Planta (2002) 215: 41–50
DOI 10.1007/s00425-001-0717-x

ORIGINAL ARTICLE

Johan Edqvist · Isabelle Farbos

Characterization of germination-specific lipid transfer proteins from *Euphorbia lagascae*

Received: 6 July 2001 / Accepted: 3 November 2001 / Published online: 2 February 2002

Abstract The endosperm of *Euphorbia lagascae* Spreng. seeds contains high levels of the epoxidated fatty acid vernolic acid (*cis*-12-epoxyoctadeca-cis-9-enoic acid). To obtain transgenic oilcrops producing high levels of vernolic acid, better knowledge of its endogenous metabolism is needed. In this paper we study the gene activities involved in the mobilization and oxidation of vernolic acid during germination. A cDNA library was constructed from mRNA isolated from germinating *E. lagascae* seeds. Over 300 cDNA clones were partially characterized by DNA sequencing. Of the sequenced cDNAs, 18% encoded proteins with a putative function related to the metabolism of lipids or fatty acids. Among these cDNAs were genes coding for lipase, thiolase, acyl-CoA reductase and epoxide hydrolase. Of the sequenced clones, 4.5% encoded lipid-transfer proteins (LTPs), indicating the high abundance of such proteins during germination. We isolated the full-length sequences of the *E. lagascae* cDNAs encoding the LTPs ElLTP1 and ElLTP2. These proteins share only 38% identity, but both show high similarity to LTPs from other plant species. Both sequences contain eight cysteine residues, which are conserved in most plant LTPs. Expression analysis revealed that both genes were specifically expressed during germination.

Keywords Endosperm · *Euphorbia* (germination, lipids) · Expressed sequence tag · Fatty acid · Germination · Lipid

Abbreviations EST: expressed sequence tag · LTP: lipid-transfer protein · RACE: rapid amplification of cDNA ends

J. Edqvist (✉) · I. Farbos
Department of Plant Biology, SLU,
Box 7080, 750 07 Uppsala, Sweden
E-mail: johan.edqvist@vbiol.slu.se
Fax: +46-18-673279

Introduction

The epoxidated vernolic acid (*cis*-12-epoxyoctadeca-cis-9-enoic acid) is the main fatty acid in the seed oil from a few plant species. In *Vernonia galamensis*, *Crepis palaestina* and *Euphorbia lagascae* the storage triacylglycerol consists of 50–75% vernolic acid (Derksen et al. 1995). Due to the reactivity of the epoxide ring, vernolic acid has several potential commercial applications, such as in stabilizers and plasticizers in polyvinylchloride production, as well as in paints and coatings (Derksen et al. 1995). The use of vernolic acid for these products has so far been hindered due to the limited and unpredictable supplies of the epoxidated plant oil. Despite the high interest in obtaining crops producing high levels of modified fatty acids, there have been limited efforts to increase our understanding of the endogenous metabolism of epoxy fatty acids during seedling development. We have initiated an effort to characterize genes involved in the metabolism of vernolic acid during germination of *E. lagascae* seeds. The increased knowledge about the enzymatic reactions involved in mobilization and oxidation of the unusual modified fatty acids will improve our potential to develop new valuable crops. We are also interested in studying how the enzymes have evolved to interact with the epoxidated fatty acids. One relatively rapid way to obtain information was to partially sequence clones of an *E. lagascae* cDNA library from the endosperm and cotyledons of germinating seeds. Among the sequenced cDNAs, we identified several that encoded proteins with key functions in lipid metabolism, such as lipid-transfer proteins (LTPs), triacylglycerol lipase, thiolase, acyl-CoA reductase and epoxide hydrolase.

In the present work, we present a characterization of the *E. lagascae* LTPs ElLTP1 and ElLTP2. LTPs are defined by their ability to facilitate transfer of phospholipids between membranes in vitro (Kader 1996, 1997). Genes and cDNAs encoding LTPs have been characterized from various plant species. Southern blot

and genome sequencing have revealed that plant LTPs are encoded by small gene families in most plants. The LTPs in higher plants have many features in common, including low molecular mass and high conservation of eight cysteine residues. These cysteine residues are known to be involved in intramolecular disulphide bonds (Simorre et al. 1991). Structural studies of LTPs from wheat and maize have shown that these four disulfide bonds connect four helical fragments to a single compact domain extended by a C-terminal tail (Gincel et al. 1994; Shin et al. 1995). The four helices form a hydrophobic cavity, which runs through the LTP (Lee et al. 1998). This cavity is the binding site for fatty acids, acyl-CoA or phospholipids.

Plant LTPs show an unusual lack of specificity. A detailed analysis of the structures of maize LTP in complex with various fatty acids suggests that the structural flexibility of the ligand-binding cavity and the predominant involvement of non-specific van der Waals interactions may explain the non-specific binding (Han et al. 2001). These investigations revealed that the detailed ligand-protein contacts are significantly different from one ligand to another. It is also clear that the lipid-binding properties differ significantly between LTPs from various sources. For instance, it was recently shown that wheat LTP had lower transfer activity and lower kinetics for fatty acid binding in comparison with a maize LTP (Guerbette et al. 1999). These differences in lipid binding were attributed to structural differences in the binding cavities of the proteins.

Plant LTPs are synthesized as precursors with signal sequences, and LTPs from several plant species have been found in the epidermal cell wall or epicuticular waxy layer (Molina and Garcia-Olmedo 1993; Pyee et al. 1994; Thoma et al. 1994). The extracellular location, the expression in epidermal cells and their ability to bind lipids and fatty acids support the hypothesis that one of the functions of plant LTPs is to transfer lipophilic material, such as cutin monomers or components for epicuticular wax formation to the outside of the cell (Sterk et al. 1991). However, the abundant expression of specific LTPs in the cotyledons has indicated a role for LTPs in lipid mobilization (Weig and Komor 1992; Soufleri et al. 1996). Here we show that the *E. lagascae* ElLTP1 and ElLTP2 are specifically expressed during germination. We propose that these germination-specific *Euphorbia* LTPs are involved in transferring lipids from the endosperm to the cotyledons, when the storage lipids in the endosperm are mobilized during germination.

Materials and methods

Plant material, bacterial strains, cloning vectors, oligonucleotides

Euphorbia lagascae Spreng. was grown in a growth chamber under the following conditions: in light (180 μmol photons m^{-2} s^{-1}) at 22 °C from 0600 to 2000 hours, followed by darkness between 2000 and 0600 hours at 20 °C. *E. lagascae* seeds were a kind gift from Prof. Sten Stymne, Department of Crop Science, SLU, Alnarp, Sweden. Cloning in *Escherichia coli* was done in strains XL1 or DH5α. The cloning vector pGEMT-easy (Promega) was used for cloning of PCR-amplified fragments. The following synthetic oligonucleotides were used for PCR, DNA sequencing and cDNA synthesis: NSLTPA1 5′-CAATCTTTAGGAAGAGATGAGGCA-3′; LTPA2 5′-CCACCATTATTATTATGTCTTTACC-3′; LTPA3 5′-TGCCTCATCTCTTCCTAAAGATTG-3′; LTP1-1 5′-ATATCTGATCTATGAACTATGACT-3′; LTP1-2 5′-GTTGCAGTTAGTGGAGAAGCTGAT-3′.

Construction of an *E. lagascae* cDNA library

Euphorbia lagascae total RNA was isolated from the endosperm and cotyledons 7 days after planting the seeds. Poly(A)$^+$ RNA was isolated from total RNA by using a biotinylated oligo dT probe and magnetic beads according to the instructions in the PolyATtract mRNA isolation system (Promega). Synthesis of cDNA from poly(A)$^+$ RNA, ligations and library amplifications were done according to the instructions in the pBluescript II XR cDNA library construction kit (Stratagene).

Preparation of nucleic acids, northern and Southern hybridizations

RNA was isolated by the guanidine hydrochloride method (Logemann et al. 1987). Total DNA was isolated according to Landgren and Glimelius (1990). For northern blot analysis, 20 μg of total RNA was dissolved in sample buffer (20 mM Mops, 1 mM EDTA, 5 mM sodium acetate, 50% formamide, 2.2 M formaldehyde, pH 7.9) and separated by electrophoresis on agarose gels (1.2% agarose, 0.7% formaldehyde). The RNA was transferred onto a nylon membrane (Hybond+; Amersham Pharmacia Biotech), and fixed to the membrane in a UV-crosslinking oven (UVC 500; Hoefer, San Francisco, Calif., USA) according to the manufacturer's instructions. Immobilized RNA was pre-hybridized, hybridized and washed before autoradiography. For re-probings, radioactivity was stripped from the membrane by immersing the membrane for a few minutes in boiling 0.1% SDS. For Southern blot analysis 20 μg DNA was digested with appropriate restriction enzymes and loaded on to an agarose gel (1%) in 1× TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). Electrophoresis, transfer of DNA to nylon filters, hybridization and washes were done according to a protocol for alkaline blotting (Sharpe et al. 1995).

PCR, reverse transcription (RT)-PCR and rapid amplification of cDNA ends (RACE)-PCR

RACE-PCR was done according to previously published methods (Frohmann et al. 1988; Schaefer 1995). Total RNA from *E. lagascae* was treated with calf intestine alkaline phosphatase (CIP) for 30 min at 37 °C. The sample was treated with proteinase K, extracted twice with phenol and precipitated with ethanol. The phosphatase-treated RNA was incubated with tobacco acid pyrophosphatase (TAP) in the following reaction: 40 μg RNA, 5 μl 10× TAP buffer (500 mM sodium acetate, pH 5.0; 1% β-mercaptoethanol; 10 mM EDTA; 0.1% Triton X-100), 10 μl 10 mM ATP, 2 μl RNA guard (AP Biotech), 1 μl TAP (5 U/μl where 1 U releases 1 nmol of inorganic phosphate in 30 min at 37 °C; Epicentre Technologies) in a total volume of 50 μl. The reaction was incubated for 1 h at 37 °C. The TAP-treated RNA was phenol-extracted and precipitated with ethanol. 10 μg of the CIP- and TAP-treated RNA was ligated to antisense RNA 107B (Bergman et al. 2000) in the following reaction: 10 μl RNA was mixed with 1 μl RNA guard (40 U/μl), 4 μg RNA 107B (0.5 μg/μl), 3 μl of 1 mM ATP, 2.5 μl RNA ligase (13 U/μl), 3 μl 10× RNA ligase buffer (500 mM Hepes-NaOH, pH 8.0; 100 mM $MgCl_2$; 100 mM DTT). Water was added to 30 μl and the reaction was incubated overnight at 16 °C. The RNA ligation was phenol-extracted and precipitated with ethanol. The RNA 107B-ligated total *Euphorbia* RNA was then used as the template in RT-PCR. Synthesis of first-strand

cDNA was performed with AMV reverse transcriptase (Promega); PCR was done using AmpliTAq Gold (Perkin Elmer). The PCRs were done in a Perkin-Elmer Gene Amp PCR System 9600 or in a PCR express (Hybaid). When PCR products were used in downstream processes, such as cloning or hybridization, they were purified with either the QIA quick PCR-purification kit (Qiagen) or the QIAEXII gel-extraction kit (Qiagen).

DNA sequencing and sequence analysis

DNA sequencing was carried out on double-stranded plasmid DNA or PCR-amplified DNA fragments by the dideoxy chain termination method (Sanger et al. 1977), according to the instructions supplied with the DYEnamic ET terminator cycle sequencing premix kit (Amersham Pharmacia Biotech) or with the Thermosequenase II kit (Amersham Pharmacia Biotech). Sequence reactions were performed in a Perkin Elmer 9600 DNA Thermal Cycler or in a PCR express (Hybaid). The sequencing reactions were analyzed on an Applied Biosystems Model 477 according to the manufacturer's recommendations. Sequence similarity searches were performed at the National Center for Biotechnology Information (NCBI) using the BLAST services (Altschul et al. 1997). Amino acid alignments and phylogenetic trees were generated using CLUSTAL X (Thompson et al. 1997). Phylogenetic trees were generated using the neighbor-joining method (Saitou and Nei 1987). Bootstrap re-sampling analysis was performed to assess branch support.

RNA in situ hybridization

Germinating seeds were collected 7 days after planting. Tissues were fixed in 3.7% formaldehyde, 5% acetic acid, and 50% ethanol for 5 h. The material was dehydrated with ethanol, cleared with Histoclear, and embedded in paraffin (Paraplast Plus). Embedded tissues were sliced into serial 8-μm sections and attached to microscope slides coated with APES (aminopropyltrietho-silane; Sigma). Paraplast was removed by immersion in Histoclear. Sections were rehydrated, incubated at 37 °C for 40 min in a solution containing 1 mg/ml Proteinase K (Sigma), 100 mM Tris (pH 7.5), 50 mM EDTA, followed by two washes in H_2O for 5 min each. After dehydration in an ethanol series, slides were air-dried before application of the hybridization solution containing 50–80 ng/slide of digoxigenin (DIG)-labelled RNA probe, 50% formamide, 300 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 10% Dextran sulfate, 100 mM DTT, 0.5 mg/ml tRNA. Probes were labeled using DIG labeling mix (Boehringer) according to the manufacturer's protocol. After incubation in a humid box at 37 °C overnight, slides were washed in 0.2× SSPE (1× SSPE: 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA, pH 7.4) for 1 h at 55 °C. After incubation with 5 μg/ml RNase A for 30 min at 37 °C, slides were washed twice in 0.2× SSPE for 30 min at 55 °C, then 5 min in 2× SSPE at 37 °C and 5 min in PBS (1.3 M NaCl, 30 mM NaH_2PO_4, 70 mM Na_2HPO_4, pH 7.4) at room temperature. Slides were incubated in 1% blocking reagent (Boehringer) in maleic buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 45 min, then in BXT (1% BSA, 100 mM Tris, 150 mM NaCl and 0.3% Triton X-100, pH 7.5) for 45 min. Anti-DIG–alkaline-phosphatase-coupled antibody (Boehringer) was diluted 1:600 in BXT solution. A volume of 150 μl was applied to each slide, the sections covered with a coverslip, and incubated for 2 h. Slides were washed twice, 25 min each time with BXT solution. Fresh Color-Substrate solution [nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100 mM Tris, 50 mM $MgCl_2$ and 100 mM NaCl, pH 9.5] was applied to each slide and incubated in the dark at room temperature. When the color reaction was complete, slides were washed twice with TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA] for 5 min and mounted with Cristal/Mount (Biomeda). Sections were examined under bright-field optics. Images were captured though the camera system and assembled using Adobe Photoshop (Adobe Systems. Mountain View, Calif., USA).

Results

Construction and sequencing of a *Euphorbia lagascae* cDNA library

The seeds of *E. lagascae* contain high levels of epoxidated fatty acids. To obtain information about the gene activities that are involved in the process in which these fatty acids are mobilized during germination, we constructed a cDNA library from mRNA isolated from germinating seeds. Germinating seeds were collected 7 days after planting. Total RNA was isolated from the cotyledons and endosperm of the seeds. The isolated RNA was enriched for poly(A)$^+$ RNA and used as a template for cDNA synthesis. After synthesis of the second strand and addition of adaptors, the cDNA was ligated into a vector. The obtained cDNA library was screened through DNA sequencing.

Expressed sequence tags (ESTs) were obtained from 313 clones. 209 of the ESTs encoded amino acid sequences with similarity to sequences in the databases. Of these ESTs, 151 were found to share similarity to genes with identified functions, while 58 ESTs were encoding proteins with similarity to proteins with unknown functions. The *E. lagascae* ESTs that encoded proteins similar to proteins with known or putative functions were classified into functional groups as shown in Fig. 1. As expected, we identified several cDNAs with similarity to plant or animal proteins directly interacting with lipids or fatty acids (Table 1).

Several genes were encoded by multiple ESTs. 14 of the ESTs were encoding non-specific LTPs. Ribosomal protein L5 and histone 2A were both encoded by four clones, while thiolase as well as aconitate hydratase were encoded by three of the sequenced cDNA clones. Aldolase, cyclophilin and coatomer zeta subunit were among the genes that were encoded by two sequenced cDNAs.

Analysis of LTP sequences

A closer comparison of the sequences encoding LTPs revealed that six of the clones encoded a protein we defined as *E. lagascae* lipid-transfer protein 1 (ElLTP1), while eight clones encoded *E. lagascae* lipid-transfer protein 2 (ElLTP2). Five of the ElLTP1 cDNAs and all eight ElLTP2 cDNAs were completely sequenced in both directions. RACE-PCR was done to obtain full-length sequences of both ElLTP1 and ElLTP2.

The ElLTP1 cDNA encodes a putative protein of 115 amino acids (Fig. 2). Analysis with the SignalP program (Nielsen et al. 1997) indicated that the N-terminal contains a signal sequence with a cleavage site between G19 and M20. The mature protein is expected to consist of 97 amino acids with a molecular weight of 9.8 kDa. Sequencing of five different ElLTP1 clones revealed that the cDNAs were identical, except for one site

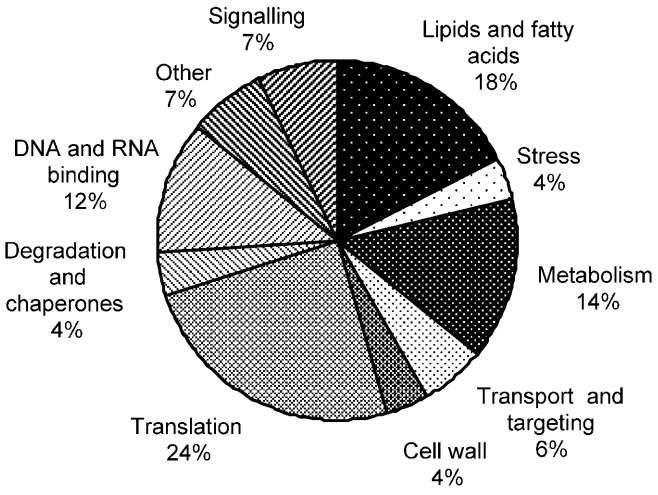

Fig. 1 Functional classification of the sequenced cDNA clones. The ESTs were classified into 10 functional groups according to their similarity to proteins with identified functions. The group *Other* included cDNAs encoding proteins such as ferredoxin, cytochrome *c* oxidase, photosystem I reaction center, NifU protein and clp5 protease. The obtained *Euphorbia lagascae* ESTs have the following accession numbers: BG487407-BG487431, BG488454-BG488500, BG507151-BG507283, BG517045-BG517134, BG606063-BG606065

Table 1 Examples of identified expressed genes with similarities to genes related to lipid metabolism

Identified genes	Accession No. of most similar protein
Lipid transfer protein 1	P82534 *Prunus domestica*
Lipid transfer protein 2	P10973 *Ricinus communis*
3-Ketoacyl-CoA-thiolase	BAA11117 *Cucurbita* sp.
4-Coumarate-CoA ligase	T46131 *Arabidopsis thaliana*
Multifunctional β-oxidation protein 2	S74209 *Rattus norvegicus*
13-Lipoxygenase	T11578 *Vigna unguiculata*
Epoxide hydrolase	T01316 *A. thaliana*
Oleosin	AAD41080 *Elaeis guineensis*
β-Ketoacyl-CoA-synthase	AAB95298 *A. thaliana*
Acyl CoA reductase	AAD38039 *Simmondsia chinensis*
AMP-binding protein	O96538 *Brassica napus*
Lipase/acylhydrolase	AAF23243 *A. thaliana*
Phospholipase D	Q41142 *R. communis*

of polymorphism in the 3′-non-coding region. Four clones contained T462, while the fifth clone contained a C in that position (Fig. 2). Our results from cDNA sequencing indicated that ElLTP1 is encoded by at least

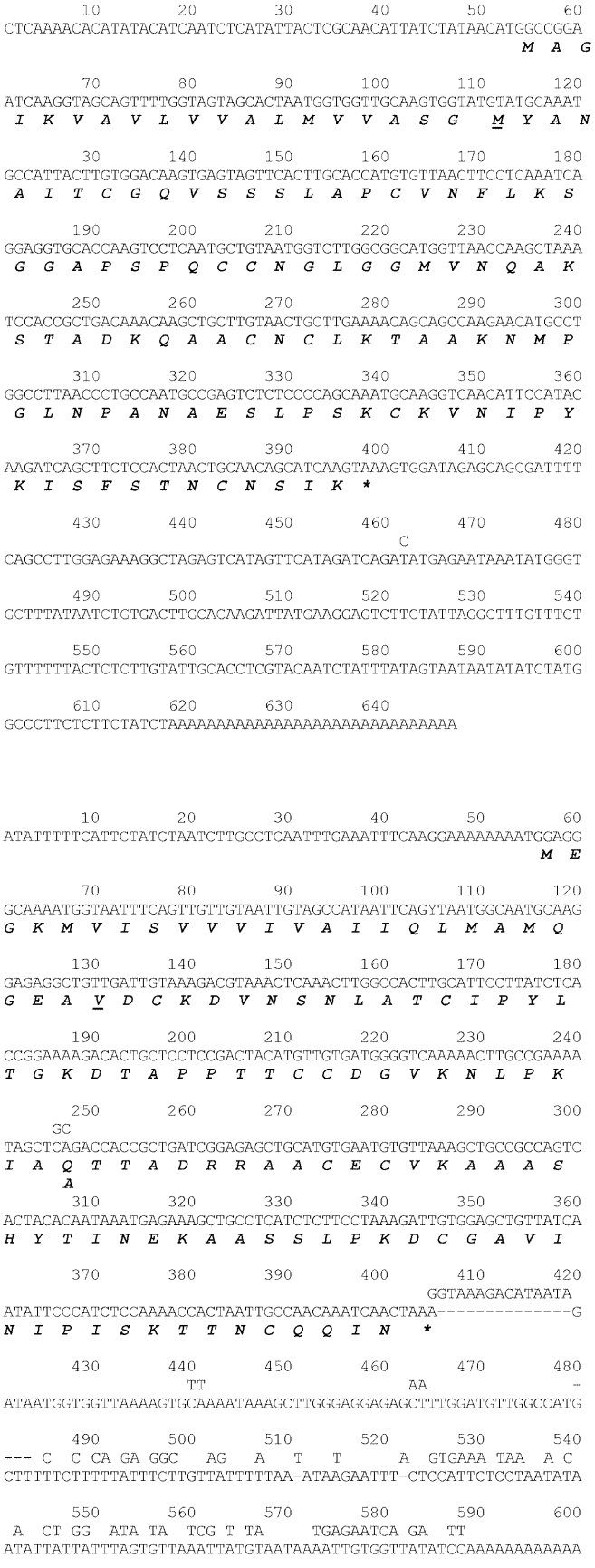

Fig. 2 Nucleotide and amino acid sequences of ElLTP1 (*top*) and ElLTP2 (*below*). Nucleotide polymorphisms identified through sequence comparisons of different cDNAs are indicated above the full-length nucleotide sequences. The underlined amino acids indicate the N-terminal of the mature proteins after cleavage of the putative signal sequences. The SignalP software calculated the location of the cleavage sites. ElLTP1 and ElLTP2 have the following sequence accession numbers: AF363505, AF363506

two genes in *E. lagascae*. The results from Southern blot hybridization also confirmed that a small gene family encodes ElLTP1 in *E. lagascae* (Fig. 3).

The ElLTP2 cDNA encodes a putative protein of 116 amino acids (Fig. 2). According to the results from SignalP analysis, the ElLTP2 cDNA also encodes a signal peptide. The expected cleavage site is between A25 and V26, yielding a mature ElLTP2 protein of 91 amino acids with a molecular weight of 9.6 kDa. When sequence information from the eight cDNAs encoding ElLTP2 was compared several sites of sequence polymorphism were revealed. The full-length sequence shown in Fig. 2 is representative for five of the clones, while the three other clones contained the variations shown above the full-length sequence. As shown in Fig. 2, these sites are mainly in the 3′-non-coding region. We also identified one site of sequence divergence in the coding region yielding variation between Q65/A65 in the mature peptide. In this paper we refer to both forms as ElLTP2. The data suggested that ElLTP2 is also encoded by at least two genes. The results from hybridization of a Southern blot with a probe against ElLTP2 also showed that several genes are encoding ElLTP2 in the *E. lagascae* genome (Fig. 3).

Sequence comparison between ElLTP1 and ElLTP2 revealed that the amino acid sequences share 38% identity and 54% similarity when the putative signal peptides are excluded. Among proteins in the public sequence databases, ElLTP1 is most similar to a *Pyrus communis* LTP (unpublished data; accession number AAF26451.1), which shares 61% identity and 79% similarity to ElLTP1. ElLTP2 is most similar to nonspecific LTP A from *Ricinus communis* (accession number S07142; Takishima et al. 1986). This protein shares 66% identity and 79% similarity with ElLTP2. A multiple amino acid sequence alignment with 17 plant LTPs is shown in Fig. 4. Conserved residues are present in all regions of the proteins. Twelve amino acids, including 8 cysteine residues, are conserved in all plant LTPs used for the alignment.

To understand more about the relationships between the plant LTPs we constructed a phylogenetic tree (Fig. 4b) of aligned amino acid sequences using the neighbor-joining method (Saitou and Nei 1987). Putative and experimentally verified signal sequences were removed before analysis. Sequences from the Brassicaceae, Rosacae, Poaceae and Euphorbiaceae form well-supported groups (over 82% bootstrap). A less well-supported group is formed by the proteins from *Cicer arietinum* and *Phaseoulus vulgaris* of the Fabaceae family (35% bootstrap). The phylogenetic tree does not indicate a close relationship between ElLTP1 and ElLTP2. ElLTP2 forms a well-supported group (100% bootstrap) with the other Euphorbiaceae sequences, LTPs A and B from *Ricinus communis*. The relationship between ElLTP1 and the other plant LTPs is less clear, although an association between ElLTP1 and the LTPs from *Prunus domestica*, *Prunus dulcis* and *Pyrus communis* of the Rosacae family is suggested (68% bootstrap).

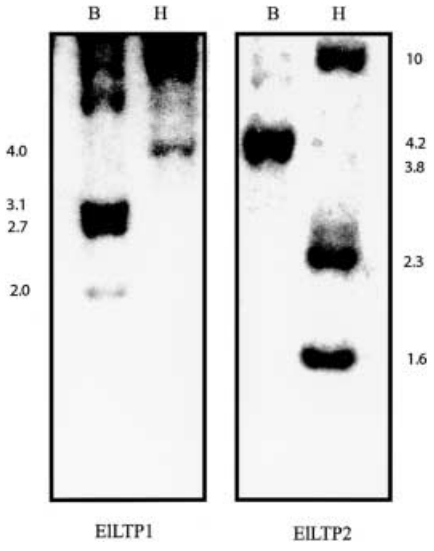

Fig. 3 Southern blot analysis of ElLTP1 and ElLTP2 was performed to estimate the copy numbers of the genes in the genome of *E. lagascae*. The genomic DNA was treated with restriction enzymes *Bgl*II (*B*) and *Hin*dIII (*H*). The numbers on the sides indicate approximate sizes in kb of the hybridizing DNA fragments

Northern blot analysis of ElLTP1 and ElLTP2 mRNAs

Total RNA was isolated from different tissues, such as leaves, germinating seeds, roots and stems of *E. lagascae* plants. The RNA was separated by electrophoresis and transferred by capillary blotting to a nylon filter. The filter was used in subsequent hybridizations with probes for ElLTP1 and ElLTP2. The results from the northern blot indicate that ElLTP1 and ElLTP2 transcripts are very abundant during germination (Fig. 5). The cellular levels of both transcripts were very low in the other tested tissues, or below the detection level after 24 h of autoradiography. After prolonged exposure for 3 days, it was possible to detect a weak hybridization signal to the probe for ElLTP1 with RNA isolated from leaves (data not shown).

To obtain a more detailed picture of the expression of ElLTP1 and ElLTP2 during germination, germinating seeds were collected 5–9 days after planting. During germination the cotyledons are carried above ground by the elongating hypocotyl. In *E. lagascae* the elongating hypocotyl forms a hook, which straightens out and pulls the cotyledons, together with the endosperm and seed coat, above ground. Five days after planting, the root had barely emerged from the seed, while at 9 days after planting the hypocotyl was elongated and the cotyledons had absorbed most of the endosperm.

In the material collected 7–9 days after planting, the hypocotyl was separated from the cotyledons. Total RNA was isolated from the fraction containing endosperm and cotyledons, as well as from the fraction of

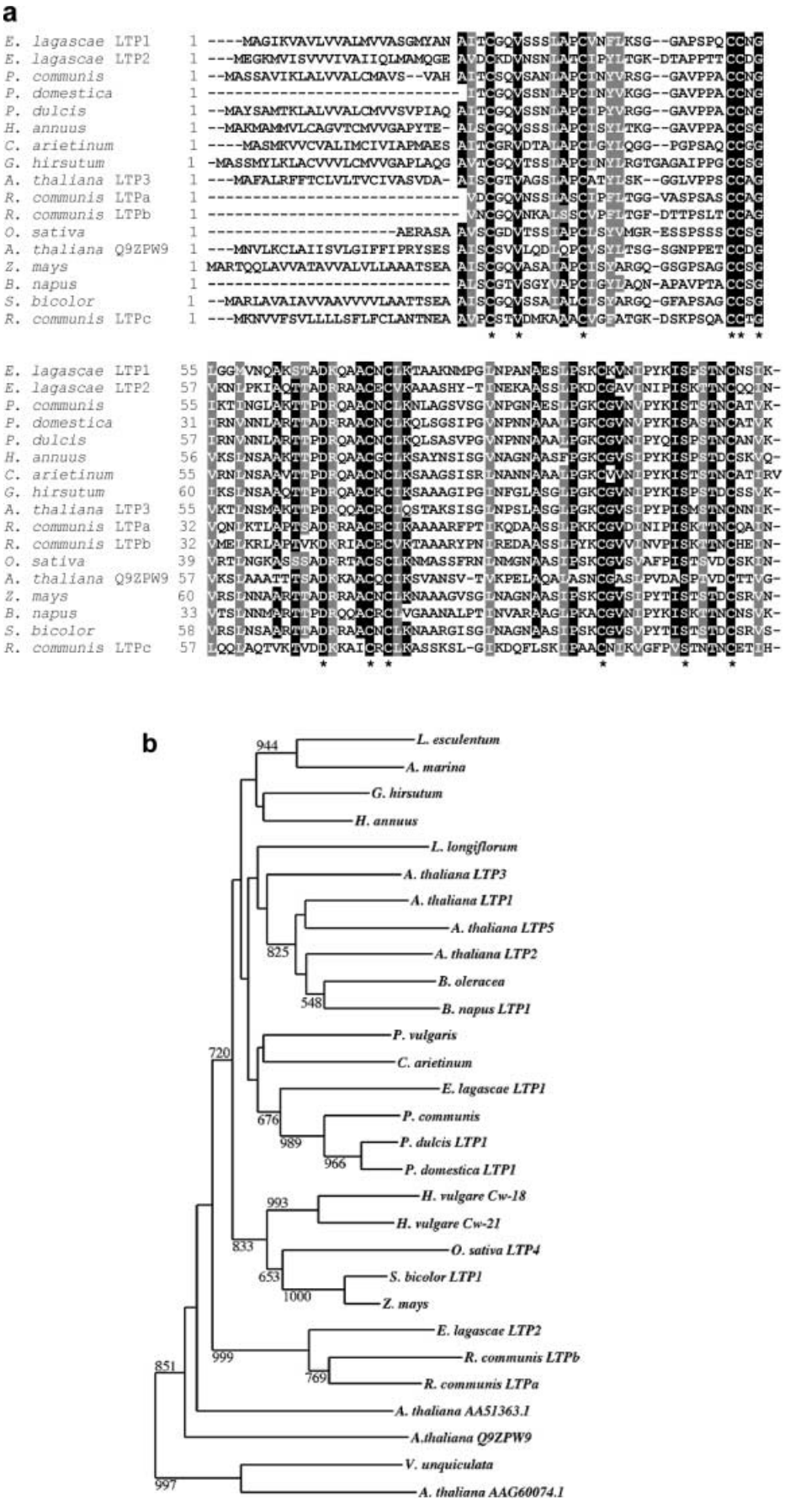

Fig. 4 Multiple sequence alignment (**a**) and phylogenetic analysis (**b**) of plant LTPs. In **a**, *black boxes* indicate that identical amino acids are present in at least 80% of the sequences, while *shaded boxes* indicate that amino acids with similar physicochemical properties are present in at least 80% of the sequences. The *stars* below the sequences indicate positions where identical amino acids are present in all sequences. In the phylogenetic tree **b**, numbers indicate the sum of 1,000 bootstrap re-samplings that support the inferred topology. Only bootstrap values over 500 are shown in the figure. The following plant LTP sequences were used for comparisons: *Arabidopsis thaliana* LTP1 Q42589, *A. thaliana* LTP2 QS7I3, *A. thaliana* LTP3 Q9LLR7, *A. thaliana* LTP5 Q9XFS7, *A. thaliana* Q9ZPW9, *A. thaliana* AAG60074.1, *A. thaliana* AAG51363.1, *Avicennia marina* AAK01293.1, *Brassica napus* LTP1 Q9S9F9, *B. oleracea* AAC63372.1, *Cicer arietinum* O23758, *Gossypium hirsutum* Q9M6B6, *Helianthus annuus* Q39950, *Hordeum vulgare* Cw-21 S45371, *H. vulgare* Cw-18 S45370, *Lilium longiflorum* AAD46693.1, *Lycopersicon esculentum* T07626, *Oryza sativa* LTP4 Q42976, *Phaseolus vulgaris* AAC49860.1, *Prunus domestica* P82534, *Prunus dulcis* Q43017, *Pyrus communis* AAF26451.1, *Ricinus communis* LTPa P10973, *R. communis* LTPb P10974, *R. communis* LTPc P10975, *Sorghum bicolor* LTP1 Q43193, *Vigna unguiculata* Q43681, *Zea mays* P19656

hypocotyls and roots. The results from the northern blot analysis showed that both ElLTP1 and ElLTP2 mRNA are most abundant in RNA isolated from cotyledons and endosperm (Fig. 6). The ElLTP1 mRNA was also detectable, but less abundant, in RNA from the fraction containing hypocotyls and roots. The expression of both genes reached a peak 6–8 days after planting, and declined at day 9. To verify that approximately equal amounts of RNA had been loaded in each well, the stripped filter was re-hybridized to a probe specific for *E. lagascae* elongation factor 1-α (Fig. 6, lower panel).

In situ hybridization analysis of ElLTP1 mRNA

Northern hybridization showed that ElLTP1 was expressed in several regions of the germinating seedlings. To more exactly pinpoint the tissues expressing ElLTP1 during germination we used RNA in situ hybridizations. Sections of germinating seeds, collected 7 days after planting, were hybridized with ElLTP1 sense and antisense transcripts. The results from hybridizations with the antisense probe indicated that expression of ElLTP1 is mainly localized to the cotyledons (Fig. 7a–c). Expression was strong over the whole cotyledons. In the hypocotyls, expression of ElLTP1 was restricted to the outer cell layers in the region close to the cotyledons (Fig. 7a, b). No expression was seen in the root tips (Fig. 7b), endosperm (Fig. 7b, c) or apical meristem (Fig. 7b). No hybridization signal was obtained with the ElLTP1 sense RNA probe (Fig. 7d).

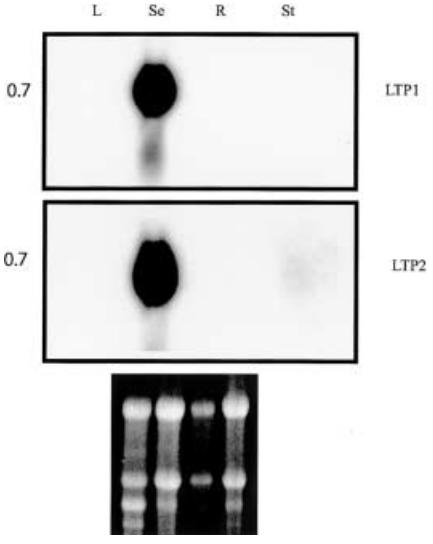

Fig. 5 Northern blot analysis of *E. lagascae* total RNA from leaves (*L*), germinating seeds (*Se*), roots (*Ro*) and stems (*St*). The blot was hybridized with probes for ElLTP1 (*top panel*) and ElLTP2 (*middle panel*). The *bottom panel* shows ethidium bromide staining of the gel before blotting. The numbers to the left indicate approximate transcript sizes in kb

Discussion

Our aim is to learn more about the breakdown of the epoxy fatty acids during germination of *E. lagascae* seeds. We intend to identify genes that encode proteins with novel activities or specificities that reflect the unusually high content of vernolic acid in this plant. Thus, we expect to identify enzymatic activities that are different from those seen in most other plants that do not store epoxidated fatty acids in the seed oil. It will be of interest to determine which amino acid substitutions the enzymes have obtained to develop specificity for the epoxidated fatty acids. Some *Euphorbia* enzymes, such as epoxide hydrolases, may also turn out to be useful as biocatalysts for the organic synthesis of epoxides (Archelas and Furstoss 1998). Moreover, we speculate that it is possible to increase the levels of epoxidated fatty acids in oil crops by transforming the crop plants with genes involved in the synthesis as well as in the degradation of the fatty acids. We approached our aims by characterizing genes expressed during germination. Even though we sequenced a relatively small number of cDNAs, we identified more than 10 different genes that are expected to play a role in the metabolism of lipids or

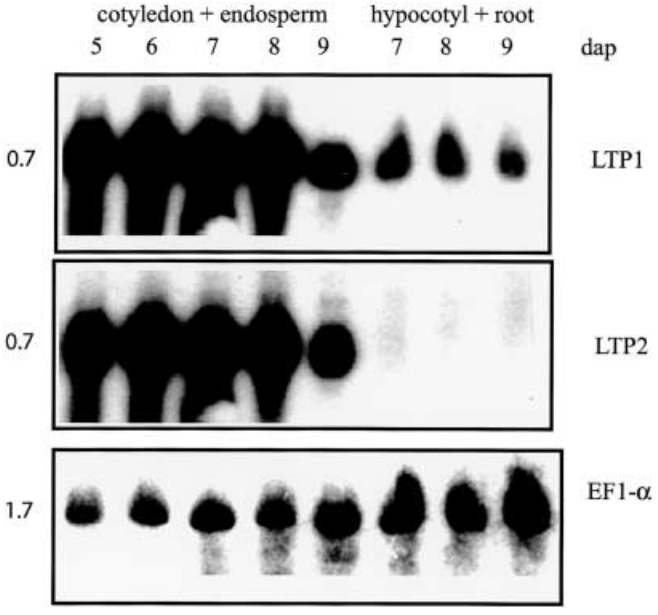

Fig. 6 Northern blot analysis of RNA isolated from germinating *E. lagascae*. RNA was isolated from germinating seeds collected 5–9 days after planting (*dap*). At 5–6 days after planting the root had barely emerged and RNA was isolated using the whole germinating seed, which mainly consisted of cotyledons and endosperm. In samples collected 7–9 days after planting, the hypocotyl was cut from the cotyledons. RNA was isolated from the fraction containing the cotyledons and the endosperm, as well as from the hypocotyl and root fraction. The *top panel* shows hybridization to a probe for ElLTP1. The *middle panel* shows hybridization to the ElLTP2 probe. To verify that approximately equal amounts of RNA had been loaded in each well the blot was also hybridized to a probe specific for *E. lagascae* elongation factor 1-α (*bottom panel*). The numbers to the left indicate approximate transcript sizes in kb

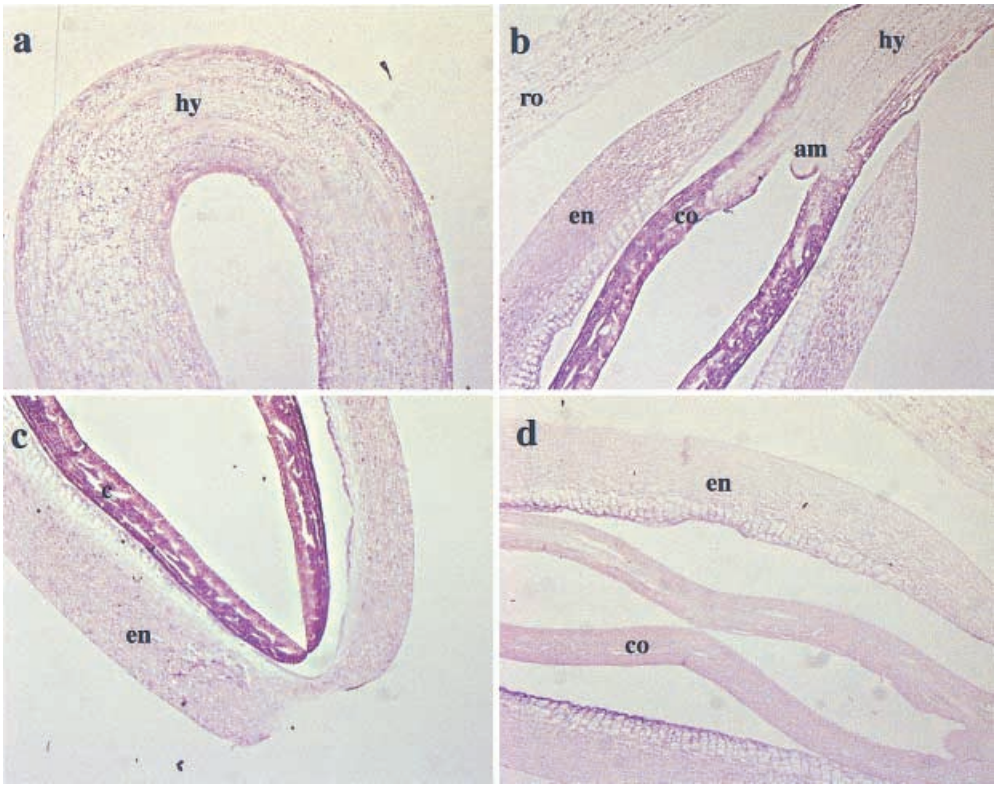

Fig. 7 RNA in situ hybridization with antisense (**a–c**) and sense (**d**) RNA probes for ElLTP1. *E. lagascae* seedlings were collected 7 days after sowing. *co* Cotyledon, *en* endosperm, *hy* hypocotyl, *am* apical meristem, *ro* root

fatty acids. Those results indicate that the obtained library will be very useful for the cloning of genes involved in the catabolism of the epoxidated fatty acids in *Euphorbia* seeds. In the present work we have characterized the *E. lagascae* LTPs ElLTP1 and ElLTP2. These proteins share only 38% identity at the amino acid level. Nevertheless, both proteins show high similarity to other plant LTPs, such as a conserved pattern of eight cysteines, which are all known to be involved in intramolecular disulphide bonds in plant LTPs (Simorre et al. 1991).

ElLTP1 and ElLTP2 contain putative signal sequences, suggesting that the mature proteins are secreted. Expression analysis revealed that both genes were specifically expressed during germination. The expression is very abundant during germination since 4.5% of the sequenced cDNA clones were encoding either ElLTP1 or ElLTP2. On the basis of the high and specific expression during germination, and low abundance in other tissues, we conclude that ElLTP1 and ElLTP2 play important roles during germination of the *E. lagascae* seeds. In *E. lagascae* seeds the oil-storing cells of the endosperm surround the cotyledons. During germination the carbon stored in the endosperm will be exported to the growing seedling in the form of carbohydrates, such as sucrose (Beevers 1980; Eastmond and Graham 2001). After germination the cells of the endosperm undergo senescence. Since the ElLTP genes, like other plant LTP genes, encode signal sequences it is reasonable to believe that the ElLTPs are located on the surface of the cotyledons. On the cotyledon surface the ElLTPs will face the oil-storing cells of the endosperm. We hypothesize that the ElLTPs bind and transfer lipids from the endosperm, possibly from senescing cells, to the cotyledons. Subsequently, the cotyledons of the germinating seedling will absorb the transferred endosperm lipids and utilize them for the synthesis of wax or cell membranes. Since cell walls and membranes are flexible structures it is not unlikely that a fraction of the LTP-transferred lipids will be converted to acetyl CoA through the β-oxidation spiral in the glyoxysomes. In conclusion, we suggest that the germination-specific ElLTPs play an important role in promoting an efficient uptake of the storage lipids by the cotyledons during germination.

Although their expression patterns are similar there are also distinct differences between the expression of ElLTP1 and that of ElLTP2. ElLTP2 is very specifically expressed in the cotyledons during germination. No traces of the ElLTP2 transcript were detected in any other tissues. ElLTP1 is also mainly expressed in the cotyledons, but there is also appreciable expression in the hypocotyls and detectable amounts of the transcripts in leaves. These distinct expression patterns together with the divergent sequences indicate that ElLTP1 and ElLTP2 may have somewhat different functions during germination.

Most plant LTPs contain a tyrosine residue near the N-terminus and another one near the C-terminus. In ElLTP1 the tyrosine near the N-terminus is replaced by phenylalanine. This substitution is also seen in LTPa, LTPb and LTPc from *R. communis* (Fig. 4). This replacement of tyrosine is only known for these four plant

LTPs. The strict substitution of tyrosine by phenylalanine supports the hypothesis that the presence of the aromatic ring in this position is essential for the activity of the protein (Douliez et al. 2000). The hydroxyl group of the C-terminal tyrosine (Tyr81) in maize LTP is known to be involved in direct contacts with the carboxylate group of fatty acid ligands (Han et al. 2001). In ElLTP2 this tyrosine is exchanged for isoleucine, which is also true for LTPa from *R. communis*. In *R. communis* LTPb there is valine present in the corresponding position. Thus, seemingly, an aliphatic amino acid can replace the aromatic side chain. In the plant LTPs, the C-terminal tyrosine is always preceded by a proline. Interestingly, in all cases where the tyrosine is replaced with another residue such as isoleucine, the proline is also replaced. For instance, in the case of ElLTP2 and *R. communis* LTPa, asparagine precedes isoleucine, yielding the sequence NI instead of the more common PY (Fig. 4). It will be of interest to determine the ligand-protein contacts in an LTP that lacks the C-terminal tyrosine.

Although the plant LTPs are non-specific in their binding to ligands, most plants express a set of LTPs with varying degrees of sequence divergence. It seems reasonable to believe that each LTP has a specific function, which may be related to the ligand-binding properties of the protein. To learn more about the plant LTPs it is very useful to compare the structural and functional properties of LTPs from various sources. Now that we have characterized the expression of the ElLTPs, it will be of high interest to continue the investigation of the function of these proteins. In particular, it will be important to analyze the lipid-binding properties to see if the *E. lagascae* LTPs have specifically evolved to interact with fatty acids, acyl-CoAs or lipids carrying epoxide groups.

Acknowledgements We thank Sten Stymne (Department of Crop Science, SLU, Alnarp, Sweden) for the original idea and also for *E. lagascae* seeds. We thank Ingrid Eriksson for excellent technical support. Kristina Blomqvist (Department of Biotechnology, KTH, Stockholm, Sweden) is acknowledged for valuable comments on the manuscript. This work was supported by Stiftelsen för Strategisk Forskning and by the Carl Trygger Foundation.

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Archelas A, Furstoss R (1998) Epoxide hydrolases: new tools for the synthesis of fine organic chemicals. Trends Biotech 16:108–115

Beevers, H (1980) The role of the glyoxylate cycle. In: Stumpf PK (ed) The biochemistry of plants, vol 4. Academic Press, London, pp 117–130

Bergman P, Edqvist J, Farbos I, Glimelius K (2000) Male sterile tobacco display abnormal mitochondrial *atp1* transcript accumulation and reduced floral ATP/ADP ratio. Plant Mol Biol 42:531–544

Derksen JTP, Cuperus FP, Kolster P (1995) Paints and coatings from renewable resources. Ind Crops Prod 3:225–236

Douliez J-P, Michon T, Elmorjani K, Marion D (2000) Structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. J Cereal Sci 32:1–20

Eastmond PJ, Graham IA (2001) Re-examining the role of the glyoxylate cycle in oilseeds. Trends Plant Sci 6:72–77

Frohmann MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNAs from rare transcripts – amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85:8998–9002

Gincel E, Simmore JP, Caille A, Marion D, Ptak M, Vovelle F (1994) Three-dimensional structure in solution of a wheat lipid-transfer protein from multidimensional ^{1}H-NMR data. Eur J Biochem 226:413–422

Guerbette F, Grosbois M, Jolliot-Croquin A, Kader J-C, Zachowski A (1999) Comparison of lipid binding and transfer properties of two lipid transfer proteins from plants. Biochemistry 38:14131–14137

Han GW, Lee JY, Song HK, Chang C, Min K, Moon J, Shin DH, Kopka ML, Sawaya MR, Yuan HS, Kim TD, Choe J, Lim D, Moon HJ, Suh SW (2001) Structural basis of non-specific lipid binding in maize lipid-transfer protein complexes revealed by high-resolution X-ray chromatography. J Mol Biol 308:263–278

Kader J-C (1996) Lipid-transfer proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 47:627–654

Kader J-C (1997) Lipid-transfer proteins: a puzzling family of plant proteins. Trends Plant Sci 2:66–70

Landgren M, Glimelius K (1990) Analysis of chloroplast and mitochondrial segregation in three different combinations of somatic hybrids produced within *Brassicaceae*. Theor Appl Genet 80:776–784

Lee JY, Min K, Cha H, Shin DH, Hwang KY, Suh SW (1998) Rice non-specific lipid transfer protein: The 1.6 Å crystal structure reveals a small hydrophobic cavity. J Mol Biol 276:437–448

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem 163:16–20

Molina A, Garcia-Olmedo F (1993) Developmental and pathogen-induced expression of three barley genes encoding lipid transfer proteins. Plant J 4:983–991

Nielsen H, Engelbrecht J, Brunak S, von Heijne G (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng 10:1–6

Pyee J, Yu H, Kolattukudy PE (1994) Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. Arch Biochem Biophys 311:460–468

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schaefer BC (1995) Revolutions in rapid amplification of cDNA ends: new strategies for polymerase chain reaction cloning of full-length cDNA ends. Anal Biochem 227:255–273

Sharpe AG, Parkin IAP, Keith DJ, Lydiate DJ (1995) Frequent nonreciprocal translocations in the amphidiploid genome of oilseed rape (*Brassica napus*). Genome 38:1112–1121

Shin DH, Lee JY, Hwang KY, Kim KK, Suh SW (1995) High-resolution structure of the non-specific lipid-transfer protein from maize. Structure 3:189–199

Simorre JP, Caille A, Marion D, Marion D, Ptak M (1991) Two- and three-dimensional ^{1}H NMR studies of a wheat phospholipids transfer protein: sequential resonance assignments and secondary structure. Biochemistry 30:11600–11608

Soufleri IA, Vergnolle C, Miginiac E, Kader J-C (1996) Germination-specific lipid transfer protein cDNAs in *Brassica napus* L. Planta 199:229–237

Sterk P, Booij H, Schellekens GA, Van Kammen A, De Vries SC (1991) Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell 3:907–921

Takishima K, Watanabe S, Yamada M, Mamiya G (1986) The amino-acid sequence of the nonspecific lipid transfer protein from germinated castor bean endosperms. Biochim Biophys Acta 870:248–255

Thoma S, Hecht U, Kippers A, Botella J, De Vries S, Somerville C (1994) Tissue-specific expression of a gene encoding a cell wall-localized lipid transfer protein from *Arabidopsis*. Plant Physiol 105:35–45

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL°X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Weig A, Komor E (1992) The lipid-transfer protein C of *Ricinus communis* L.: isolation of two cDNA sequences which are strongly and exclusively expressed in cotyledons after germination. Planta 187:367–371