

Four 9-kDa proteins excreted by somatic embryos of grapevine are isoforms of lipid-transfer proteins

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Four 9-kDa small extracellular proteins produced by embryogenic cultures in the absence of auxin have been purified from the extracellular medium of grapevine somatic embryo cultures through cation-exchange chromatography and hydrophobic-interaction chromatography. The partial amino-acid sequences reflect high similarities between the four proteins as well as with the sequences established for carrot, spinach, millet and maize nonspecific lipid-transfer proteins. All these sequences show conservation of three cysteines at positions 4, 14 and 30–32, as well as glycine, valine, tyrosine and lysine residues at positions 5, 7, 17 and 37, respectively. *In-vitro* lipid-transfer assays reveal that the four proteins catalyze the transfer of phosphatidylcholine from liposomes towards mitochondria with an efficiency similar or higher than that of a purified maize lipid-transfer protein.

Somatic embryogenesis, i. e. the ability of cultured somatic cells to form embryos, has been extensively studied in carrot as a model system for the determination of factors controlling this process [1–3]. It has been established that this phenomenon requires two main steps including the induction of the embryogenic potential by exogenous auxin, allowing the emergence of proembryogenic masses (PEMs) consisting of groups of 10–50 cells and the transfer of the cultures to auxin-free medium which leads to the formation of somatic embryos from PEMs through the successive stages of embryo development in plants (globular, heart-shaped and torpedo forms). This multistep process, initially established for carrot cells, has been successfully used for other plants [4], including grapevine [Krull, W. R. & Narragansett, R. I. (1985) US patent number 4.532.733] [6].

Recent observations showed that several specific proteins, secreted into the culture medium, play a key role in the process of somatic embryo development in carrot [2, 7]. We previously presented evidence for strong similarities between grapevine and carrot somatic embryogenesis as far as the influence of auxin on extracellular protein patterns and embryo differentiation is considered [8]. Moreover, several extracellular proteins and glycoproteins which were evidenced in grapevine culture medium shared similarities in terms of molecular masses and enzymic activities with those described in the carrot system [8].

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Abbreviations. LTP, lipid-transfer protein; ns, nonspecific; PEMs, proembryogenic masses; –aux, auxin-free medium; +aux, auxin-supplemented medium.

Note. The novel amino-acid sequence data published here have been submitted to the EMBL sequence data bank(s).

One of the main characteristics of the culture medium of grapevine embryo suspensions is the abundance of a protein band corresponding to one or several proteins with a molecular mass in the range 10 kDa. We showed that the corresponding protein(s), totally under the negative control of auxin, is (are) recognized by a maize lipid-transfer-protein-(LTP)-specific polyclonal antibody [8]. Furthermore, experiments performed on total proteins harvested from auxin supplemented (+aux) or auxin free (–aux) culture medium showed that lipid-transfer activity was restricted to the –aux condition. This situation is similar to that described for embryogenic carrot-cell cultures where a 10-kDa protein accumulates and exhibits lipid-transfer activity as well as immunological cross-reactivity with the maize LTP-specific polyclonal antibody [9]. A cDNA corresponding to this protein has been isolated by screening an expression library [9]. The derived protein sequence exhibits 54% similarity with the nonspecific LTP (nsLTP) purified from maize seeds [10]. Other nsLTP have been described in spinach leaves [11], castor bean endosperm [12] as well as barley aleurone layers of developing, mature and germinating caryopses [13]. These nsLTP are basic proteins (isoelectric point about 9) with a molecular mass of about 9 kDa; they share high amino-acid-sequence similarities and they facilitate, *in vitro*, the intermembrane transfer of various phospholipids [14–16].

In the present paper, we describe the purification and the characterization of several extracellular peptides corresponding to the 10-kDa region of gels. Based on the results of this purification, the *in-vitro* phospholipid-transfer activities, the immunological cross-reactivities with maize and carrot antisera and the analysis of partial amino-acid sequences, we conclude that at least three isolipid-transfer proteins are

secreted into the extracellular medium of grapevine somatic embryo culture.

MATERIALS AND METHODS

Plant material and culture conditions

The embryogenic grapevine cell suspensions derived from the rootstock 41B (*Vitis vinifera* cv. Chasselas x *Vitis berlandieri*) was provided by Moët et Chandon. Undifferentiated growth of the cells was maintained by weekly subcultures in a modified Murashige and Skoog medium as already described [17].

Embryogenic differentiation was initiated according to the protocol described previously [17]. After 15 days of incubation, the medium was collected by first separating the PEMs or embryos with glass-particle filters numbers 2 and 4, respectively (Pyrex). The conditioned medium was cleared by passing it through glass-fiber membranes (GF/C, Whatman) under vacuum and concentrated 100–200-fold using the Amicon ultrafiltration system (Amicon W. R. Grace and Co.) with Amicon membranes YM 3 (cut-off 3 kDa). Protein contents were determined according to the method of Lowry [18].

Protein purification

Purification was performed using a HPLC system (Waters) including a M 440 ultraviolet detector, two model 510 HPLC pumps and a model 680 automated gradient controller. Samples were injected by a sample injection valve (model 7010 from Rheodyne) equipped with a 4.5-ml loop. Elution of proteins was monitored at 280 nm. The purification method was as follows:

Step 1

A 100-fold-concentrated 15-day-old culture medium was loaded onto a cation-exchange column (model SP 5PW from Waters), previously equilibrated with 25 mM sodium phosphate, pH 6.5 (flow rate, 0.8 ml/min). About 4 mg protein was applied to the column. Basic proteins were eluted by a linear gradient of 0–0.3 M NaCl over 30 min. Fractions containing the 10-kDa proteins were then submitted to the second step of purification.

Step 2

Samples (1 vol.) were diluted with a sample buffer (1 vol.; 3.6 M ammonium sulfate, 100 mM sodium phosphate, pH 7) and loaded onto a hydrophobic column (HIC PH-214 from Shodex). The mobile phase was a 1.8 M ammonium sulfate, 100 mM sodium phosphate, pH 7, (flow rate, 1 ml/min). Bound proteins were eluted by a linear gradient of 1.8–0 M ammonium sulfate. The fractions corresponding to the different peaks were collected and desalted using a Sephadex G-25 M column (PD-10 from Pharmacia). After determination of the protein concentrations, the fractions were concentrated using a SpeedVac concentrator (model SVC 100H from Savant Instrument Inc.).

Determination of the lipid-transfer activity

The protein fractions were tested for their lipid-transfer activity by incubating them for 30 min at 30°C with maize-

purified mitochondria and a preparation of radioactive liposomes [10]. Radiolabeling was performed with 257 nmol exchangeable [³H]phosphatidylcholine (2.7×10^9 Bq/M) and 680 nmol nontransferable cholesteryl [^{1-¹⁴C}]oleate (1.0×10^9 Bq/M). After centrifugation, mitochondria were resuspended in 1% Triton X-100, and the ratio ³H/¹⁴C was determined. Transfer activity was expressed relative to the transferred [³H]phosphatidylcholine after correction for residual radioactivity of the non-exchangeable cholesteryl [^{1-¹⁴C}]oleate. The specific activity is expressed as nmol of phosphatidylcholine transferred $\cdot$ min⁻¹ $\cdot$ mg protein⁻¹.

Amino acid sequence analysis

Analyses were carried out on a 470-A gas/liquid phase sequencer (ABI). Phenylthiohydantoin derivatives of amino acids were analysed using a 120A on-line analyser. Sequence analysis was using the University of Wisconsin Genetic Computer Group (UWGCG) package [19] and carried out on a microVAX 3400.

Electrophoresis and electroblotting

Proteins exhibiting a cationic electrophoresis mobility were separated on polyacrylamide gel slabs (10.1 cm width, 7.2 cm length and 1.00 mm thickness), carried out without SDS. The stacking and the resolving acidic (pH 6.5) gels consisted of 4.7% and 13.6% acrylamide, respectively. The buffer tank contained an 0.14 M acetic acid/0.35 M β -alanine, pH 4.5. As indicator, methyl green was added to color sample in 15% glycerol. They were placed in the sample slots, then subjected to a 20 mA/gel current with reversed polarities.

Electrotransfer to Immobilon poly(vinylidene fluoride) transfer membrane (Millipore Corporation) was performed at room temperature for at least 3 h at 60 V. The transfer buffer was a 25 mM Tris/HCl, pH 9.4, 20% methanol. After transfer, the sheet was rinsed twice with 10 mM sodium phosphate, 120 mM NaCl, 2.7 mM KCl, pH 7.4 (NaCl/P_i) containing 0.05% Tween 20. It was then soaked for 1 h at room temperature in blocking solution (NaCl/P_i containing 3% bovine serum albumin).

For immunological analysis, the Immobilon sheets were incubated for 1 h at 37°C with 1/1000-diluted antibody raised to maize phospholipid transfer protein [20] in 0.05% Tween-20/NaCl/P_i buffer. After incubation with a alkaline-phosphatase-anti-(rabbit immunoglobulin) (Bio-rad Laboratories), the enzymic activity was revealed using the AP Color Development Reagents (BCIP, NBT; Bio-rad Laboratories).

RESULTS

Purification of the basic proteins of 9 kDa

About 960 ml of a 15-day-old –aux culture medium was concentrated 100-fold. After loading the concentrate onto a cation-exchange column, several peaks, detected at 280 nm, were eluted (Fig. 1). SDS/PAGE analysis of each peak revealed that only two main peaks, F₁ and F₂, contained a major band at 9 kDa (data not shown).

The proteins contained in these two peaks were separated by electrophoresis under non-denaturing conditions and electrotransferred on an Immobilon sheet. An antiserum raised against a maize lipid-transfer protein revealed a single band in each of the two samples (Fig. 2), but the two bands

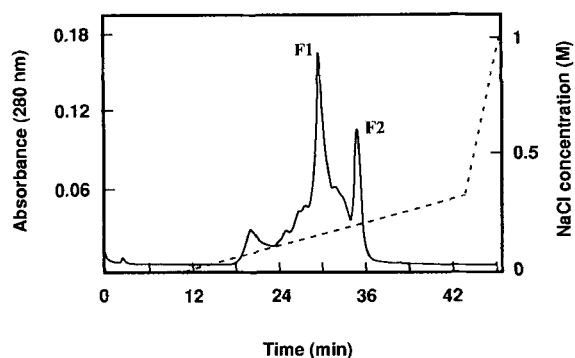


Fig. 1. Elution pattern of extracellular proteins secreted by embryos in a 15-day-old $-aux$ medium separated on a SP 5PW cation-exchange column. About 4 mg protein was applied to the column previously equilibrated in 25 mM sodium phosphate, pH 6.5. Proteins were eluted with a linear gradient of 0–0.3 M of NaCl (---) over 30 min at a flow rate of 0.8 ml/min. Absorbance was determined at 280 nm. The two fractionated peaks were identified as F_1 and F_2 for further processing.

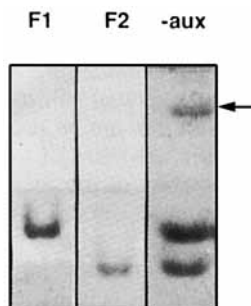


Fig. 2. Comparison of the immunoreactivities of the fractions F_1 , F_2 and of total proteins from a 15-day-old $-aux$ medium with an antiserum raised against a maize lipid-transfer protein. After concentration, proteins were separated under non-denaturing conditions by gel electrophoresis under conditions of cationic separation. After migration, proteins were electrotransferred onto an Immobilon membrane and revealed as described in Material and Methods. Samples F_1 , F_2 and $-aux$ medium proteins were loaded at 3, 1 and 15 μ g, respectively. The arrow indicates the 25-kDa protein band. No immunostaining was observed when primary antibody was omitted from the experimental protocol or preimmune serum was used (data not shown).

migrated to different positions, showing differences in their net electrical charges. These two bands were also found in the unfractionated proteins from embryo culture medium (Fig. 2). A third band, indicated in Fig. 2, exhibiting a non-specific cross reaction with the LTP antibody, corresponds to a 25 kDa protein identified previously as a cationic peroxidase [8].

The fractions corresponding to peaks F_1 and F_2 were then submitted to the second purification step, i.e. a hydrophobic-interaction chromatography. The elution profile (Fig. 3) reveals three peaks, P_1 , P_2 and P_3 , in the F_1 fraction, whereas only one peak, P_4 , was recovered in the F_2 fraction (data not shown).

The four proteins have been analyzed by positive-ion ESI mass spectrometry by formation of multiply protonated molecules. The ESI mass spectrums of the proteins P_1 – P_4 showed that the four proteins exhibit molecular masses in the range 9.1–9.3 kDa.

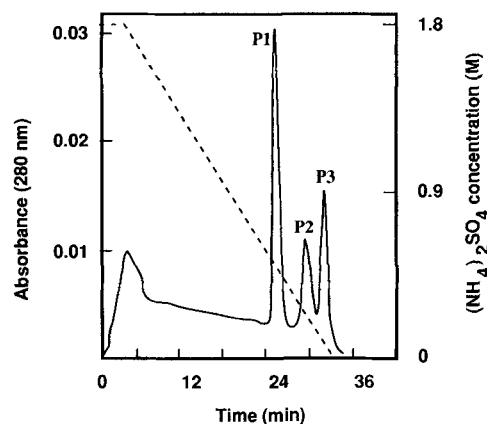


Fig. 3. Elution profile of the fraction F_1 from the hydrophobic-interaction PH 214 hydrophobic column. About 1.5 mg protein was applied to the column previously equilibrated in 1.8 M ammonium sulfate, 100 mM sodium phosphate, pH 7. The column was eluted with a linear gradient of 1.8–0 M ammonium sulfate (---) at a flow rate of 1 ml/min. The three major peaks detected at 280 nm were named P_1 , P_2 and P_3 .

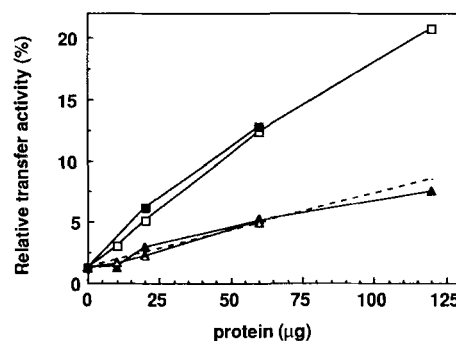


Fig. 4. Transfer activity of the proteins P_1 ($\square$), P_2 ($\blacksquare$), P_3 ($\triangle$) and P_4 ($\blacktriangle$) as a function of the amount of protein. The transfer activity is expressed relative to the initial [^3H]phosphatidylcholine transferred from liposomes labelled with an exchangeable [^3H]phosphatidylcholine and a nontransferable cholesteryl [^{14}C]oleate towards mitochondria. The activity of the maize LTP (----) is followed as control.

Lipid-transfer assays

The four proteins and, as control, maize-seedling-purified LTP [10] were assayed for their lipid-transfer activity. As shown in Fig. 4, the four proteins were able to transfer phosphatidylcholine from liposomes towards mitochondria. The estimated specific activity of transfer is in good agreement with those reported in the literature for other LTPs (about $10 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein). The proteins P_1 and P_2 exhibit high specific activities whereas the proteins P_3 , P_4 and the maize LTP are four-times-less active.

Amino-acid sequences

The partial amino-acid sequences of the four proteins are shown in Fig. 5. The homogeneity of the proteins was confirmed by the presence of only one N-terminal amino acid in each sample. The proteins P_1 and P_2 exhibit the same N-terminal sequences, whereas the amino-acid compositions of the proteins P_3 and P_4 are different. Nevertheless, these sequences present similarities among themselves and also with protein sequences established for carrot [9], spinach [21],

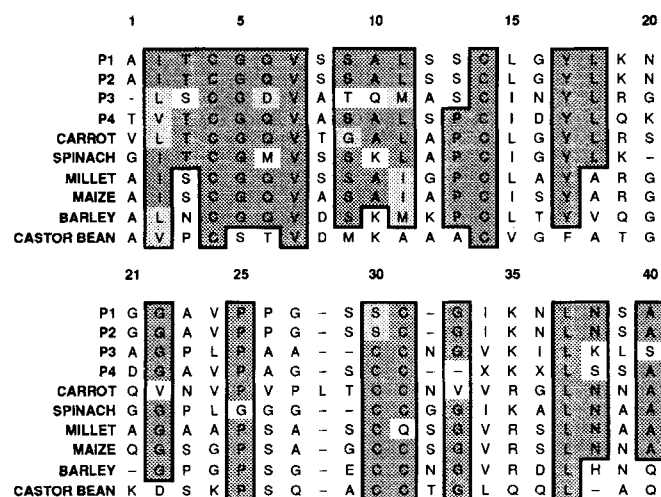


Fig. 5. Partial amino-acid sequences of the proteins P₁, P₂, P₃ and P₄ and comparison with four already established plant LTP sequences. Optimal alignment was made using the (UWCGC) package [19] as described in Material and Methods. The amino-acid residues are presented in the standard one-letter code, where X designates a non-identified residue. Dashes indicate regions where a sequence has been expanded to allow optimal sequence alignment. Boxes show homologous amino acids in at least eight of the sequences, including the replacements by substitute amino acids (⊠). The sequences presented are those of nsLTPs from carrot [9], spinach leaf [21], millet [22], maize seed [23], barley [24] and castor bean [25].

millet [22], maize [23], barley [24] and castor bean [25] nsLTP, despite the fact that the region over residues 21–31 is of lower similarity [14]. Almost all sequences show conservation of three cysteines at positions 4, 14 and 30/31, as well as glycine, valine, tyrosine and lysine residues at positions 5, 7, 17 and 37, respectively.

DISCUSSION

Many plant species are able to induce embryo development from proliferating unorganized cells. This alternative developmental pathway is strongly under the control of plant growth factors and more especially under the control of auxin. While somatic embryo development has been widely described morphologically [4], few information exist concerning the molecular events that occur during embryo formation. In the last few years, a special interest has been focused on macromolecules excreted in embryogenic cell-culture media [26, 27]. It has been particularly demonstrated that specific proteins are secreted by embryogenic carrot cells, some of them playing an important role in the development of somatic embryos [28]. In agreement with the demonstration that carrot cells secrete a 10-kDa protein identified as a nsLTP [9], we show here that grapevine cells secrete large amounts of nsLTP. The ability of these proteins to catalyze *in vitro* transfer of phospholipids and their amino-acid sequences led us to conclude that these proteins belong to a family of at least four isolipid transfer proteins. The homogeneity of the molecular masses of grapevine nsLTP, close to those of other nsLTP already isolated (see Kader [14] for a review), reflects the high similarity of the amino-acid sequences. Two proteins of the first fraction possess the same first 40 amino acids. The differences observed in their hydrophobic characteristics and in their molecular masses could

reflect differences in the rest of the sequence or post-translational modifications.

The existence of isoforms of LTP in the extracellular medium differentiates the grapevine somatic embryogenesis system from the carrot model where only one LTP gene has been characterized [9]. In contrast, it is consistent with the observations of Douady et al. [10] and Kader et al. [11] who found that after carboxymethyl-Sepharose chromatography, the lipid-transfer activity from maize seeds and spinach-leaf extracts were detected in two and three groups of fractions, respectively. The existence of LTP isoforms has recently been confirmed by Arondel et al. [29] who described the presence of multiple LTP-encoding mRNA and by Tsuboi et al. [25] who purified four organ-specific isoforms of LTP from castor-bean seedlings.

Nothing is known about the *in vivo* function of the extracellular nsLTP and their biological role has actually been widely discussed [15]. Based on its extracellular location, Sterk et al. [9] proposed an involvement of LTP in the transport of cutin monomers to the developing cuticle, deposited at the outer surface of epidermal cells, but direct evidence in favour of such a role still has to be provided. The question that arises from our results concerns the signification of the existence of isoforms of LTP in the embryo extracellular medium. Future developments concerning the expression of the corresponding genes and eventual substrate-specificity determination of the four proteins are in progress. Interestingly, differences in the specific activities of the different isoforms (Fig. 4) as well as variations in the relative amounts of the four isoforms with the time of culture (unpublished data) has been already observed.

Auxin, one of the primary element in the induction of somatic embryogenesis, negatively controls the secretion of various proteins in the extracellular medium. Isoforms of LTP are a typical example of such proteins. The mechanisms by which auxin exerts this negative control on the excretion of LTP remain to be explored. Further work will be devoted to investigate whether the changes in the protein levels in the extracellular medium can be attributed to an auxin effect on gene expression or secretion of the proteins.

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