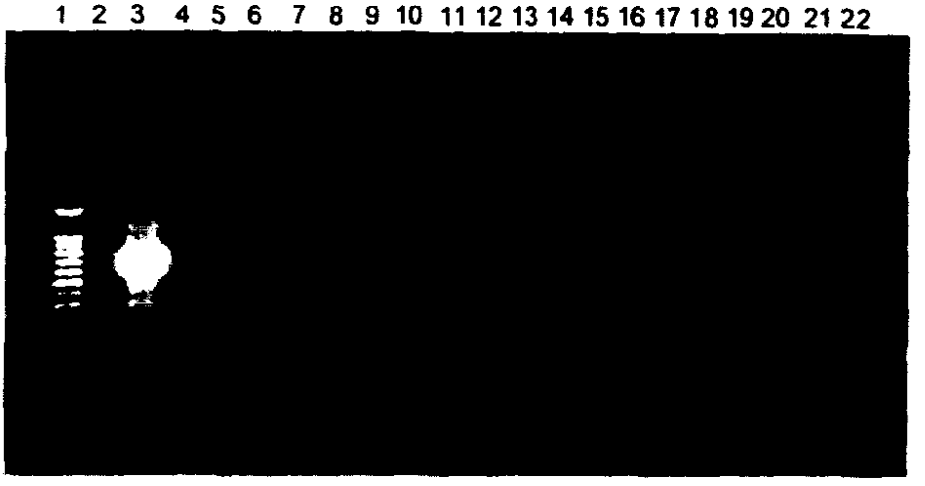


Lane
1. 100 bp molecular weight marker
2. Negative control: non transformed grape vine
3. Positive Control: Plasmid known to contain the *npt*II gene
4. Positive Control: Agrobacterium strain containing the construct used for transformation
5-23. The unknown samples in order 1-19

Putative positive samples:
9, 11, 12, 13, 16, 18, 19

Gel 2

200 ng of DNA and an annealing temperature of 55°C were used in the PCR

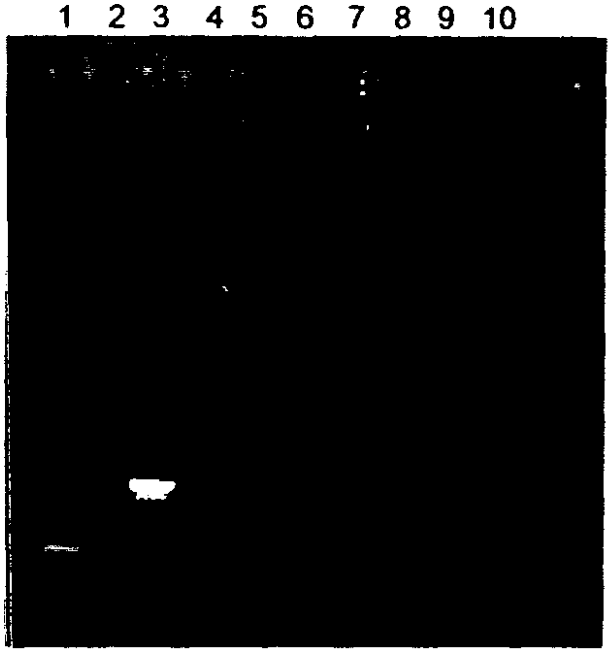


Lane
1. 100 bp molecular weight marker
2. Negative control: non-transgenic grapevine
3. 3. Positive Control: Plasmid known to contain the *npt*II gene
4-22. The unknown samples in order 1-19

Putative positives
16, 19

Gel 3

100 ng of DNA and an annealing temperature of 50°C were used in the PCR. The reason for doing this PCR was to see whether the faint bands observed in certain samples might be better amplified at a lower temperature. A sample which had been negative in the previous PCRs was also included.



1. 100 bp molecular weight marker
2. Negative control: non-transgenic grapevine
3. Positive Control: Plasmid known to contain the *npt*II gene
4. 10
5. 11
6. 13
7. 14
8. 15
9. 16
10. 19

Conclusions

The lower annealing temperature has resulted in non-specific amplification as shown by the presence of a 400 bp band in the non-transformed as well as unknown samples. However, once again, samples 11, 13, 16 and 19 had faint bands corresponding to the expected size of the *npt*II PCR product.

BENEFITS TO SOUTH AFRICAN PARTNER

For the immediate future the results obtained in this project will not be beneficial to the South African table grape industry as plantlets will be under experimentation for the next few years. In the interim benefits should be regarded as purely of academic value. Another factor is the strong consumer resistance to GMO's. Recently, the ARC had a number of requests from South African table grape export companies for written statements that no genetic manipulation is used in the breeding programme of this institution and that none of the ARC's cultivars were developed by using these techniques. Bearing this in mind it seems impossible that the ARC or any South African growers will benefit financially from the plants obtained in this project for the foreseeable future. However, personnel benefitted by training and personal growth in this discipline.

Literature

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MURASHIGE, T. & SKOOG, F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15: 473 - 497.

NITSCH, J.P. & NITSCH, C. 1969. Haploid plants from pollen grains. *Science* 163, 85-87.

PERRIN, M., MARTIN, D., JOLY, D., DEMANGEAT, G., THIS, P. & MASSON, J.E. 2001. Medium-dependent response of grapevine somatic embryogenic cells. *Plant Science* 161, 107-116.

Fig 1. Watery callus on left, with embryogenic callus on the right

Fig 2. Close-up of embryogenic callus with developing embryos

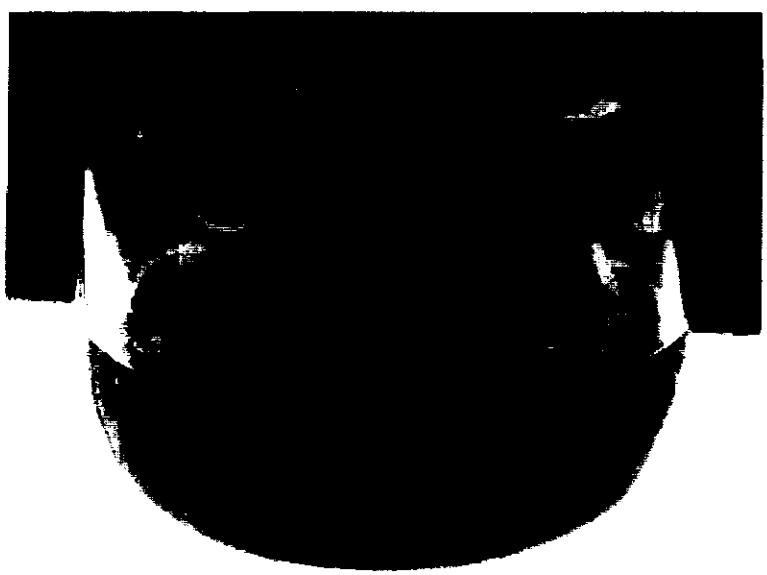

Fig 3. Germination of embryos and development of plantlets

Fig 4. Multiplication of putatively transformed plantlets

ADDENDUM

Somatic Embryo Induction medium (NITSCH & NITSCH based)

1 MACRO ELEMENTS (In 500ml distilled water)		mg/litre
KNO3	23,75g	950
NH4NO3	18,0g	720
MgSO4.7H2O	4,625g	185
CaCl2.6H2O	4,150g	166
KH2PO4	1,70g	68
Take 20ml per litre medium		

2 MICRO ELEMENTS (In 500ml distilled water)		
MnSO4.4H2O	1,89g	18,9
H3BO3	1,0g	10
ZnSO4.4H2O	1,0g	10
NaMoO4.2H2O	0,025g	0,25
CuSO4.5H2O	0,0025g	0,025
Take 5ml per litre medium		

3 Fe & EDTA (In 500ml distilled water. Store in DARK)		
FeSO4.7H2O	2,785g (Dissolve in warm distilled water)	27.8
Na2EDTA	3,725g	37,3
Take 5ml per litre medium		

4 VITAMINS (In 250ml distilled water)		
Glycine	0,1g	2,0
Nicotinic acid	0,25g	5,0
Pyridoxine HCl (Vitamin B6)	0,025g	0,5
Thiamine HCl (Vitamin B1)	0,025g	0,5
Folic acid	0,025g Dissolve in 1 M NaOH	0,5
D-Biotin	0,0025g Dissolve in 1 M NaOH	0,05
Take 5ml per litre medium		

ADD THE FOLLOWING WHEN PREPARING MEDIUM

6 SUCROSE	20g/litre
7 GELRITE	2g/litre
8 INOSITOL	0,1g/litre
9 BA	Depending on concentration either 1 µM, 4,5 µM or 9 µM
10 2,4 D	Depending on medium either 5 µM, 10 µM or 20 µM

pH = 5,7

MAINTENANCE MEDIUM FOR SOMATIC EMBRYOS

1 MACRO ELEMENTS (In 500ml distilled water) Nitsch		mg/litre
KNO3	23,75g	950
NH4NO3	18,0g	720
MgSO4.7H2O	4,625g	185
CaCl2.6H2O	4,150g	166
KH2PO4	1,70g	68
Take 20ml per litre medium		

2 MICRO ELEMENTS (In 500ml distilled water) Murashige & Skoog		
MnSO4.4H2O	2,23g	22,3
H3BO3	0,62g	6,2
ZnSO4.4H2O	0,86g	8,6
NaMoO4.2H2O	0,025g	0,25
CuSO4.5H2O	0,0025g	0,025
CoCl2.6H2O	0,0025g	0,025
KI	0,083g	0,83
Take 5ml per litre medium		

3 Fe & EDTA (In 500ml distilled water. Store in DARK)		
FeSO4.7H2O	2,785g (Dissolve in warm distilled water)	27,8
Na2EDTA	3,725g	37,3
Take 5ml per litre medium		

4 VITAMINS (Dissolved in 250ml distilled water)		
Nicotinic acid	0,25g	5,0
Pyridoxine HCl (Vitamin B6)	0,025g	0,5
Thiamine HCl (Vitamin B1)	0,025g	0,5
Folic acid	0,025g Dissolve in 1 M NaOH	0,5
D-Biotin	0,0025g Dissolve in 1 M NaOH	0,05
Take 5ml per litre medium		

5 AMINO ACIDS (In 500ml distilled water) Bouquet & Davis		
Glutamine	5,0g	100
Cysteine	5,0g	100
Phenylalanine	0,5g	10
Glycine	0,1g	2
Take 10ml per litre medium		

ADD THE FOLLOWING WHEN PREPARING MEDIUM

6 SUCROSE	60 g/litre	
7 GELRITE	2 g/litre	
8 MESO-INOSITOL	0,05g/litre	
9 CASEIN HYDROLYSATE	0,1g/litre	
10 ACTIVATED CHARCOAL	2,5 g/litre	
11 NOA		10 µM
12 BA		1 µM
13 IAA		20 µM

pH 5,8

EXPENDITURE : 1 October 2003 to 31 March 2004

	R	c
Travel	222	15
Postage and courier	285	96
Research consumables	2 410	49
Cleaning materials	183	86
Laboratory equipment	2 053	00
Chemicals	8 887	27
Glassware	3 672	84
Mite control	75	79
Maintenance (Growth tunnel repairs)	11 793	00
Sub total	**29 584**	**36**
15% Indirect costs	4 437	65
TOTAL	**34 022**	**01**

The exchange rate over this period averaged about R 6,70 to the US dollar

R 34 022,01 equals $ 5 077,91

TOTAL EXPENDITURE AND IMBURSEMENTS OVER THE EXTEND OF THE PROJECT (US DOLLAR)

PERIOD	INCOME (Imbursement)		EXPENDITURE	
1 October 2000 to 30 September 2001	16 675	00	16 877	49
1 October 2001 to 30 September 2002	10 200	60	16 119	78
1 October 2002 to 30 September 2003	17 250 6 119	00 78	19 341	19
1 October 2003 to 31 March 2004	00	00	5 077	91
TOTAL	**50 245**	**38**	**57 416**	**37**

Total expenditure – total income = $ 7 170,99