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Mannose and xylose cannot be used as selectable agents for *Vitis vinifera* L. transformation

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Summary

Only a few selectable marker systems for genetic engineering of grapevine have been studied in the past and only resistance to antibiotics has been used to recover transgenic vines. Since the acceptance of antibiotic resistances is small mannose and xylose were evaluated as selectable agents for the genetic transformation of grapevine. Survival of suspension cells and the ability to develop embryos from embryogenic calli were estimated in the presence of mannose and xylose. Embryogenic calli obtained from anther cultures of *Vitis vinifera* cv. Chardonnay were able to grow and to produce embryos even if mannose or xylose were the only source of carbohydrates.

K e y w o r d s *:* mannose, xylose, carbohydrate source, selectable agent, somatic embryogenesis, *Vitis vinifera.*

Introduction

A number of selectable agents, other than antibiotics, were recently tested for positive selection following the transformation of various plant species (HALDRUP *et al.* 1998; NEGROTTO *et al.* 2000; JOERSBO 2001). Different carbon sources were used as selectable agents, like mannose and xylose (HALDRUP *et al.* 1998, 2000; ODD and TAGUE 1998; JOERSBO *et al.* 1999, 2000; NEGROTTO *et al.* 2000; ZHANG *et al.* 2000; LUCCA *et al.* 2001; WRIGHT *et al.* 2001; HE *et al.* 2003). Indeed, mannose inhibits germination and development of a wide range of plants such as *Arabidopsis* and tomato (FERGUSON and STREET 1958; HEROLD and LEWIS 1977; PEGO *et al.* 1999; GIBSON 2000). Mannose is an important carbohydrate in mannitol metabolizing plants (STOOP *et al.* 1996) with mannose-6P being the biosynthetic precursor. In plant species, such as celery, in which an important part of carbon is translocated as mannitol, phosphomannose isomerase (PMI) activity is high (RUMPHO *et al*. 1983). In species in which mannitol is not translocated, PMI is often expressed at low or undetectable levels. Application of exogenous mannose is often lethal to these species. While mannose is readily taken up and phosphorylated by hexokinase, in these plants mannose-6-P is not further converted to fructose-6-P due to the deficiency in PMI. The accumulation of mannose-6-P results in the sequestration of cellular orthophosphate which is required for ATP production. In addition, this accumulation inhibits phosphoglucose isomerase, blocking glycolysis. PMI activity converts mannose-6-P to fructose-6-P, an intermediate of glycolysis that positively supports plant growth and that does not sequestrate cellular phosphate. The *E. coli pmi* gene encoding phosphomannose isomerase has been used in the transformation of some plant species (ODD and TAGUE 1998; JOERSBO *et al.* 1999, 2000, 2001; ZHANG *et al.* 2000; LUCCA *et al*. 2001; WRIGHT *et al.* 2001; HE *et al.* 2003). Another plant selection system is based on xylose isomerase. Various plant species such as potato cannot use D-xylose as a carbon source, but can use D-xylulose (HALDRUP *et al.* 1998, 2000). The gene encoding the D-xylose ketol-isomerase, from *Thermoanaerobacterium thermosulfurogenes* catalyses the isomerisation of D-xylose to D-xylulose, which positively supports the growth of transformed cells (HALDRUP *et al.* 1998).

Though genetic engineering is particularly promising for the improvement of grapevine cultivars, only a few selectable marker systems based on antibiotic or herbicide resistance have been studied (REUSTLE *et al.* 2002). Efficient genetic engineering implies the development of *in vitro* systems for genetic transformation and plant regeneration. The regeneration of grape plants has been obtained by both organogenesis and embryogenesis. Regeneration from somatic embryos has been used for genetic transformation of major grape species including *Vitis vinifera*, *V. riparia*, *V. rupestris* and interspecific hybrids. Transgenic grapes can be generated by the transformation of embryogenic callus obtained from different tissues including zygotic embryos (SCORZA *et al.* 1995), leaves (SCORZA *et al.* 1996; DAS *et al.* 2002), unfertilized ovules (YAMAMOTO *et al.* 2000), ovaries (MOTOIKE *et al.* 2001*)* and anther filaments. The latter is the most widely used (LE GALL *et al*. 1994; MARTINELLI and MANDOLINO 1994; NAKANO *et al.* 1994; KRASTANOVA *et al.* 1995; PERL *et al.* 1996; FRANKS *et al.* 1998; MOZSÄR *et al.* 1998; BARBIER *et al.* 2000; GÖLLES *et al.* 2000; HARST *et al.* 2000; SPIELMANN *et al.* 2000; COUTOS-THEVENOT *et al*. 2001; IOCCO *et al.* 2001; LI *et al.* 2001; MARTINELLI *et al.* 2002).

Grapevine cultivars have been successfully transformed using *Agrobacterium* inoculation, but microprojectile bombardment has also led to the production of transgenic plants (KIKKERT *et al.* 1996; TORREGROSA *et al.* 2002). Genes that are frequently used to select transformed plant tissues or cells include *nptII*, *hpt* and *bar* encoding for neomycin phosphotransferase, hygromycin phosphotransferase and phosphinothricin acetyl transferase, respectively. They confer resistance to antibiotics (kanamycin and hygromycin respectively) or herbicide (phosphinotricin). For grapevine genetic engineering, kanamycin resistance was found to be an efficient selection marker system but antibiotic or herbi-

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cide resistance markers are no longer acceptable in genetically engineered plants.

In this report, we evaluate the possibility of using mannose and xylose as selectable agents for the genetic transformation of grapevine. We estimate the effect of mannose and xylose on growth and death of cell suspensions of *Vitis vinifera* cv. Chardonnay; the ability to produce embryos from embryogenic callus of the same cultivar is also evaluated.

Material and Methods

Embryogenic callus development: Anther-derived embryogenic callus from *Vitis vinifera* cv. Chardonnay (clone 96) was obtained as described by MAURO *et al.* (1986). Some embryogenic callus appeared two months after the dissection of anthers. For long-term maintenance, subcultures were performed every three weeks on MPM medium containing sucrose (20 $g.l^{-1}$) (PERRIN *et al.* 2001). The cultures were maintained at 25 ±0.5 °C under 16 h light per 24 h (50 µmol $quanta.m^{-2}.s^{-1}$, Osram Biolux).

Culture media for selection: Embryogenic calli were transferred onto MPM medium containing the various carbohydrate sources at different concentrations: sucrose (20 $g.l^{-1}$), mannose (10 or 20 $g.l^{-1}$), xylose (10 or 20 $g.l^{-1}$), or no sugar at all. Agar (7 $g.l^{-1}$), MES (0.7 $g.l^{-1}$) and distilled water were mixed in bottles and autoclaved (115 °C for 30 min). Filter-sterilised (0.22 µm) solutions of the concentrated media (MPM macronutrients, micronutrients, vitamins, ferric citrate and hormones) containing sugar were added and extensively homogenised with the agar solution melted at 55-60 °C, before pouring (PERRIN *et al.* 2001).

In addition, for generation of embryos, embryogenic calli were grown on MPM medium containing sugars supplemented with 2.5 $g.l^{-1}$ activated charcoal, added to the melted agar solution before autoclaving (MA medium).

Cell suspension culture: Cell suspensions from Chardonnay were initiated from embryogenic calli after mechanical dissociation and cultured in MPM liquid medium. Cultures were shaken at 115 rpm and 25 °C in the dark. The cell suspensions (30 ml) were subcultured every 3 weeks by adding 70 ml fresh MPM liquid medium in a 250 ml flask.

Evaluation of the effects of mannose and xylose on embryogenic calli: In a first series of experiments we estimated the effects of mannose and xylose on callus growth. Embryogenic callus pieces, 4-6 mm in diameter, were transferred onto MPM medium containing the different carbohydrate sources at various concentrations. Embryogenic calli were subcultured every three weeks and were maintained at 25 ±0.5 °C under 16 h light per 24 h (50 µmol $quanta.m^{-2}.s^{-1}$, Osram Biolux). Development of the calli was visually estimated after 3, 6 and 9 weeks.

In a second series of experiments, we evaluated the effects of mannose and xylose on the ability of embryogenic callus to produce embryos. After removal of all the embryos visible on the calli, embryogenic callus pieces, 4-6 mm in diameter, were transferred onto MA media with different concentration of mannose, xylose and sucrose and maintained at 25 ± 0.5 °C in the dark. Differentiation of embryos was observed after 3, 6 and 9 weeks. Each experiment of the two series was repeated at least three times.

Evaluation of the effects of mannose and xylose on cells in suspension: In a third series of experiments, we quantitatively estimated the effect of mannose and xylose on growth and death of cells in cell suspensions. The initial cell suspension grown in MPM liquid medium containing sucrose was washed three times with MPM liquid medium without any carbohydrate source. Then, an aliquot of 0.5 or 1 ml cell suspension was added to 6 ml MPM liquid medium containing sucrose (20 $g.l^{-1}$) or mannose (20 $g.l^{-1}$) or xylose (20 $g.l^{-1}$) or no carbohydrate source. These cultures were grown under shaking (115 rpm) in the dark, at 25 °C and were not subcultured during 30 d**.** After 18 and 30 d, packed cell volume was measured after 20 min for each cell suspension in a 5 ml pipette. This experiment was repeated twice, with one replicate per treatment.

Quantification of cell death: For comparison between the different suspension cells, a volume of the cell suspension which gave the smallest volume of settled cells (C_1) was used. For the other cell suspensions (*e.g.* C_n), the volume used was 1/N, where N is the ratio of the volume of settled cells of C_n to the volume of settled cells of C_1. After sedimentation the cells were resuspended in the same volume of PBS (phosphate buffered saline). One volume of aqueous 0.025 % Trypan blue solution was added to one volume cell suspension for each sample. Immediately after homogenisation, the cells were sedimented and washed with 0.5 ml PBS and with 0.5 ml 1% aqueous SDS in order to release the Trypan blue not entered into dead cells. To extract the Trypan blue, 1 ml of a 50 % methanol : 1 % SDS solution was added to an equal volume of cells and incubated for 30 min at 50 °C. Then, cells were centrifuged at 11,000 g for 2 min. The optical density of the supernatant was measured spectrophotometrically at 595 nm. Data were compared using a Student's test.

Results

Effects of mannose and xylose on the growth of embryogenic calli: Embryogenic calli obtained from Chardonnay anthers were white or pale yellow, friable and soft (Fig. 1 A). When subcultured on MPM medium containing sucrose (20 $g.l^{-1}$), embryogenic calli were growing and only 10-15 % of them turned brown. On MPM medium containing mannose at 10 $g.l^{-1}$ or 20 $g.l^{-1}$, no browning of the calli was observed even after 9 weeks and growth was comparable to that of the calli grown on sucrose (Fig. 1 C). Similarly, calli grew at a similar rate on MPM medium containing xylose at the two concentrations (10 and 20 $g.l^{-1}$; Fig. 1 D). On the other hand, calli subcultured on MPM medium without any carbohydrate source did not grow and progressively turned brown after 3 weeks (Fig. 1 B).

Effects of mannose and xylose on the ability of embryogenic calli to produce embryos: To test the ability of embryogenic calli to develop somatic embryos in the presence of mannose or

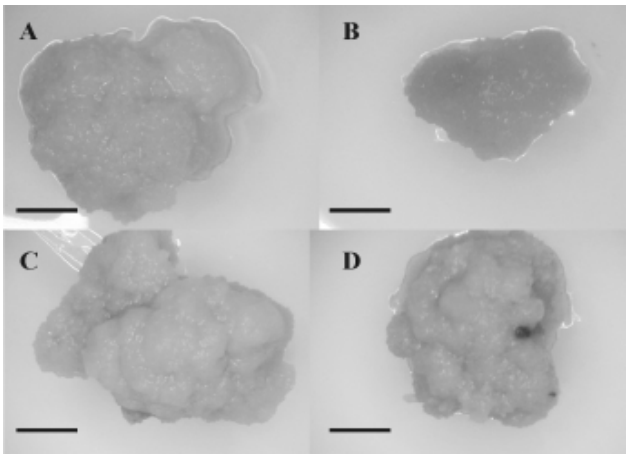

Fig. 1: Embryogenic callus growth on MPM medium in the presence of sucrose (20 g·l^{-1}; A), no sugar (B), mannose (20 g·l^{-1}; C) or xylose (20 g·l^{-1}; D). The picture was taken 6 weeks after transfer onto the different media (bars denote 2 mm).

xylose, we subcultured them on MA medium containing charcoal. After 2 or 3 weeks on MA containing sucrose (20 g·l^{-1}), somatic embryos grew on the calli (Fig. 2 A). The same results were obtained on calli grown on MA containing mannose (10 or 20 g·l^{-1}; Fig. 2 C) or xylose (10 or 20 g·l^{-1}; Fig. 2 D). On the contrary when embryogenic calli were subcultured on MA medium without sugar, we never ob-

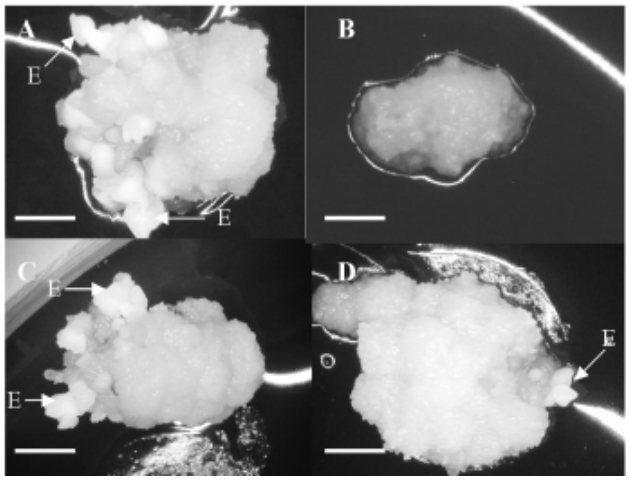

Fig. 2: Development of embryos from callus grown on MA medium in the presence of sucrose (20 g·l^{-1}; A), no sugar (B), mannose (20 g·l^{-1}; C) or xylose (20 g·l^{-1}; D). The picture was taken 3 weeks after transfer onto the different media. E: Embryos (bars denote 2 mm).

served any production of somatic embryos, and the calli progressively turned brown (Fig. 2 B).

Effects of mannose and xylose on growth and death of cells in suspension: When cultured in liquid MPM medium with sucrose (20 g·l^{-1}), the volume of cells settled after 30 d was 2.8 times higher compared to the volume of settled cells at the onset of the experiments (Fig. 3). When cultured in MPM medium with mannose (20 g·l^{-1}) or xylose (20 g·l^{-1}) the increase in cell volume was more than twofold after 30 d. On the contrary,

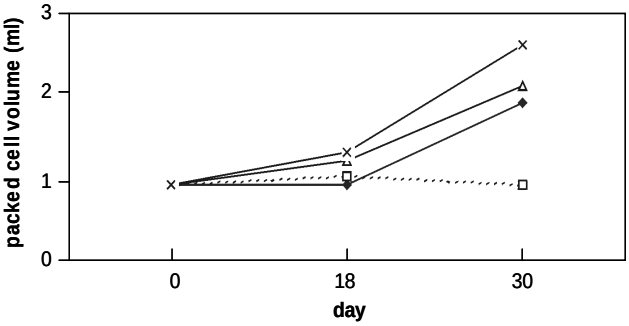

Fig. 3: Growth of Chardonnay cell suspension in MPM medium containing sucrose (20 g·l^{-1} —◆—) mannose (20 g·l^{-1}—△—), xylose (20 g·l^{-1} —×—) or no sugar (---□---). The experiment was repeated twice, with one replicate per treatment. One of the experiments is presented.

for cells cultured in MPM medium without sugar the volume of settled cells was constant from the onset to the end of the experiment (Fig. 3).

After 30 d of culture, cell death evaluated by Trypan blue coloration was maximum when the cells were cultured without carbohydrate source. In the presence of sucrose, cell death was not significantly different from that observed in the absence of sugar. However, when cells were cultured in the presence of either mannose or xylose cell death was significantly reduced (Table).

Table

Cell death of Chardonnay cell suspensions after 30 d of culture in the presence of the different sugars (concentration: 20 g·l^{-1}). Trypan blue coloration was used to estimate cell death by evaluation of the optical density measured spectrophotometrically at 595 nm

	no sugar	sucrose	mannose	xylose
Absorbance at 595 mm	0.409	0.388	0.331*	0.314*
Standard deviation	0.01	0.03	0.01	0.02

*p <0.05

Discussion

Like xylose mannose is a carbohydrate which many plant species are unable to metabolize. The synthesis of mannose-6-P and xylose-6-P results in the sequestration of cellular phosphate required for a large number of critical cellular functions. This phosphate sequestration serves as a basis for the use of mannose or xylose in non-antibiotic-based selections for the production of transgenic plants: for mannose, sugar beet (*Beta vulgaris*) (Joersbo *et al.* 2000), maize (*Zea mays*) and wheat (*Triticum aestivum*) (Wright *et al.* 2001), cassava (*Manihot esculenta*)(Zhang *et al.* 2000) and rice (*Oryza sativa*) (Lucca *et al.* 2001; He *et al.* 2003); for xylose, potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum*) (Haldrup

et al. 1998*)*. For all these plant species, selection with mannose or xylose is possible because plant growth, growth of embryogenic calli, germination, or shoot formation is severely inhibited in the presence of mannose and xylose (NEGROTTO *et al*. 2000; HALDRUP *et al*. 1998; ODD and TAGUE 1998; LUCCA *et al*. 2001). Our results show that embryogenic calli of *Vitis vinifera* cv. Chardonnay are able to grow and to produce embryos even if mannose or xylose is the only carbohydrate source.

Our results suggest that phosphorylation of mannose and xylose by hexokinases is not lethal for grape cells. The non-toxicity of mannose can be explained by the three following hypotheses. First, grape cells may express high PMI activity. VITRAC et *al.* (2000) recently suggested the presence of a PMI activity in *Vitis vinifera* cv. Gamay Fréaux. No toxicity was observed for cells of this cultivar in the presence of 18 $g \cdot l^{-1}$ mannose. Furthermore, PMI expression in "non-mannitol plants" is quite variable. While mannose is quite toxic to the growth of plants such as maize and wheat (WRIGHT *et al*. 2001), feeding mannose to leaves of *Nicotiana tabacum* caused minimal metabolic impairment (EDWARDS and WALKER 1983). This indicates that *N. tabacum* must be capable for at least limited mannose metabolism; further it can be suggested that tobacco cell suspension cultures are able to grow, even if slowly, on mannose as the sole carbohydrate source (EDWARDS and WALKER 1983). The second hypothesis is that we selected by mannose toxicity mutant cells with increased PMI activity, as was reported for *N. tabacum* (BARB *et al.* 2003). A third possibility is that the decrease in available phosphate resulting from the sequestration of the cellular phosphate could be compensated by the phosphate present in the medium we used to grow our grapevine calli and cells.

The non-toxicity of xylose suggests that grape cells also possess a xylose isomerase activity as described for barley and potato (HALDRUP *et al*. 1998). But in the case of potato, the endogenous xylose isomerase activity was not high enough to support the growth of potato stem segments; xylose as the sole carbon source appeared to be too toxic for the development of plants (HALDRUP *et al*. 1998, 2000). The selection of a mutant cell which can metabolise xylose and the compensation of the cellular phosphate by phosphate present in the medium might also be possible.

Under our conditions, mannose and xylose were not toxic for *Vitis vinifera* cv Chardonnay. This result is confirmed by the fact that the presence of each sugar does not increase death in cell suspensions; cell death was even reduced in the presence of mannose or xylose.

In conclusion, mannose and xylose can't be used as selectable agents for the transformation of Chardonnay embryogenic calli or cell suspensions. These results are in agreement with those reported by REUSTLE et *al.* (2002), who investigated mannose as a selectable marker system to regenerate genetically modified Seyval blanc grapevines. They observed that none of the regenerated embryos was transgenic and concluded that the selecting action of mannose (10-15 $g \cdot l^{-1}$) was not strong enough to be efficiently used for grapevine transformation. We are now testing other selection systems for genetic engineering of grapevine.

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