

## Overexpression of the *VvLTP1* gene interferes with somatic embryo development in grapevine

Julie François<sup>A</sup>, Magali Lallemand<sup>A</sup>, Pierrette Fleurat-Lessard<sup>A</sup>, Laurent Laquittaine<sup>A</sup>,  
Serge Delrot<sup>A,B</sup>, Pierre Coutos-Thévenot<sup>A</sup> and Eric Gomès<sup>A,C</sup>

<sup>A</sup>Laboratoire de Physiologie, Biochimie et Biologie Moléculaire Végétales, UMR CNRS-Université de Poitiers 6161,  
40 Avenue du recteur Pineau, 86022 Poitiers, France.

<sup>B</sup>Present address: Institut des Sciences de la Vigne et du Vin, UMR 1287 Ecophysiologie et Génomique  
Fonctionnelle de la Vigne, Université de Bordeaux 2, INRA, Domaine de la Grande Ferrade, BP 81, 33883  
Villenave d'Ornon, France.

<sup>C</sup>Corresponding author. Email: eric.gomes@univ-poitiers.fr

**Abstract.** Grapevine (*Vitis vinifera* L.) embryos have an early developmental pattern which differs from the one observed in model angiosperms such as *Arabidopsis*, in that the plane of divisions show variations from one individual to another. Furthermore, the protoderm (the first tissue to differentiate) does not form in one step but rather, gradually with time during globule formation. In *Arabidopsis*, expression pattern of a particular lipid transfer protein (LTP) isoform, *AtLTP1*, appears to be related to protoderm establishment, and is considered as a molecular marker of its differentiation. To investigate whether a similar role for LTPs in the development of grapevine embryos, we investigated the expression pattern of *VvLTP1*, a *Vitis* homologue of *AtLTP1*, in somatic embryo development. Expression of the *GUS* reporter gene under the control of the *VvLTP1* promoter demonstrated that this LTP isoform is a marker of protoderm formation, and confirmed that this tissue forms sequentially over time. Ectopic expression of *VvLTP1* under the control of the 35S promoter led to grossly misshapen embryos, which failed to acquire bilateral symmetry and displayed an abnormal epidermal layer. These results indicate that a correct spatial or temporal expression, or both, of this gene is essential for grapevine embryo development.

**Additional keywords:** grapevine, lipid transfer protein, somatic embryogenesis, protoderm.

### Introduction

Discovered in the late 1950s in carrot cell suspensions (Steward *et al.* 1958), somatic embryogenesis (SE) is the capacity of somatic cells to form *in vitro* complete, viable embryos, through a process that resembles to zygotic embryogenesis. SE has been viewed as a tool for elite germplasms large-scale propagation of economically relevant crops. It has indeed been successfully used to do so in several plant species such as coffee (Ducos *et al.* 2003), date palm (Fki *et al.* 2003), Norway spruce (*Picea abies* L.; Nehra *et al.* 2005) or the medicinal plant Siberian ginseng (*Eleutherococcus senticosus* M.; Kang *et al.* 2006). SE also constitutes a good model to study the molecular and cellular regulations of the earliest developmental events in the life of higher plants (Zimmerman 1993; Chugh and Khurana 2002). In addition, SE plays an important role in genetic engineering of several crops: transgenic sugarcane (*Saccharum sp.* L.; Snyman *et al.* 2006), rubber tree (Blanc *et al.* 2006) or grapevine (*Vitis vinifera* L.; Mauro *et al.* 1995; Coutos-Thévenot *et al.* 2001) were regenerated thanks to SE.

Changes in expression of extracellular proteins have been associated with induction and initiation of SE in cell suspension cultures (Chugh and Khurana 2002). These secreted proteins encompass acidic class IV chitinases (De Jong *et al.* 1992),

$\beta$ -1,3-glucanases (Helleboid *et al.* 2000), arabinogalactan proteins (Stacey *et al.* 1990; Egertsdotter and von Arnold 1995) and also lipid transfer proteins (LTPs) (Sterk *et al.* 1991; Coutos-Thévenot *et al.* 1993; Blanckaert *et al.* 2002). LTPs are small (7–13 kDa) extracellular peptides, which seem to be ubiquitous in higher plants, where they are encoded by complex multigenic families, as exemplified by *Arabidopsis thaliana* (L.) Heynh and *Oryza sativa* L. (Kader 1996; Vignols *et al.* 1997; Arondel *et al.* 2000). All LTPs identified to date are synthesised as pre-proteins, with a secretion signal peptide in their N-terminus, which is cleaved in the mature protein (Kader 1996). They are characterised by a conserved motif of eight cysteine residues, which are involved in intramolecular disulfide bridges and stabilise the three-dimensional structure of these proteins, constituted by a bundle of four  $\alpha$ -helices partly surrounded by the C-terminus turns (Kader 1996). A hydrophobic tunnel is present inside the protein and can accommodate various hydrophobic molecules including fatty acids, phospholipids, cutin monomers, and jasmonic acid (Sterk *et al.* 1991; Zachowski *et al.* 1998; Douliez *et al.* 2000; Buhot *et al.* 2004).

LTP expression has been tightly associated with the establishment of the protoderm, the first differentiated tissue that appears during angiosperm zygotic and SE

(Sossountzov *et al.* 1991; Sterk *et al.* 1991; Vroemen *et al.* 1996; Zeng *et al.* 2006). The correct formation of the protoderm, which restricts the expansion of the cells at the surface of young globular embryos, is essential for the continuation of the developmental process (Dodeman *et al.* 1997). In dicotyledones that shows a regular cell division pattern (i.e. reproducible with high consistency between individuals) during globular embryo formation, the differentiation of the protoderm occurs at a precise developmental stage of the embryo proper: 16 cells in the model plant *A. thaliana*, 64 cells in *Daucus carota* L. (Mansfield and Briarty 1991; Sterk *et al.* 1991). In these plants, mRNA *in situ* hybridisation and promoter-*GUS* ( $\beta$ -glucuronidase) fusion expression analysis demonstrated that *LTP* genes are specifically expressed in protodermal cells of early globular embryos, and not in the internal cells (Sterk *et al.* 1991; Vroemen *et al.* 1996). Carrot (*Daucus carota* L.) *ts11* mutant embryos, which express the *EP2* *LTP* gene in all cells of their somatic globular embryos, have a morphologically aberrant protoderm, and are blocked early in their development (De Jong *et al.* 1993). *Arabidopsis knolle* mutant embryos, which show a uniform expression of the *AtLTP1* gene in all their cells, have major morphological defects (Vroemen *et al.* 1996). Thus, correct cell-specific expression pattern of *LTP* genes might be necessary for correct embryo development in plant species with regular globular embryo cell division pattern.

Regularity of cell division pattern during early embryogenesis is not, however, common to all flowering plants (Natesh and Rau 1984). In some dicotyledones, like, for example, cotton (*Gossypium hirsutum* L.) or grapevine, the plane of cell divisions that leads to the globular embryo differs from one individual to another (Pollock and Jensen 1964; Vallade *et al.* 1987). It is not possible to establish a clear cell fate map during early embryogenesis. The protoderm cell layer do not form in a synchronous manner at a precise stage, but rather by a series of localised periclinal divisions spread all over the development of the globular embryo, and little is known about the role of cell-specific *LTP* expression during zygotic or SE of these species. Accumulation of four *LTPs* isoforms in the culture medium of 41B grapevine cells during SE has been reported, even though neither kinetic of expression nor cell-specific expression patterns were studied (Coutos-Thévenot *et al.* 1993). In this cell suspension, SE, as well as *LTP* synthesis, are triggered by auxin starvation. We have shown that a particular isoform, VvLTP1 (*Vitis vinifera* L. *LTP1*, previously named P1) exhibit the highest expression level when the cells were submitted to auxin starvation (Gomès *et al.* 2003). We have recently reported the isolation of a genomic clone encoding VvLTP1, which contains its promoter region (Laquitaine *et al.* 2006).

The present work was conducted to better understand the role of VvLTP1 expression during grapevine somatic embryo development. The upstream region (2.8 kb) of the VvLTP1 gene translational start was fused to the *GUS* ( $\beta$ -glucuronidase) reporter gene to monitor the tissue-specific expression pattern of VvLTP1 during somatic embryo development. Accumulation of the VvLTP1 protein in the embryo culture media has been monitored over time during SE, to check whether high level of expression was associated with one (or more) specific

development stage(s). Finally, ectopic expression of the VvLTP1 gene under the control of the strong 35S promoter was realised to test the phenotypical effects of VvLTP1 overexpression on embryo development.

## Materials and methods

### Grapevine cell suspension culture and transformation

Grapevine (*Vitis vinifera* L.) cells (embryogenic line derived from the 41B rootstock) were established in the presence of 5  $\mu$ M NOA ( $\beta$ -naphtoxyacetic acid) at 22°C in the dark on glycerol-maltose medium (GM, Coutos-Thévenot *et al.* 1992), pH 5.8, and subcultured weekly under orbital shaking (110 rpm). For SE trials, the cells were washed three times in the same culture medium, without auxin (GM<sub>0</sub>) and inoculated at 0.1  $\mu$ L packed cell volume per mL of GM<sub>0</sub> culture medium. Cell suspensions were cultured for 18 days at 26°C, in the dark, under orbital shaking (110 rpm). In order to express VvLTP1 ectopically in 41B embryogenic cells, the VvLTP1 open reading frame (Gomès *et al.* 2003) was subcloned in the *Xho*I and *Xba*I restriction sites of the *pFB8* binary vector (Atanassova *et al.* 1995), downstream the strong 35S promoter. To generate embryos expressing the *GUS* ( $\beta$ -glucuronidase) reporter gene under the control of the VvLTP1 promoter (*pVvLTP1*, GenBank number DQ136029), a 2815-bp fragment was subcloned in the *Sac*I and *Bam*HI restriction sites of the *pBin19* binary vector (Frisch *et al.* 1995), in front of the *GUS* coding region. Embryogenic cells were transformed with these constructs using an *Agrobacterium* co-cultivation method (Mauro *et al.* 1995). The transgenic cells were selected and subcultured in the GM medium supplemented with paromomycin at 2  $\mu$ g mL<sup>-1</sup> and cefotaxime at 200  $\mu$ g mL<sup>-1</sup>.

### HPLC protein analysis and quantification

Media flasks containing 200 mL of GM<sub>0</sub> were inoculated as described above and cultured in the same culture conditions. The suspension cell cultures were filtered at different times on 0.45  $\mu$ m pore size filter. This conditioned culture medium was loaded onto a cationic exchange column (Selfpack POROS 8.5 by 0.5 cm; SP Sepharose, fast flow; Applied Biosystem, Foster City, CA, USA) previously equilibrated with 25 mM sodium phosphate buffer (pH 6.5) mounted on a HPLC system (Perkin Elmer Series 200 chain; Perkin Elmer, Waltham, MA, USA) controlled by the TotalChrom Chromatography Software. The bound proteins were eluted in one pick by the same buffer containing 0.5 M NaCl, in one step, and absorbance was monitored at 280 nm. The collected fractions, containing all the cationic proteins of the 200 mL of conditioned culture medium were further analysed by reverse-phase chromatography (Perkin Elmer Series 200, C4 Alltech Prosphere 300 column, 5  $\mu$ m). After equilibration in buffer A (0.1% trifluoroacetic acid, 1% acetonitrile; 98.9% water), elution was performed (temperature, 40°C; flow rate, 0.8 mL min<sup>-1</sup>) by two steps of linear gradient from 0 to 40% of buffer B (0.1% trifluoroacetic acid, 80% acetonitrile, 19.9% water) in 30 min and 40–100% of B in 5 min. Eluted proteins were monitored at 220 nm (to increase sensibility). The peak corresponding to VvLTP1 was identified by its R<sub>f</sub> (reference to front) value. Quantification of protein amounts by the

TotalChrom Chromatography Software was performed using a calibration curve established with previously purified VvLTP1 protein as a standard.

#### *RNA extraction and northern blot analysis*

RNA extraction and northern blotting were performed as described previously (Laquitaine *et al.* 2006), with no changes. For each extraction, three independent cell cultures were pooled before grinding. Radiolabelled probes were synthesised by random priming, using the Ready-to-Go DNA labelling beads kit and [<sup>32</sup>P]-dCTP (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). A 250-bp fragment corresponding to the 5'UTR of *VvLTP1* was used as matrix. Hybridisation signals were recorded with a Storm Phosphor Imager (Molecular Dynamics, Uppsala, Sweden). The 28S ribosomal RNA from *A. thaliana* was used as a probe to normalise hybridisation signals between the different lanes.

#### *β-Glucuronidase (GUS) histochemical assay*

Somatic embryos were incubated 2 min in 70% ethanol and submerged 30 min in fixation buffer (MES 10 mM pH 5.6, mannitol 300 mM, formaldehyde 0.3%). Samples were then washed three times with 50 mM sodium phosphate buffer (pH 7.0) and infiltrated 10 min with the GUS substrate buffer [NaPO<sub>4</sub> 50 mM (pH 7.0), K<sub>3</sub>Fe(CN)<sub>6</sub> 0.5 mM, K<sub>4</sub>Fe(CN)<sub>6</sub> 0.5 mM, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid 1 mM]. GUS enzymatic activity was allowed to proceed for 1 h at 37°C, and stopped by washing three times the embryos with 50 mM sodium phosphate (pH 7.0). Whole-mount observation of the GUS-stained embryos was done with a Euromex (Arnhem, The Netherlands) ZE-1657 stereomicroscope. For optical microscopy observation, GUS-stained embryos were further processed as described below.

#### *Photonic microscopy*

Samples were fixed by submersion 45 min in 0.5% glutaraldehyde, 1.5% paraformaldehyde, 0.05 M sodium phosphate (pH 7.4) and rinsed by 0.1 M sodium phosphate (pH 7.0) supplemented with sucrose 7.5% (w/v). Tissues dehydration was achieved by gradually increasing ethanol percentage from 20 to 100%. Dehydrated samples were then impregnated with the LR-white epoxy resin (London Resin Co. Limited, London, UK) by successive treatments: 15 min into resin: ethanol 1:2 (v/v), twice; 20 min into resin: ethanol 2:1 (v/v), twice; and 16 h into pure resin. The resin was polymerised for 24 h at 60°C. Polymerised samples were cut with a Leica (Göttingen, Germany) EMUC6 ultracut microtome for either toluidine blue (0.5% toluidine blue in 1% borax, pH 8.8) coloration or direct GUS coloration. Observation were made using a Zeiss (Göttingen, Germany) Axioplan microscope. In the case of GUS coloration, 2–3 μm thick slices were made to get enough signal. Such thickness can lead to the presence of ridges observed at high magnification.

For SE kinetic monitoring, the developing embryos were observed as whole-mount under a Euromex ZE-1657 stereomicroscope.

#### *Scanning electron microscopy*

Somatic embryos were fixed 1 h at room temperature in a 2.5% (w/v) glutaraldehyde solution (in 10 mM sodium phosphate buffer, pH 6.0) and washed twice in 10 mM phosphate buffer. Dehydration was achieved by successive incubations: 2 × 2 min in 50% (v/v) acetone, 2 × 5 min 70% (v/v) acetone, 2 × 15 min 90% (v/v) acetone. The samples were then desiccated in a CO<sub>2</sub> critical point dryer (BALZERS, CPD 030) and coated with 25 nm gold (BAL-TEC, SCD 005). Observations were made with a JEOL (Tokyo, Japan) JSM-840A Scanning electron microscope.

### **Results**

#### *VvLTP1 promoter activity during 41B somatic embryo development*

To localise *VvLTP1* expression during SE, the *GUS* gene was placed under control of the *VvLTP1* promoter region. Histochemical GUS activity was performed, and the GUS-stained tissues were observed by photonic microscopy. At day 0 just before shifting the cells to auxin-free culture medium to induce SE (see Materials and methods), GUS activity was observed rather uniformly in the proembryogenic masses (PEMs, Fig. 1A). At globular stage, expression of the *GUS* gene was restricted to the external cell layer (Fig. 1B). At early globular stage (6 days after SE initiation), GUS activity was not present in all peripheral cells of the embryos but only in a localised area, usually with regularly shaped and organised cells (Fig. 1C). At the heart stage (10–12 days after SE initiation), GUS activity was detected in all the protoderm cells (Fig. 1D, E). In mature (Fig. 1F) or germinated embryos (Fig. 1H), the cotyledons and the hypocotyl exhibit GUS coloration; whereas most of the root was not stained, except for the very tip (Fig. 1E, F). On the cotyledons of germinated embryos, GUS staining was detected mostly on the abaxial side (Fig. 1I).

#### *Expression kinetics of VvLTP1 during SE in wild-type and p35S::VvLTP1 cell suspension*

In order to compare *VvLTP1* mRNA accumulation in wild-type and *p35S::VvLTP1* embryos, RNA gel blot analysis were performed after shifting the PEMs to auxin-free medium (Fig. 2A, B). In wild-type 41B cells, *VvLTP1* mRNA accumulation followed a mildly accentuated bell-shape curve, with maximal hybridisation signals at days 12–14 post initiation of SE. The signal then dropped on day 18 so that it was only half that measured on day 14. In transgenic embryos, the levels of *VvLTP1* mRNA detected were higher than those measured in wild-type embryos at any time of the culture, with the exception of day 10, strongly suggesting an overexpression of the *VvLTP1* gene. To confirm the overexpression, the VvLTP1 protein was quantified by HPLC analysis in the cell suspension culture media, as a function of time, after shifting the cells to the auxin-free medium (Fig. 3). In wild-type embryos cultures, the amount of VvLTP1 protein secreted in the culture media increased slowly from 0.05 to 0.15 μg mL<sup>-1</sup> between the 6th and 12th day after SE initiation, reached its maximum (0.62 μg mL<sup>-1</sup>) at day 14 and dropped by half between the 16th and the 18th day. In the *p35S::VvLTP1* embryos, the amounts of VvLTP1 protein secreted are always higher than the ones measured for wild-type embryos. Moreover, the protein kept on accumulating between the 16th and

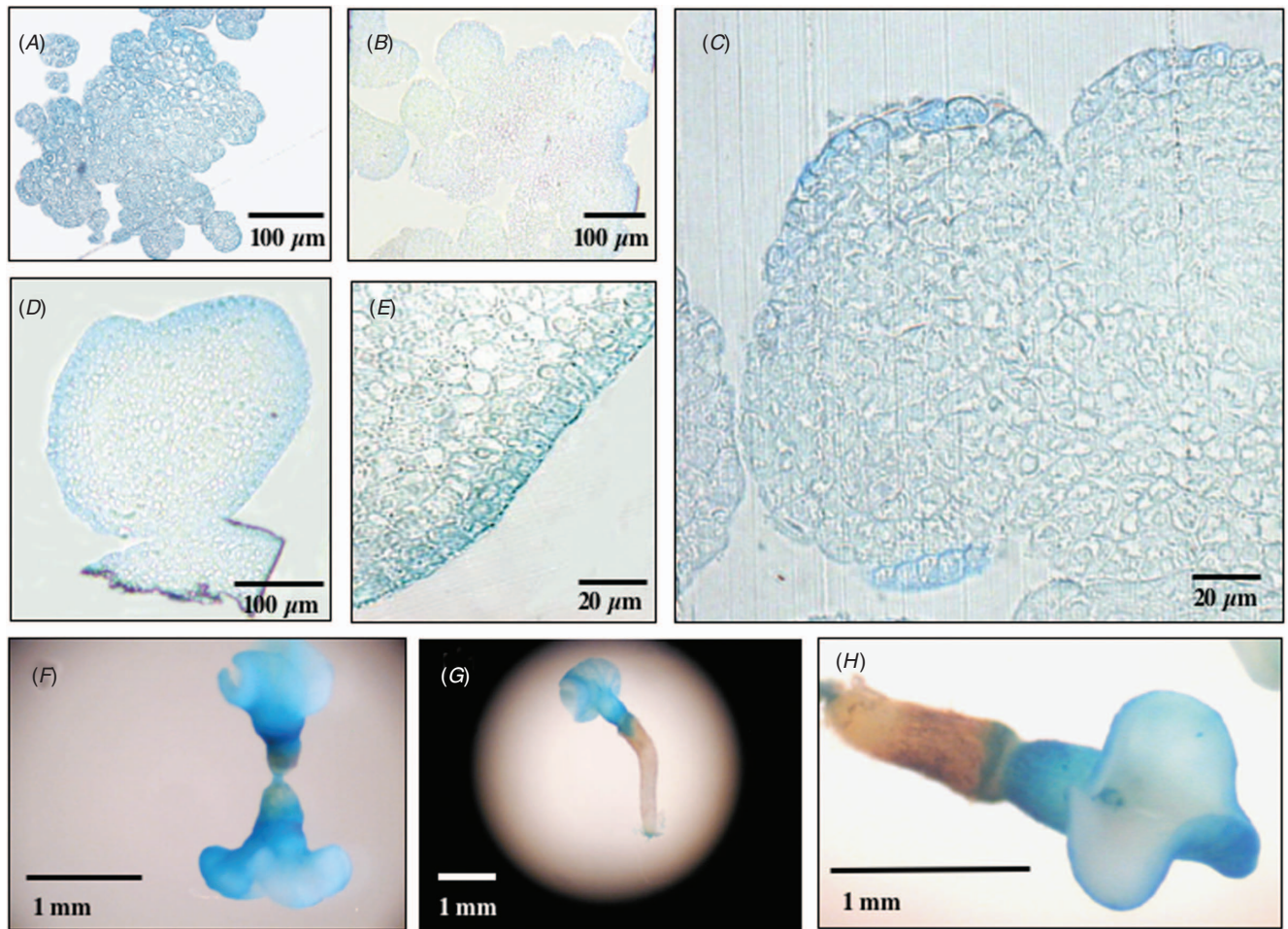


**Fig. 1.** Activity of the GUS reporter gene in transgenic 41B embryos of grapevine containing the *VvLTP1* promoter-*GUS* fusion. (A–E) Semi-thin sections of various developmental stages. (A) Proembryogenic masses. (B) Early globule formation, general view. (C) Detailed view of a globular embryo with blue patches of GUS activity clearly visible in the peripheral cell layer. (D) Heart-shaped embryo with epidermal GUS activity. (E) Detail of GUS labelling in heart stage embryo epidermis. (F–H) Whole mounts of (F) mature and (G, H) germinated embryos, observed under the stereomicroscope.

18th days, to reach  $1.89 \mu\text{g mL}^{-1}$ , compared with  $0.33 \mu\text{g mL}^{-1}$  for the wild-type embryos at the same time.

#### *Somatic embryo formation in wild-type and p35S::VvLTP1 cell suspensions*

In order to compare somatic embryos development, wild-type and *p35S::VvLTP1* transgenic PEMs were shifted from auxin-containing to auxin-free culture media, and observed every two days under the stereomicroscope (Fig. 4). Six days after initiating SE, globular-shaped embryos of similar appearance were formed in both wild-type and *VvLTP1* overexpressing embryos. At the 8th day, most of the wild-type embryos undergo the globular-heart shape transition, whereas most of the transgenic ones retained their globular shape. Two days later, heart-shaped embryos were visible in the wild-type cell culture, but not in the transgenic one where the embryo enlarged uniformly, retaining their globular shape and failing to acquire their bilateral symmetry. Between the 12th and the 16th days, the wild-type embryos switched from the heart to the torpedo stage (day 16) and to mature embryos, with fully

formed cotyledons and hypocotyl-root axis were visible at the 18th day. During the same period, the transgenic embryos simply elongates along their root-hypocotyl axis, still failing to develop cotyledons and to acquire bilateral symmetry. Moreover, secondary embryos started to bud from the first transgenic ones (Fig. 4, white stars). Embryos derived from cells transformed with the empty vector (*VvLTP1* coding region absent, negative control), showed a development identical to wild-type ones (Fig. 4, *pFB8*), indicating that the observed phenotype was not due to *Agrobacterium* transformation and subsequent selection process.

#### *Scanning surface electron microscopy of wild-type and p35S::VvLTP1 somatic embryos*

As demonstrated by promoter-*GUS* fusion, *VvLTP1* expression was gradually restricted to the protoderm during the transition from globular to heart stage transition, whereas the rest of the embryos (i.e. internal cells) did not appear to have detectable GUS activity. Considering that, as previously shown in several plant species, the correct formation of the protoderm is essential for the

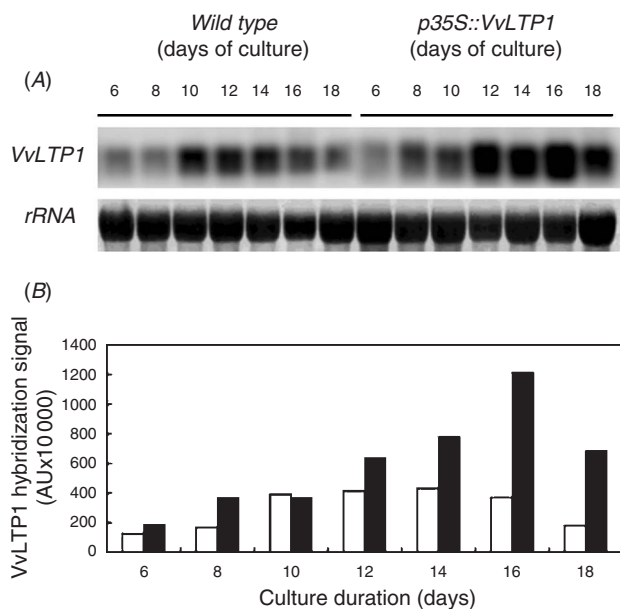


**Fig. 2.** RNA gel blot analysis of *VvLTP1* in both wild-type (white bars) and *p35S::VvLTP1* transgenic (black bars) 41b grapevine cells as a function of culture time in the auxin-free medium. (A) *VvLTP1* and 18S ribosomal (*rRNA*) hybridisation signals. (B) Signal quantification with the phosphorimager.

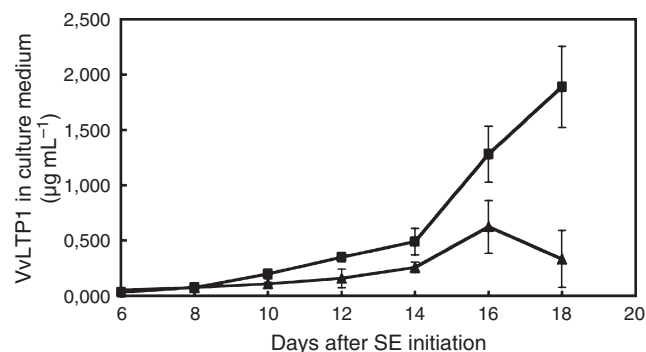


**Fig. 3.** Quantification of *VvLTP1* protein in the culture medium of wild-type (▲) and *p35S::VvLTP1* transgenic (■) grapevine cells, as a function of time. Quantification was performed by HPLC, using a calibration curve established with pure *VvLTP1* protein, as described in Materials and methods.

proper development of the embryos, ectopic expression of *VvLTP1* under the control of the strong constitutive 35S promoter was expected to disturb the protoderm organisation in the transgenic embryos. Scanning surface electron microscopy was then used to compare the protoderm of wild-type and *p35S::VvLTP1* embryos (Fig. 5). Ten days after triggering SE, when the wild-type embryos reached the heart stage, the surface of wild-type embryos appeared to be smooth at low magnification (Fig. 5A), but a transgenic embryo of the same age exhibited an irregular, bumpy surface (Fig. 5B). Higher magnification observations showed that the protoderm of the wild-type embryos is constituted by isodiametric, regularly packed cells (Fig. 5C). In contrast, the protoderm of the transgenic embryo shows severe morphological alterations, with irregular, more

densely packed cells (Fig. 5D). Protuberant domes were also visible on the cell surfaces of the *p35S::VvLTP1* embryos. Moreover, optical microscopy performed on semi-thin sections (Fig. 6) showed that in addition to this protoderm defect, the transgenic embryos did not exhibit an organised apical region, as neither cotyledon primordia nor apical meristem were visible.

## Discussion

This work was conducted in order to better understand the role of LTPs during somatic embryo development in grapevine, a plant species with an irregular cell division pattern during early embryo developmental stages (i.e. globule formation). This led us to analyse the expression kinetic of *VvLTP1* gene by monitoring transcripts levels and protein accumulation during SE. *VvLTP1* expression levels are not constant during embryo formation: the accumulation pattern of *VvLTP1* mRNA exhibits a bell-shape curve, with a maximum around 12 and 14 days after SE initiation, a period of time within which the embryos undergo the globular-heart shape transition, and shift from radial to bilateral symmetry. This is in agreement with the results already obtained on plant species with regular embryo cell division patterns such as carrot or *Arabidopsis* (Stern *et al.* 1991; Vroemen *et al.* 1996). Maximal protein accumulation in the embryo culture media occurs 2 days later, at the 16th day. This time-lag probably reflects the delay between transcription, translation, and finally secretion through the secretory pathway.

Promoter-GUS studies allowed us to map the expression pattern of *VvLTP1*, at the tissue level. The results clearly indicate a shift in the *VvLTP1* promoter activity pattern, from a mostly uniform expression in all cells of the PEMs to a protoderm specific expression. As soon as the protoderm appears in the globular embryos, the localisation of the GUS activity is restricted to the protodermal cells; exhibiting a patchwork pattern for *VvLTP1* expression between the differentiated and non-differentiated portions of the globules periphery. This fits with the role of *VvLTP1* as a marker of protodermal cell layer differentiation during grapevine somatic embryo development, as demonstrated for angiosperms with regular division patterns such as carrot or *Arabidopsis* (Stern *et al.* 1991; Vroemen *et al.* 1996). However, unlike what has been demonstrated for those latter species, the expression is not detected in all cells of the outer layer, but only in the area with smooth, regularly packed, periclinally oriented cells – in other words, in the embryo surface area containing established protodermal cells. The grapevine situation is closer to the one described in gymnosperms, in which the protoderm cell layer is less clearly defined during early embryogenesis and forms through a series of periclinal divisions spread over time (Singh 1978; Sabala *et al.* 2000). Later, at the heart stage, all the peripheral cells show GUS activity, indicating that the protoderm is fully developed, as described for SE in angiosperm model plants such as *Arabidopsis* or carrot (Chugh and Khurana 2002). The precise role of LTP proteins in the protoderm formation is not yet known, but it has been postulated they may act as carriers for cutin deposition in protodermal cells (Kader 1996). When the embryo reaches maturity and germinates, *VvLTP1* promoter is active mainly in the aerial part-to-be of the young plants, i.e. cotyledons and hypocotyl; whereas no activity was

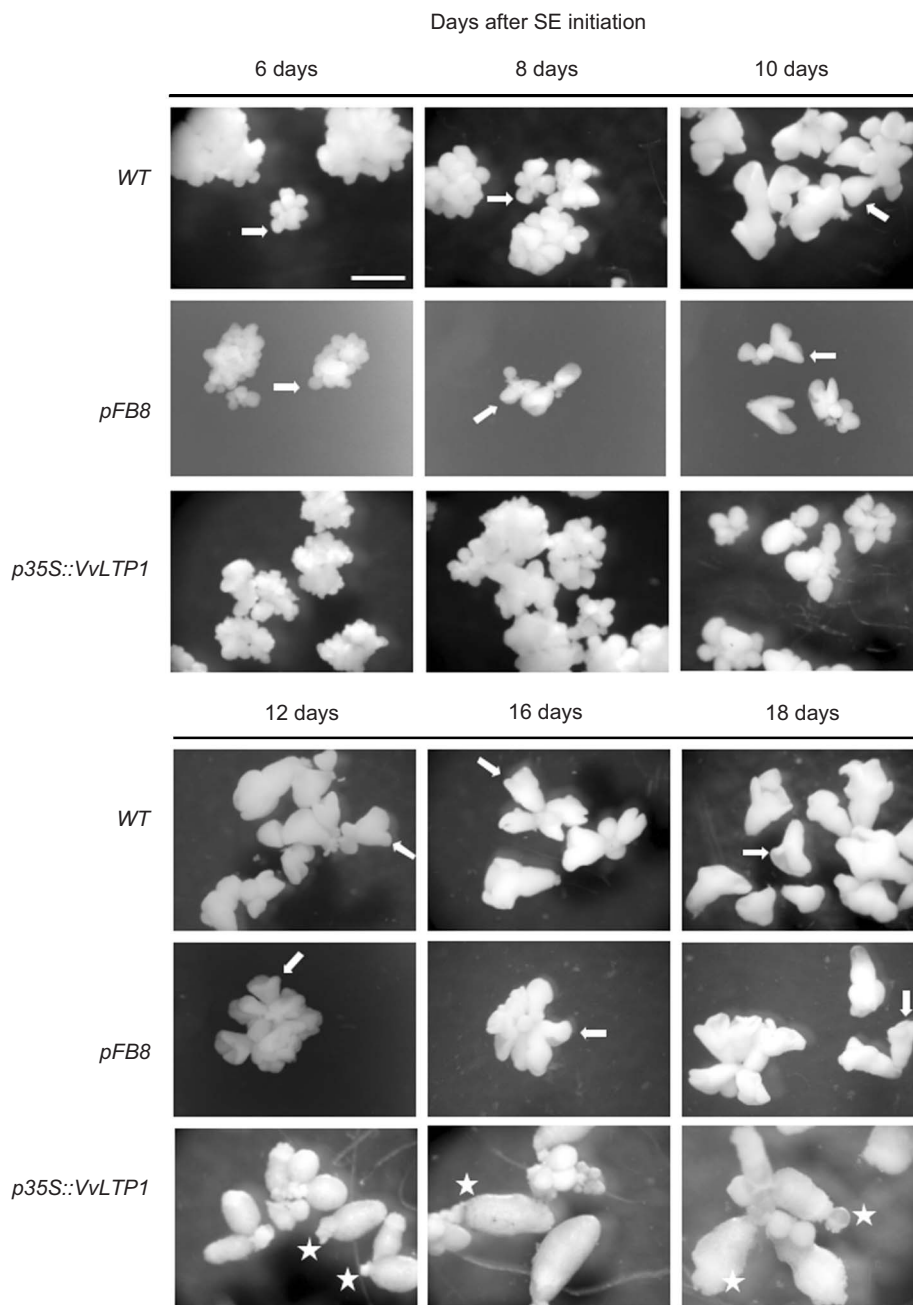


**Fig. 4.** Development kinetics of 41B somatic embryos in grapevine. Proembryogenic masses subcultured in glycerol-maltose media (see Materials and methods) in the presence of auxin ( $5 \mu\text{M}$  NOA) were shifted to auxin-free medium and somatic embryogenesis (SE) was allowed to proceed for 18 days. Pictures were taken at the most characteristic developmental stages for both wild-type (WT), empty vector control (pFB8) and *VvLTP1* overexpressing embryos (p35S::VvLTP1). In both wild-type and pFB8 picture series, the arrows indicate a typically shaped embryo for each developmental stage: globular (6 days), transition (8 days), heart (10 days), late heart (12 days), torpedo (16 days) and mature (18 days). In the *VvLTP1* overexpressing embryo picture series, \* indicate the presence of secondary embryos.

detected in roots, with the exception of the root tip. Such an expression pattern is common for *LTP* genes (Sossountzov *et al.* 1991; Thoma *et al.* 1994; Kader 1996), even though a root-specific *LTP* isoform has been isolated in *Phaseolus vulgaris* L. (Choi *et al.* 1996).

To further analyse the role of the *VvLTP1* gene in grapevine SE, the corresponding cDNA has been ectopically overexpressed under the control of the strong, constitutive 35S promoter, which confers high expression levels and very little cell specificity (Benfey and Chua 1990). P35S::VvLTP1

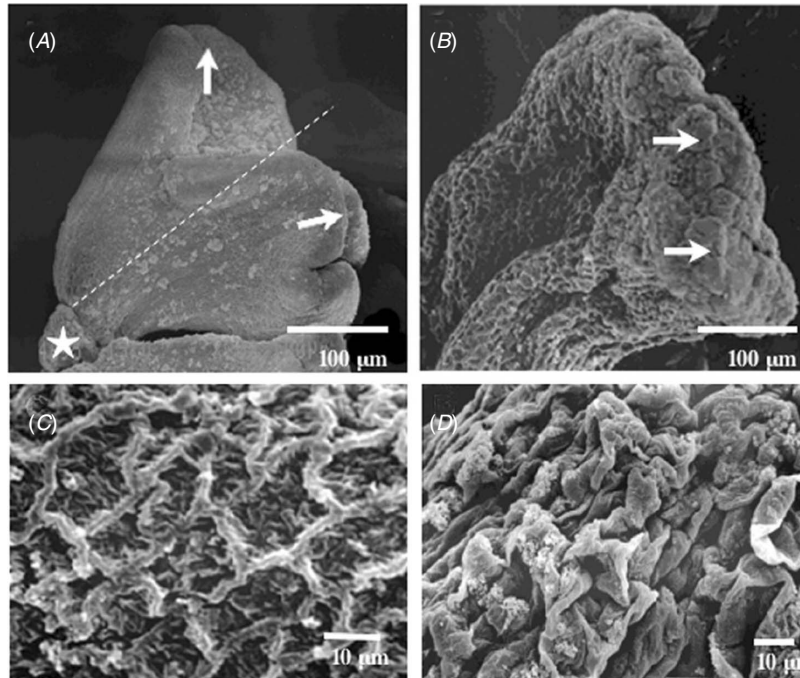


**Fig. 5.** Surface scanning electron microscopy of 10 days old wild-type (left) and *p35S::VvLTP1* (right) somatic embryos in grapevine. (A) Wild-type embryo with a regularly shaped, smooth-looking surface, correctly formed cotyledon primordia (arrows), and a bilateral symmetry (dashed line). The stars indicate the basal area of the embryo. (B) Transgenic embryo showing drastic shape changes and apex disorganisation, with aborted leaf primordia, and no bilateral symmetry. (C) Detailed view of regularly-shaped and well ordered epidermal cells in a wild-type embryo. (D) Detailed view of irregularly-shaped epidermal cells with erratic arrangement in a transgenic embryo.

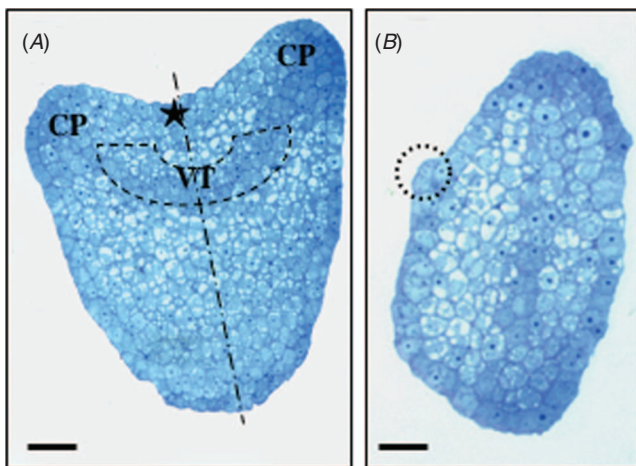


**Fig. 6.** Comparative shape of 12-day-old wild-type and *p35S::VvLTP1* embryos of grapevine. (A) Longitudinal section in a wild-type embryo showing its heart shape with cotyledon primordia (CP) and vascular traces (VT). The dashed line indicates the embryo axis and the star the apical region. (B) Section in a transgenic embryo showing its irregular shape, with outgrowths on the epidermis indicating secondary embryo initiation (dotted-line circle), absence of apical organisation, and cotyledon primordia. Glutaraldehyde fixation, Spurr resin embedding, semi-thin sections, toluidine blue staining. Scale bars are 100 µm.

somatic embryos were grossly misshaped, displaying an abnormally formed epidermis with an irregular, bumpy surface. Such phenotypes, particularly the irregular epidermis, have been linked to an incorrect spatial and temporal expression pattern of LTP in mutants embryos with a defective protoderm like the *A. thaliana* mutants *raspberry* (Yadegari *et al.* 1994) and *knolle* (Jürgens *et al.* 1991) or the carrot *ts11* mutant (De Jong *et al.* 1992). In both *knolle* or *ts11* mutants, the expression of a LTP gene (*AtLTP1* and *EP2*, respectively) which is normally restricted to the protoderm cell layer, is expressed uniformly in globular embryos (De Jong *et al.* 1993; Vroemen *et al.* 1996), and their disorganised epidermal cell layer is very similar to the one observed for *p35S::VvLTP1* embryos. Beside this, *p35S::VvLTP1*, also failed to achieve the transition from the globular to heart-shape stage and kept elongating along the root-hypocotyl axis, without developing any cotyledonary structure nor a proper apical meristem. In this way, they are very much like the *gurke* mutant of *Arabidopsis*, which is basically reduced to a root-hypocotyl axis (Torres-Ruiz *et al.* 1996). The expression profile of *AtLTP1* in the *gurke* background has not been described, but considering our results, it is very likely to be disturbed. The use of the 35S promoter to drive *VvLTP1* expression has indeed two effects; loss of tissue-specific expression, and increased protein amounts in the embryo culture media. To try to distinguish between them,

we have performed a SE trial in which the embryos were transferred to a fresh culture media daily in order to prevent VvLTP1 protein accumulation (data not shown). This did not, however, lead to *p35S::VvLTP1* embryo rescue, indicating that cell-specific expression of the *VvLTP1* gene, rather than its expression level, was responsible for the phenotype.

Our results provide the first functional evidence that *VvLTP1* is not only a marker, but might also be related to epidermal layer and embryo development in a species with an irregular cell division pattern during early embryo developmental stages. This later point, however, can only be assessed using a *Vvltpl* knockout mutant derived from T-DNA or transposon insertion lines; tools which are not currently available on grapevine. The development pattern of grapevine embryos is closer to the one of gymnosperm somatic embryos, in which the protodermal cell layer forms gradually over time. Changes in level of expression and especially modification of the cell-specific pattern of expression of this gene completely destroy the structure and development of somatic embryos.

### Acknowledgements

This work was supported by The University of Poitiers, the 'Centre National pour la Recherche Scientifique' and the 'Conseil Régional Poitou-Charentes'. We acknowledge the 'Service Interdisciplinaire de Microscopie et d'Imagerie Scientifique', UFR 'Sciences Fondamentales et Appliquées' for excellent technical assistance.

### References

- Arondel V, Vergnolle C, Cantrel C, Kader J (2000) Lipid transfer proteins are encoded by a small multigene family in *Arabidopsis thaliana*. *Plant Science* **157**, 1–12. doi: 10.1016/S0168-9452(00)00232-6
- Atanassova R, Favet N, Martz F, Chabbert B, Tollier M-T, Monties B, Fritig B, Legrand M (1995) Altered lignin composition in transgenic tobacco expressing *O*-methyltransferase sequences in sense and antisense orientation. *The Plant Journal* **8**, 465–477. doi: 10.1046/j.1365-3113X.1995.8040465.x
- Benfey PN, Chua NH (1990) The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. *Science* **250**, 959–966. doi: 10.1126/science.250.4983.959
- Blanc G, Baptiste C, Oliver G, Martin F, Montoro P (2006) Efficient *Agrobacterium tumefaciens*-mediated transformation of embryogenic calli and regeneration of *Hevea brasiliensis* Müll Arg. plants. *Plant Cell Reports* **24**, 724–733. doi: 10.1007/s00299-005-0023-3
- Blanckaert A, Belingheri L, Sautiere PE, Vasseur J, Hilbert JL (2002) 9-kDa acidic and basic nsLTP-like proteins are secreted in the culture-medium conditioned by somatic embryogenesis in *Cichorium* L. *Plant Physiology and Biochemistry* **40**, 339–345. doi: 10.1016/S0981-9428(02)01382-7
- Buhot N, Gomès E, Milat M-L, Ponchet M, Marion D, Lequeu J, Delrot S, Coutos-Thévenot P, Blein J-P (2004) Modulation of the biological activity of a tobacco LTP1 by lipid complexation. *Molecular Biology of the Cell* **15**, 5047–5052. doi: 10.1091/mbc.E04-07-0575
- Choi DW, Song JY, Oh MH, Lee JS, Moon J, Suh SW, Kim SG (1996) Isolation of a root-specific cDNA encoding a ns-LTP-like protein from the roots of bean (*Phaseolus vulgaris* L.) seedlings. *Plant Molecular Biology* **30**, 1059–1066. doi: 10.1007/BF00020816
- Chugh A, Khurana P (2002) Gene expression during somatic embryogenesis – recent advances. *Current Science* **83**, 715–729.
- Coutos-Thévenot P, Goebel-Tourand I, Mauro MC, Jouanneau JP, Boulay M, Deloire A, Guern J (1992) Somatic embryogenesis from grapevine cells. I-Improvement of embryo development by changes in culture conditions. *Plant Cell, Tissue and Organ Culture* **29**, 125–133. doi: 10.1007/BF00033617
- Coutos-Thévenot P, Jouenne T, Maes O, Guerbette F, Grosbois M, Le Caer JP, Boulay M, Deloire A, Kader J, Guern J (1993) Four 9-kDa proteins excreted by somatic embryos of grapevine are isoforms of lipid-transfer proteins. *European Journal of Biochemistry* **217**, 885–889. doi: 10.1111/j.1432-1033.1993.tb18317.x
- Coutos-Thévenot P, Poinssot B, Yean H, Buffard D, Esnault R, Hain R, Boulay M (2001) Tolerance of grapevine 41B rootstock in *in vitro* transgenic plants expressing the stilbene synthase *vst1* gene under the control of a fungi inducible PR 10 promoter. *Journal of Experimental Botany* **358**, 949–959.
- De Jong AJ, Cordewener J, Lo Schiavo F, Terzi M, Vandekerckhove J, Van Khammen A, de Vries SC (1992) A carrot somatic embryo mutant is rescued by chitinase. *The Plant Cell* **4**, 425–433. doi: 10.2307/3869444
- De Jong AJ, Heidstra R, Spaijk HP, Hartog MV, Meijer EA *et al.* (1993) *Rhizobium* lipooligosaccharides rescue a carrot somatic embryo mutant. *The Plant Cell* **5**, 615–620. doi: 10.2307/3869804
- Dodeman VL, Ducreux G, Kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. *Journal of Experimental Botany* **48**, 1493–1509.
- Douliez JP, Michon T, Marion D (2000) Steady-state tyrosine fluorescence to study the lipid-binding properties of a wheat non-specific lipid-transfer protein (nsLTP1). *Biochimica et Biophysica Acta* **1467**, 65–72. doi: 10.1016/S0005-2736(00)00197-8
- Ducos JP, Alentorn R, Reano JF, Kanchmanai C, Deshayes A, Pétiard V (2003) Agronomic performance of *Coffea canephora* P. trees derived from large-scale somatic embryo production in liquid medium. *Euphytica* **131**, 215–223. doi: 10.1023/A:1023915613158
- Egertsdotter U, von Arnold S (1995) Importance of arabinogalactan proteins for the development of somatic embryos of Norway spruce (*Picea abies*). *Physiologia Plantarum* **93**, 334–345. doi: 10.1111/j.1399-3054.1995.tb02237.x
- Fki L, Masmoudi R, Drira N, Rival A (2003) An optimised protocol for plant regeneration from embryogenic suspension cultures of date palm. *Plant Cell Reports* **21**, 517–524.
- Frisch DA, Harris-Haller LW, Yokubaitis NT, Thomas TL, Hardin SH, Hall TC (1995) Complete sequence of the binary vector Bin 19. *Plant Molecular Biology* **27**, 405–409. doi: 10.1007/BF00020193
- Gomès E, Sagot E, Gaillard C, Laquittaine L, Poinssot B, Sanjeouand H-Y, Delrot S, Coutos-Thévenot P (2003) Non specific lipid-transfer protein genes expression in grape (*Vitis* sp.) cells in response to fungal elicitor treatments. *Molecular Plant-Microbe Interactions* **16**, 456–464. doi: 10.1094/MPMI.2003.16.5.456
- Helleboid S, Chapman A, Hendriks T, Inze D, Vasseur J, Hilbert JL (2000) Cloning of beta-1,3-glucanases expressed during *Cichorium* somatic embryogenesis. *Plant Molecular Biology* **42**, 377–386. doi: 10.1023/A:1006344024877
- Jürgens G, Mayer U, Torres-Ruiz RA, Berleth T, Miséra S (1991) Genetic analysis of pattern formation in the *Arabidopsis thaliana*. *Development* **1**, 27–38.
- Kader J-C (1996) Lipid-transfer protein in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**, 627–654. doi: 10.1146/annurev.arplant.47.1.627
- Kang TJ, Lee WS, Choi EG, Kim JW, Kim BG, Yang MS (2006) Mass production of somatic embryos expressing *Escherichia coli* heat-labile enterotoxin b subunit in Siberian ginseng. *Journal of Biotechnology* **121**, 124–133. doi: 10.1016/j.jbiotec.2005.07.020
- Laquittaine L, Gomès E, François J, Marchive C, Pascal S, Hamdi S, Atanassova R, Delrot S, Coutos-Thévenot P (2006) Molecular basis of ergosterol-induced protection of grape against *Botrytis cinerea*: induction of type I lipid transfer protein promoter activity, WRKY, and stilbene synthase gene expression. *Molecular Plant-Microbe Interactions* **19**, 1103–1112. doi: 10.1094/MPMI.19.1103
- Mansfield SG, Briarty LG (1991) Early embryogenesis in *Arabidopsis thaliana*. II. The developing embryo. *Canadian Journal of Botany* **69**, 461–476. doi: 10.1139/b91-063

- Mauro MC, Toutain S, Walter B, Pinck L, Otten L, Coutos-Thévenot P, Deloire A, Barbier P (1995) High efficiency regeneration of grapevine plants transformed with the GLFV coat protein gene. *Plant Science* **112**, 97–106. doi: 10.1016/0168-9452(95)04246-Q
- Natesh S, Rau MA (1984) The embryo. In 'Embryology of angiosperm'. (Ed. BM Johri) pp. 377–443. (Springer-Verlag: Berlin)
- Nehra NS, Becwar MR, Rottmann WH, Pearson L, Chowdhury K *et al.* (2005) Forest biotechnology: innovative methods, emerging opportunities. *In Vitro Cellular & Developmental Biology. Plant* **41**, 701–717. doi: 10.1079/IVP2005691
- Pollock EG, Jensen WA (1964) Cell development during early embryogenesis in *Capsella* and *Gossypium*. *American Journal of Botany* **51**, 915–921. doi: 10.2307/2440240
- Sabala I, Elfstrand M, Farbos I, Clapham D, von Arnold S (2000) Tissue-specific expression of Pa18, a putative lipid transfer protein gene, during embryo development in Norway spruce (*Picea abies*). *Plant Molecular Biology* **42**, 461–478. doi: 10.1023/A:1006303702086
- Singh H (1978) Embryology of gymnosperms. In 'Handbuch der Pflanzenanatomie'. (Eds W Zimmerman, Z Carlquist, P Ozeda, ED Wulff) pp. 187–241. (Borntraeger Science Publisher: Berlin)
- Snyman SJ, Meyer GM, Richards JM, Haricharan N, Ramgareeb S, Hockett BI (2006) Refining the application of direct embryogenesis in sugarcane: effect of the developmental phase of leaf disc explants and the timing of DNA transfer on transformation efficiency. *Plant Cell Reports* **25**, 1016–1023. doi: 10.1007/s00299-006-0148-z
- Sossountzov L, Ruiz-Avila L, Vignols F, Jolliot A, Arondel V *et al.* (1991) Spatial and temporal expression of a maize lipid transfer protein gene. *The Plant Cell* **3**, 923–933. doi: 10.2307/3869155
- Stacey NJ, Roberts K, Knox JP (1990) Patterns of expression of the JIM4 arabinogalactan-protein epitope in cell cultures and during somatic embryogenesis in *Daucus carota* L. *Planta* **180**, 285–292. doi: 10.1007/BF00194009
- Sterk P, Booi H, Schellekens GA, Van Kammen A, De Vries SC (1991) Cell-specific expression of the carrot EP2 lipid transfer protein gene. *The Plant Cell* **3**, 907–921. doi: 10.2307/3869154
- Steward FC, Mapes MO, Mears K (1958) Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. *American Journal of Botany* **45**, 705–708. doi: 10.2307/2439728
- Thoma S, Hecht U, Kipper A, Botella J, De Vries SC, Somerville C (1994) Tissue specific expression of a gene encoding a cell wall-localized lipid transfer protein from *Arabidopsis*. *Plant Physiology* **105**, 35–45. doi: 10.1104/pp.105.1.35
- Torres-Ruiz RA, Lohner A, Jürgens G (1996) The GURKE gene is required for normal organization of the apical region in the *Arabidopsis* embryo. *The Plant Journal* **10**, 1005–1016. doi: 10.1046/j.1365-313X.1996.10061005.x
- Vallade J, Alabouvette J, Chabber AM (1987) The development of the zygotic embryo of *Vitis vinifera* L. *Vitis* **26**, 215–224.
- Vignols F, Wigger M, Garcia-Garrido JM, Grellet F, Kader JC, Delseny M (1997) Rice lipid transfer protein (LTP) genes belong to a complex multigene family and are differentially regulated. *Gene* **195**, 177–186. doi: 10.1016/S0378-1119(97)00137-6
- Vroemen CW, Langeveld S, Mayer U, Ripper G, Jürgens G, Van Kammen A, de Vries SC (1996) Pattern formation in the *Arabidopsis* embryo revealed by position-specific lipid transfer protein gene expression. *The Plant Cell* **8**, 783–791. doi: 10.2307/3870281
- Yadegari R, de Palva GR, Laux T, Koltunow AM, Apuya N, Zimmerman JL, Fisher RL, Harada JJ, Goldberg RB (1994) Cell differentiation and morphogenesis are uncoupled in *Arabidopsis* *raspberry* embryos. *The Plant Cell* **6**, 1713–1729. doi: 10.2307/3869903
- Zachowski A, Guerbette F, Grosbois M, Jolliot-Croquin A, Kader J-C (1998) Characterisation of acyl binding by a plant lipid-transfer protein. *European Journal of Biochemistry* **257**, 443–448. doi: 10.1046/j.1432-1327.1998.2570443.x
- Zeng F, Zhang X, Zhu L, Tu L, Guo X, Nie Y (2006) Isolation and characterization of genes associated to cotton somatic embryogenesis by suppression subtractive hybridization and microarray. *Plant Molecular Biology* **60**, 167–183. doi: 10.1007/s11103-005-3381-x
- Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. *The Plant Cell* **5**, 1411–1423. doi: 10.2307/3869792

Manuscript received 24 December 2007, accepted 2 May 2008