

Research article

Isolation and characterization of multiple abundant lipid transfer protein isoforms in developing sesame (*Sesamum indicum* L.) seeds

Ah Mi Choi^a, Saet Buyl Lee^b, Sung Ho Cho^c, Inhwon Hwang^d,
Cheol-Goo Hur^e, Mi Chung Suh^{b,*}

^a School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea

^b Department of Plant Biotechnology, Agricultural Plant Stress Research Center and Biotechnology Research Institute, College of Agriculture and Life Sciences, Chonnam National University, Gwangju 500-757, Republic of Korea

^c Department of Biology, Inha University, Incheon 402-751, Republic of Korea

^d Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea

^e National Center for Genome Information, Korea Research Institute of Bioscience and Biotechnology, Yuseong, Taejeon 305-600, Republic of Korea

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Abstract

Sesame (*Sesamum indicum*) is an important oilseed crop; approximately 50% of the seed dry weight is storage oil. In a previous report, developing sesame seed expressed sequence tags (ESTs) revealed that ESTs encoding lipid transfer protein (LTPs) were one of the most abundant groups of sesame ESTs. LTP functions in the transfer of wax or cutin monomers and in the defense response against pathogen attack. To study the biological role of the abundant LTP isoforms in developing seeds, 122 ESTs out of 3328 sesame ESTs were analyzed against *Arabidopsis* and rice proteome databases. LTP fraction, which was partially purified from developing sesame seeds, actively transferred fluorescent phospholipids and bound to fatty acids. Full-length cDNAs of five out of 21 LTP isoforms were isolated and named *SiLTP1*–*SiLTP5*. The predicted amino acid sequences of the five *SiLTPs* harbor typical characteristics of LTPs, including conserved arrangement of cysteine residues. Northern blot analysis revealed that the five *SiLTP* isoforms were most abundantly expressed in developing seeds, but were also detected in flower tissues. Also, *SiLTP3* and *SiLTP4* transcripts were expressed in leaves and seed-pot walls, respectively. In addition, *SiLTP2* and *SiLTP4* transcripts were significantly induced in 6-day-old sesame seedlings by application of NaCl, mannitol, and abscisic acid (ABA). Transient expression of green fluorescent protein (GFP)-fusion constructs in *Arabidopsis* protoplasts revealed that *SiLTP1* and *SiLTP2* were secreted by different pathways. Taken together, the abundant LTPs in developing sesame seeds are involved in lipid transfer into the extracellular matrix. Possible biological roles of *SiLTPs* related to organ-specific expression and abiotic stresses are discussed.

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1. Introduction

Sesame (*Sesamum indicum*) belongs to the *Pedaliaceae* family. Sesame seed is an important source of edible oil and is widely used as a dietary supplement. The seed contains

50–60% oil, which has excellent stability due to the presence of natural antioxidants such as sesamol, sesamin and sesamol. After oil extraction, the remaining meal contains 35–50% protein, which is rich in tryptophan and methionine, and is used as poultry feed [5]. As an initial approach to identify genes involved in accumulation of storage products and in biosynthesis of antioxidant lignans, 3328 expressed sequence tags (ESTs) were recently obtained from developing sesame seeds. ESTs encoding lipid transfer protein (LTPs) were one of the most abundant groups of sesame ESTs [35].

Abbreviations: ABA, abscisic acid; EST, expressed sequence tag; GFP, green fluorescent protein; LTP, lipid transfer protein.

* Corresponding author. Tel.: +82 62 530 2183; fax: +82 62 530 2047.

E-mail address: mcsuh@chonnam.ac.kr (M.C. Suh).

Lipid transfer proteins (LTP) were initially defined by their ability to facilitate the transfer of phospholipids between membranes *in vitro* and *in vivo* [17]. In higher plants, LTPs are small (7–10 kDa), abundant, basic proteins that contain eight conserved cysteine residues involved in four disulfide bridges. Plant LTPs have been classified into eight groups (LTP1–LTP8) based on the number of amino acids between the fourth and fifth cysteine residues in the core of motif [1]. X-ray crystallography and nuclear magnetic resonance analyses revealed that LTPs contain a hydrophobic pocket that can accommodate fatty acids or lysophospholipid molecules [34]. Since plant LTPs were initially observed in spinach leaves, maize coleoptiles and barley aleurone layers, they have been identified as ubiquitously expressed in seedlings, leaves, stamen, tapetum and microspores, as well as in somatic and zygotic embryos [17]. Several immunolocalization and LTP::GFP fusion analyses have demonstrated that LTPs localize to the cell wall or are predominantly expressed in the epidermis [4,36]. This localization data suggests that LTP plays a role in cutin and wax assembly [30]. When leaves of tree tobacco were dried at periodic intervals, cuticular wax accumulation and LTP expression were increased [3]. Some LTPs have also displayed antifungal and antiviral properties, suggesting another biological role for these proteins [14,21,28]. In fact, increasing the level of an LTP in transgenic plants such as tobacco and wheat enhances the resistance of the plant toward the pathogen [26,31]. LTPs are also reported to be involved in long-distance signaling during systemically acquired resistance in *Arabidopsis* [23] and in cell-wall loosening during pollen-pistil interaction [15]. Finally, several LTP genes are responsive to environmental changes such as salt, drought, abscisic acid (ABA) or cold treatment [19,25,37,39,41]. The variety of expression patterns, as well as the presence of multiple isoforms in *Arabidopsis* and rice genome, suggests that multiple LTP isoforms might be associated with diverse functions that have yet to be discovered [1,39].

Soluble proteins are targeted for delivery to the ER by the presence of specific signal sequences that are removed as the nascent protein enters the ER lumen. ER-transported polypeptides are subsequently secreted to the apoplast by the default pathway or delivered to the ER and then transported to the Golgi apparatus, where they are further sorted for translocation to the cell exterior or to cellular compartments such as protein storage vacuoles, lytic vacuoles, the plasma membrane, or the cell wall [10,40]. Although there is some evidence of intracellular LTPs in glyoxysome matrix of castor (*Ricinus communis*) seed and protein storage vacuoles of cowpea (*Vigna unguiculata*) [6,38], most plant LTPs are secreted to the cell wall by unidentified pathways.

In this study, 122 LTP ESTs, the most abundant in developing sesame seed ESTs, were analyzed against *Arabidopsis* and rice proteome databases by the BLASTX program. LTP fraction, which was partially purified from developing sesame seeds, was examined for the ability to transfer fluorescent phospholipids and to bind to fatty acids. Five LTP isoforms were isolated and their mRNA expression patterns were analyzed in various sesame tissues and in 6-day-old seedlings

after abiotic stresses and plant hormone application. Finally, the subcellular localization of two major sesame LTP isoforms, *SiLTP1* and *SiLTP2*, was investigated by introducing LTP::GFP fusion constructs into *Arabidopsis* protoplasts.

2. Methods

2.1. Plant materials and treatment

Sesame (*Sesamum indicum* L. cultivar, Yangbaeck) was obtained from National Crop Experiment Station, Rural Development Administration, South Korea. Sesame were grown in a greenhouse at $26^{\circ}\text{C} \pm 3^{\circ}\text{C}$. Developing seeds were harvested between 5 and 25 days after flowering. Six-day-old sesame seedlings were incubated in MS medium containing 300 mM NaCl, 300 mM mannitol, 10 μM GA₃ or 10 μM ABA for 12 h and 24 h. Plant tissues were immediately utilized or frozen in liquid nitrogen after harvesting and stored at -70°C until use. *Arabidopsis* (*Arabidopsis thaliana* ecotype Columbia-0) was grown in soil in the *Arabidopsis* room under a photocycle of 16 h of light (24°C) and 8 h of darkness (22°C).

2.2. EST processing and isolation of five LTP isoforms

Plasmid DNA was prepared by alkaline lysis [33] and column purification (Qiagen). Nucleotide sequencing and EST sequence processing were carried out as described in Suh et al. [35]. Pre-processed ESTs were clustered into contigs and singletons using StackPack2.2 (provided by SANBI, <http://www.sanbi.ac.za>). BLASTX searches against NR, NCBI, *Arabidopsis*, and rice databases were performed at a threshold of an E-value $>1.0\text{E}-10$. Contigs and singletons were selected that contained LTP or a described lipid transfer protein. The longest clone from each of five LTP contigs containing at least 3 ESTs was sequenced and analyzed (<http://www.ncbi.nlm.nih.gov>; <http://www.ebi.ac.uk/parasites/webtools.html>).

2.3. Purification of LTP from sesame seeds

Eight grams of developing sesame seeds (15–25 days old) were ground in 300 ml of homogenization buffer containing 100 mM Tris–Cl (pH 7.2), 1 mM EDTA, 400 mM sucrose, 4 mM cysteine, and 8 mM β -mercaptoethanol and then filtered through two layers of miracloth (Calbiochem). The homogenate was centrifuged at $10,000 \times g$ for 15 min. The supernatant was collected, and its pH was adjusted to 5.1 with diluted HCl. After centrifugation at $10,000 \times g$ for 15 min, the pH of the supernatant was increased to 7.2 with 2 M Tris-base, and ammonium sulfate was added to achieve 40% saturation. After incubation at 4°C for 16 h, the suspension was centrifuged at $10,000 \times g$ for 15 min, and the ammonium sulfate concentration in the supernatant was increased to 80% saturation. After incubating the mixture at 4°C for 2 h, the mixture was centrifuged at $10,000 \times g$ for 15 min, and the pellet was resuspended in 10 mM Tris-acetate buffer (pH 7.2)

and 8 mM β -mercaptoethanol. Sesame LTP fraction was then dialyzed for 1 day against the same buffer and used for phospholipid transfer assays and fatty acid binding assays. The protein concentration was measured using bovine serum albumin as a standard [2].

2.4. Fatty acid binding and phospholipid transfer assays

Fluorescent fatty acid binding assays were carried out as described in Zachowski et al. [42]. Dialyzed LTP (8 nmol) was mixed with 8 mM β -mercaptoethanol and 10 mM MOPS buffer, pH 7.2 (final volume of 1.5 mL), in a thermostated fluorimeter cuvette at 25 °C. 1-Pyrenedodecanoic acid (0.2 μ M, from a 0.5 M stock solution in ethanol) was added while the mixture was stirred, and the fluorescence intensity was recorded at 378 nm (excitation at 343 nm) for monomer and at 437 nm (excitation at 343 nm) for excimer on a Perkin-Elmer LS-5 spectrofluorimeter.

Di-oleoylphosphatidylcholine (DOPC) and 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC) were purchased from Avanti Biochemical Corp. (USA). The donor lipid, NBD-PC (20 μ M), was dissolved in ethanol, dried under a stream of nitrogen gas, and resuspended in 10 mM Tris-acetate buffer (pH 7.4). The acceptor lipid, DOPC (1 mM), was dissolved in toluene/ethanol (4/1, v/v), dried under a stream of nitrogen gas, and resuspended in 10 mM Tris-acetate buffer (pH 7.4). Small unilamellar vesicles were produced by sonication with a tip sonicator (Branson sonifier, 250) at 0 °C for 15 min. For phospholipid transfer measurements, 10 μ L each of donor and acceptor vesicles were mixed with 2 mL of 10 mM Tris-acetate buffer (pH 7.4) and 100 μ L of protein solution. Fluorescence was excited at 475 nm and measured at 530 nm in a Perkin Elmer LS-5 spectrophotometer equipped with a thermostated cuvette holder (25 °C) and a mixing device. Fluorescence yield of NBD-PC in acceptor vesicles was determined as described by Zachowski et al. [42]. At the high concentration in the donor membranes, NBD fluorescence is self-quenching, so that fluorescence is minimal at the start of the experiment. When labeled lipid molecules are transferred into acceptor membrane, self-quenching is relieved and fluorescence emission from NBD increases.

2.5. Isolation of total RNAs and Northern blot analysis

Sesame tissues were ground to a fine powder in liquid nitrogen with a prechilled mortar and pestle. The powder was transferred to new 50 mL tubes containing 15 mL of lysis buffer (100 mM Tris-HCl, pH 7.5, 1% *N*-laurylsarcosine, 4 M Guanidine thiocyanate, 25 mM EDTA, and 100 mM β -mercaptoethanol) and 15 mL of PCI (phenol:chloroform:isoamyl alcohol, 25/24/1, v/v/v), vigorously mixed, and centrifuged at $10,000 \times g$ for 15 min. After PCI extraction was repeated, the upper phase was transferred to a mixture of 75 μ L of glacial acetic acid and 0.6 volume of isopropanol and incubated at -20 °C for 1 h. After centrifugation at $10,000 \times g$ for 30 min at 4 °C, the pellet was dissolved in DEPC-treated

water. An equal volume of 4 M LiCl was added, and the sample was incubated at -20 °C for 1 h. After centrifugation at $10,000 \times g$ for 30 min at 4 °C, the RNA pellet was cleaned up with PCI and finally dissolved in DEPC-treated water.

For Northern blot analysis, 20 μ g of total RNAs were fractionated on 1.0% agarose-formaldehyde gels in MOPS buffer, transferred to Tropilon-plus™ membranes (Tropix, USA) and then cross-linked by UV irradiation according to the manufacturer's instructions. Probes were synthesized by PCR using biotin-labeled dCTP (Tropix, USA) and T7 and T3 primers, and purified with the PCR purification kit (Qiagen, Germany). Hybridization was carried out at 68 °C for 16 h as described by the manufacturer (Tropix, USA). After hybridization, the membranes were washed twice with $2 \times$ SSC/0.1% SDS for 20 min at room temperature and twice with $0.1 \times$ SSC/0.1% SDS for 1 h at 65 °C. The signal was visualized using the Southern-star™ biotin-labeled DNA detection kit (Tropix, USA).

2.6. Transient expression of the LTP::smGFP construct in Arabidopsis protoplast

To construct LTP::smGFP, *SiLTP1* (MC01026C12) and *SiLTP2* (MC01022A05) cDNAs were amplified by PCR using gene-specific primers (LTP26-F1: 5'-GCGGGATCCGTTTGAAGATGAAGAAG-3', LTP26-R1: 5'-CTCGGATCCTACATGTGGGGAAGGC-3', LTP22-F1: 5'-GCGGGATCCGACAAGAGACATTTTC-3' and LTP22-R1: 5'-CTCGGATCC AATGAACCTTGGAGCA-3') and purified using the nucleic acid purification kit (Qiagen, Germany). The purified DNA fragments were digested with BamHI restriction enzyme and subcloned into the pBIN35S-smGFP vector under the control of the CaMV35S promoter [9].

Two- and 3-week-old *Arabidopsis* leaves were cut into small squares (5–10 mm²) with a razor blade and transferred to 30 mL of enzyme solution (5 mM MES-KOH, pH 5.7, 1% cellulose R-10, 0.3% macerozyme R-10, 500 mM mannitol, and 1 mM CaCl₂). After infiltration at 15 mmHg for 2 min, the plant tissue was incubated in the dark at 22 °C for 5 ~ 7 h with gentle agitation (50 ~ 75 rpm). Protoplasts were filtered through 100- μ m mesh, and intact protoplasts were isolated on a 21% sucrose gradient by spinning at $50 \times g$ for 5 min. The protoplasts were transferred to a new 50 mL tube and washed with two volume of W5 solution (1.5 mM MES-KOH, pH 5.7, 154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, and 5 mM glucose). After centrifugation at $200 \times g$ for 5 min, the protoplasts were resuspended in 10 mL of W5 solution and incubated at 4 °C for at least 2 h. W5 solution was removed and the protoplasts were diluted in MMg solution (5 mM MES-KOH, pH 5.7, 400 mM mannitol, and 15 mM MgCl₂) to 2×10^5 protoplasts mL⁻¹.

For transient expression analysis, 10 μ L of the plasmid DNA ($\sim 2 \mu$ g μ L⁻¹) was added to 300 μ L of protoplast solution (5×10^6 protoplasts mL⁻¹). An equal volume of PEG solution [400 mM mannitol, 100 mM Ca(NO₃)₂, and 40% PEG 8000] was added. The protoplasts were mixed carefully and incubated at room temperature for 30 min. After incubation, the mixture was diluted with 10 mL of W5 solution.

Protoplasts were recovered by centrifugation at $50 \times g$ for 5 min, resuspended in 3 mL of W5 solution, and incubated at 22 °C in the dark. Images were captured with a cooled charge-coupled device camera using a Zeiss (Jena, Germany) Axioplan fluorescence microscope [16].

For Western blot analysis, the transformed protoplasts and protoplast incubation medium were separated by spinning at $50 \times g$ for 5 min. To recover the proteins in the culture medium, cold TCA (100 μ L) was added to the medium (1 mL), and protein aggregates were precipitated by centrifugation at $10,000 \times g$ at 4 °C for 5 min [18]. To prepare cell extracts, transformed protoplasts were subjected to repeated freeze-thaw cycles in ice-cold homogenization buffer (25 mM Hepes, pH 7.7, 1 mM $MgCl_2$, 250 mM sucrose, and 1 mM DTT) supplemented with protease inhibitors (1 μ g mL^{-1} pepstatin A, 1 μ g mL^{-1} aprotinin, 0.5 μ g mL^{-1} leupeptin, and 100 μ M phenylmethylsulfonyl fluoride), followed by brief sonication for 3 s at 50% output (repeated three times). Whole-cell extracts were subjected to low-speed centrifugation ($8000 \times g$) at 4 °C for 5 min to collect cellular debris containing large organelles such as mitochondria. The supernatant was subjected to high-speed centrifugation ($80,000 \times g$) for 30 min at 4 °C to separate microsomal (pellet) and cytoplasmic fractions (soluble supernatant). Whole-cell extracts were assayed by protein gel blot analysis using anti-GFP (Clontech, Palo Alto, CA) and the ECL detection system (Amersham).

3. Results

3.1. Multiple LTP isoforms were isolated in developing sesame seed ESTs

In a previous report [35], 3328 ESTs were obtained from a cDNA library of 5- to 20-day-old immature sesame seeds. From a comparative analysis of sesame and *Arabidopsis* seed ESTs, one striking difference was that the LTP transcript levels were 10-fold lower in *Arabidopsis* seeds than in sesame seeds. Up to 122 LTP ESTs comprise 3.7% of the total sesame seed ESTs. They were clustered into 14 contigs and 7 singletons by StackPack program and analyzed by BLASTX against *Arabidopsis* and rice proteome databases. The most abundant sicn123 contig contains 37 ESTs, and 14 contigs harbor more than one EST. The sicn443 and sicn334 contigs and the MC01006H03, MC01037E02, and MC01040D11 singletons were not matched in rice databases at a threshold of an E-value $>1.0E-10$, but they have sequence homology with *Arabidopsis* protease inhibitor/seed storage/LTP family protein (Table 1). This result suggests that LTP genes exist as a multigene family in sesame and that they are abundantly expressed in developing sesame seeds.

3.2. LTP fraction, which was partially purified from developing sesame seeds, was able to bind to fatty acids and to transfer phospholipids

Because LTP ESTs are highly abundant in developing sesame seeds, we analyzed whether LTP fraction, which

was partially purified from developing sesame seeds, was able to bind to fatty acids and transfer fluorescent phospholipids. The LTP fraction was obtained from developing sesame seeds (15–25 days old) at 40%–80% saturation of ammonium sulfate. For fatty acid binding assays, the LTP fraction was mixed with 1-pyrenedodecanoic acid, and the fluorescence intensity was measured on a spectrofluorimeter at 378 nm (excitation at 343 nm) for monomer and at 437 nm (excitation at 343 nm) for excimer. As shown in Fig. 1A, the upper curve represents the first binding with the acyl-chain of fluorescent fatty acids; the lower curve is the second binding. When the buffer was mixed, background fluorescence was detected (Fig. 1B). For fluorescent phospholipid transfer assays, the LTP fraction was added to NBD-PC donor vesicles. When the acceptor vesicles (DOPC) were added 1 min after addition of sesame LTP fraction, the transfer of fluorescent phospholipid began. In addition, the transfer of fluorescent phospholipid started again when additional LTP fraction was added (Fig. 2). This result indicates that LTPs partially purified from developing sesame seeds can bind to fluorescent fatty acids and transfer fluorescent phospholipids.

3.3. Five LTP cDNAs were isolated from developing sesame seed ESTs

To obtain full-length LTP cDNAs, the longest clones, MC01026C12, MC01022A05, MC01006B03, MC01026G08 and MC01017A02, were selected from five clusters, sicn123, sicn524, sicn331, sicn561, and sicn442, respectively. They were sequenced and named *SiLTP1*, *SiLTP2*, *SiLTP3*, *SiLTP4*, and *SiLTP5*, respectively. The nucleotide and deduced amino acid sequences of the five LTP cDNAs are shown in Fig. 3. The deduced amino acid sequences of all five LTP isoforms contain common features of plant LTPs; they are low molecular weight proteins with a molecular mass of about 7–9 kDa, have similar hydropathic profiles that may reflect conserved secondary structural features in plant LTPs, have eight conserved cysteine residues that form four disulfide bridges, and have a putative signal peptide (Fig. 4A). When the deduced amino acid sequences of these five sesame LTP cDNAs were analyzed by BLASTP against the non-redundant (NR) GenBank protein database, well-matched LTP clones from a variety of plant sources were obtained. The deduced amino acid sequences of sesame and other LTPs were aligned using CLUSTALW and a phylogenetic tree was constructed with TreeView (<http://clustalw.genome.ad.jp>). As shown in Fig. 4B, the five sesame LTP isoforms grouped into three major clusters, the *SiLTP1* cluster, the *SiLTP2* cluster, and the *SiLTP3*–*SiLTP4*–*SiLTP5* cluster. The *SiLTP1* cluster was classified as non-specific LTP type 2 (nsLTP2), a type which was previously defined based on the conserved cysteine pattern. The other clusters were classified as non-specific LTP type 1 (nsLTP1), which includes the original LTPs that have *in vitro* lipid-binding properties.

Table 1
Clusters and singletons encoding LTPs from developing sesame seed ESTs

Contig ID	# of EST	Clones studied in this paper	Members in Contig	Arabidopsis Gene ID	E-value	Rice gene ID	E-value	Description in Arabidopsis and Rice
sicn123	37	MC01026C12	MC01007C03, MC01011B07, MC01012F06, MC01013H08, MC01020A02, MC01020E08, MC01020G12, MC01020H12, MC01021D02, MC01021G11, MC01021H10, MC01024A09, MC01024D10, MC01025G09, MC01026B12, MC01026C12, MC01027A05, MC01027G04, MC01030G01, MC01032G07, MC01033A07, MC01033D11, MC01033F02, MC01037A07, MC01039A03, MC01039H01, MC01039H03, MC01040D01, MC01040D04, MC01045E07, MC01046H10, MC01047E07, MC01053H05, MC01055A05, MC01091E04, MC01091F06, MC01091H02	AT5G38170	1.8E-20	Os05g47700	3.7E-19	[Arabidopsis] protease inhibitor/seed storage/lipid transfer protein (LTP) family protein, [Rice] non-specific lipid-transfer protein AKCS9 precursor
sicn524	20	MC01022A05	MC0122A05, MC01006D05, MC01010E02, MC01011B08, MC01012B05, MC01016F06, MC01017G12, MC01025B07, MC01028B03, MC01028B04, MC01029C02, MC01034A01, MC01034F01, MC01035A08, MC01038B08, MC01041D01, MC01053C10, MC01055E09, MC01058A04, MC01058H03	AT5G01870	8.3E-25	Os11g02379	2.6E-26	[Arabidopsis] putative similar to lipid transfer protein 6 from <i>Arabidopsis thaliana</i> , [Rice] non-specific lipid-transfer protein 1 precursor
sicn443	13		MC01006B12, MC01006G12, MC01009B10, MC01010H01, MC01011D11, MC01021G10, MC01025G08, MC01031H12, MC01033G10, MC01044H07, MC01046B02, MC01057A11, MC01059B11	AT5G54740	9.3E-04			[Arabidopsis] protease inhibitor/seed storage/lipid transfer protein (LTP) family protein similar to 2S seed storage proteins from <i>Arabidopsis thaliana</i>
sicn535	11		MC01002G09, MC01029A10, MC01030C05, MC01031F05, MC01037B01, MC01044E03, MC01050E11, MC01053A06, MC01056E01, MC01058H07, MC01059E12	AT5G55410	3.9E-17	Os07g18750	2.3E-15	[Arabidopsis] similar to protease inhibitor/seed storage/lipid transfer protein (LTP) family protein from <i>Arabidopsis thaliana</i> , [Rice] Protease inhibitor/seed storage/LTP family protein
sicn331	6	MC01006B03	MC01006B03, MC01007E07, MC01019E04, MC01025A10, MC01050C11, MC01057C05	AT2G38540	1.9E-21	Os11g02400	1.2E-26	[Arabidopsis] non-specific lipid transfer protein 1 (LTP1), [Rice] non-specific lipid-transfer protein precursor
sicn561	6	MC0126G08	MC01015D09, MC01024H10, MC01026G01, MC01026G08, MC01026H01, MC01029D08	AT5G01870	8.2E-23	Os11g02400	2.4E-27	[Arabidopsis] putative, similar to lipid transfer protein 6 from <i>Arabidopsis thaliana</i>
sicn582	5		MC01015C08, MC01025B09, MC01043F04, MC01047G10, MC01048G12	AT2G38540	2E-21	Os12g02320	1.1E-27	[Arabidopsis] non-specific lipid transfer protein 1 (LTP1), [Rice] non-specific lipid-transfer protein precursor
sicn124	4		MC01007D10, MC01038F08, MC01043C04, MC01048C11	AT5G38170	1.7E-20	Os05g47700	3.6E-19	[Arabidopsis] protease inhibitor/seed storage/lipid transfer protein (LTP) family protein, [Rice] non-specific lipid-transfer protein AKCS9 precursor
sicn442	3	MC0117A02	MC01017A02, MC01025B08, MC01031F03	AT2G38540	2.7E-20	Os11g02400	2.6E-25	[Arabidopsis] non-specific lipid transfer protein 1 (LTP1), [Rice] non-specific lipid-transfer protein precursor

(continued on next page)

Table 1 (continued)

Contig ID	# of EST	Clones studied in this paper	Members in Contig	Arabidopsis Gene ID	E-value	Rice gene ID	E-value	Description in Arabidopsis and Rice
sicn387	2		MC01045G08, MC01052G04	AT4G30880	8E-26	Os01g58660	6.5E-20	[Arabidopsis] protease inhibitor/seed storage/lipid transfer protein (LTP), [Rice] protein expressed protein
sicn321	2		MC01016G05, MC01055H07	AT5G48485	8.8E-20	Os07g18750	4.5E-16	[Arabidopsis] protease inhibitor/seed storage/lipid transfer protein (LTP) family protein, [Rice] Protease inhibitor/seed storage/LTP family protein
sicn334	2		MC01012A07, MC01029G01	AT4G30880	3E-01			[Arabidopsis] protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
sicn210	2		MC01030G04, MC01030G05	AT1G27950	1.5E-31	Os08g42040	5.8E-26	[Arabidopsis] lipid transfer protein-related, low similarity to lipid transfer protein <i>Picea abies</i> , [Rice] Protease inhibitor/seed storage/LTP family protein
sicn317	2		MC01049H06, MC01054D07	AT1G70250	4.8E-14	Os06g47200	5.6E-13	[Arabidopsis] putative, similar to receptor serine/threonine kinase PR5K, [Rice] Protease inhibitor/seed storage/LTP family protein
MC01056E02	1		MC01056E02	AT5G48485	1.9E-12	Os07g18750	4.9E-13	[Arabidopsis] protease inhibitor/seed storage/lipid transfer protein (LTP) family protein, [Rice] Protease inhibitor/seed storage/LTP family protein
MC01006H03	1		MC01006H03	AT1G36150	2.5E+00			[Arabidopsis] protease inhibitor/seed storage/lipid transfer protein (LTP) family protein low similarity to glucoamylase S1/S2 (Precursor) from <i>Saccharomyces cerevisiae</i>
MC01020D06	1		MC01020D06	AT3G51590	4.7E-12	Os11g02400	2.5E-16	[Arabidopsis] putative, similar to lipid transfer protein E2 precursor <i>Brassica napus</i>
MC01037E02	1		MC01037E02	AT3G22600	7.7E-08			[Arabidopsis] protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
MC01040D11	1		MC01040D11	AT1G73550	1.9E-01			[Arabidopsis] similar to protease inhibitor/seed storage/lipid transfer protein (LTP) family protein, [Rice] non-specific lipid-transfer protein precursor
MC01046G02	1		MC01046G02	AT5G64080	8.3E-25	Os03g09230	3.3E-17	[Arabidopsis] protease inhibitor/seed storage/lipid transfer protein (LTP) family protein, [Rice] Protease inhibitor/seed storage/LTP family protein
MC01051H05	1		MC01051H05	AT5G59310	4.6E-15	Os11g02350	3.2E-18	[Arabidopsis] lipid transfer protein 4 (LTP4), identical to lipid transfer protein 4 from <i>Arabidopsis thaliana</i> , [Rice] non-specific lipid-transfer protein 4 precursor

Developing sesame seed ESTs were clustered using StackPack (<http://www.sanbi.ac.za/CoDES>). Groups that contain only one sequence were classified as singletons.

Sesame seed ESTs were analyzed by BLASTX against *Arabidopsis* and rice proteome databases.

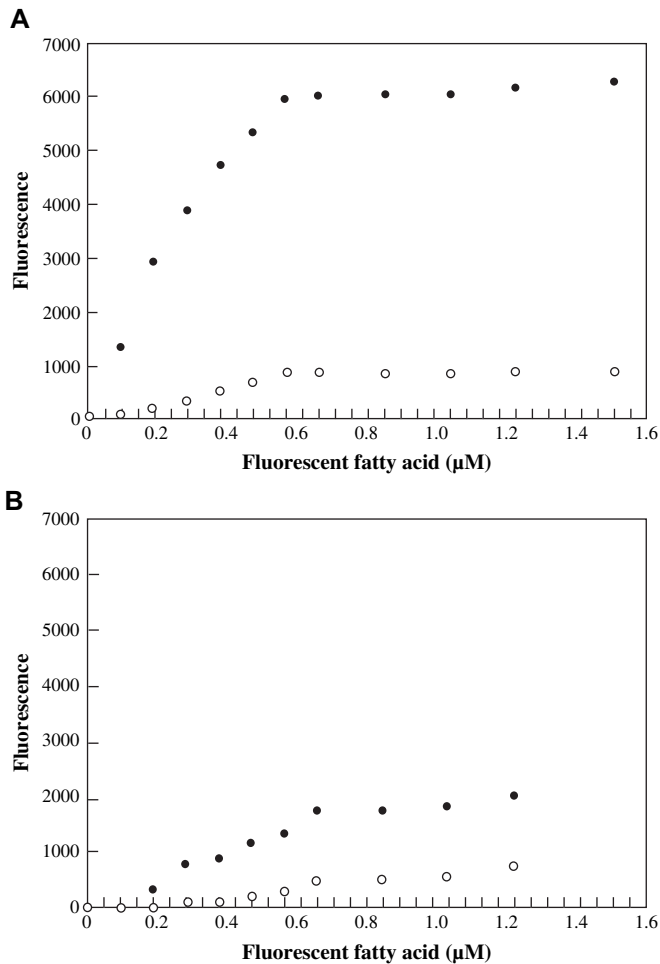


Fig. 1. Association of 1-pyrenedodecanoic acid with buffer (A) or crude LTP extracts from developing sesame seeds (B). 1-pyrenedodecanoic acids were introduced at a final concentration of 1.3 mM. Fluorescence intensity recorded at 378 nm (excitation at 343 nm) for monomer and at 473 nm (excitation at 343 nm) for excimer.

3.4. Expression of five sesame LTP transcripts was tissue-specific and the *SiLTP2* and *SiLTP4* transcripts were induced by exogenous abiotic stresses and plant hormones

To investigate the mRNA expression of these five sesame LTP isoforms, Northern blot analyses were carried out. Total RNA was extracted from roots, stems, leaves, flowers, seeds, and seed coats, run on 1% agarose gels containing 17% formaldehyde, and transferred onto nylon membranes. The membranes were hybridized with biotin-labeled cDNA probes. These five *SiLTP* isoforms were most abundantly expressed in developing seeds, but were also detected in flower tissues. *SiLTP3* and *SiLTP4* transcripts were also expressed in leaves and seed-pot walls, respectively (Fig. 5).

To determine whether expression of these five sesame LTP isoforms was regulated by abiotic stresses and plant hormones, Northern blot analysis was performed with 6-day-old sesame seedlings that were incubated in MS medium containing 300 mM NaCl, 300 mM mannitol, 10 μM gibberellic acid

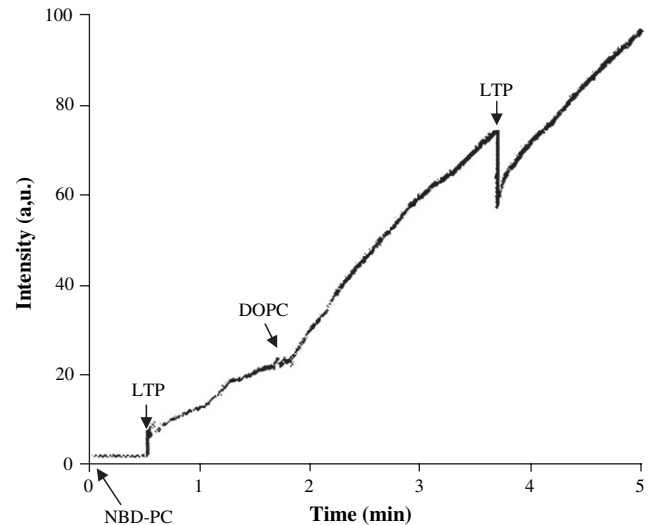


Fig. 2. Transfer of fluorescent phospholipids by crude LTP extracts from developing sesame seeds. NBD-PC and DOPC were used as donor and acceptor vesicles, respectively. Fluorescence was excited at 475 nm and measured at 530 nm in a Perkin Elmer LS-5 spectrophotometer equipped with a thermostated cuvette holder (25 °C) and a mixing device.

(GA₃) or 10 μM ABA for 12 h and 24 h. The expression level of *SiLTP2* and *SiLTP4* transcripts increased significantly with exogenous application of 300 mM NaCl and 300 mM mannitol. *SiLTP2* and *SiLTP4* transcripts also increased approximately 2-fold with ABA treatment. However, GA₃ did not significantly alter the expression of the *SiLTP2* and *SiLTP4* transcripts. Expression levels of *SiLTP1*, *SiLTP3*, and *SiLTP5* transcripts were not significantly influenced by NaCl, mannitol, ABA or GA₃ (Fig. 6). This result suggests that the *SiLTP2* and *SiLTP4* isoforms are responsive to salt- or water-stresses.

3.5. The most abundant LTPs, *SiLTP1* and *SiLTP2*, were secreted

To investigate the subcellular localization of *SiLTP1* and *SiLTP2*, an *in vivo* targeting experiment was performed. The entire *SiLTP1* or *SiLTP2* cDNA without the termination codon was fused to the smGFP (soluble modified green fluorescent protein) gene, and the resulting constructs were introduced into *Arabidopsis* protoplasts. After incubation, the transformed *Arabidopsis* protoplasts were observed under a fluorescence microscope. Protein extracts were harvested from the protoplast incubation medium (M), from the insoluble fraction (D), which contained large organelles such as mitochondria or endoplasmic reticulum (ER), from the microsomal fraction (P), and from the cytoplasmic fraction (S); these extracts were analyzed by Western blot using anti-GFP antibody.

When *Arabidopsis* protoplasts transformed with the *SiLTP1::smGFP* or *SiLTP2::smGFP* constructs were visualized under a fluorescence microscope, most of the green fluorescent signals were secreted out of the cells, whereas the fluorescent signal from the *Arabidopsis* protoplasts transformed with control smGFP constructs was detected in the cytosol (data not shown). In Western blot analysis, recombinant

SiLTP1

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1      gc
3      acgagggcacacacacacataaatacacattgtgctgtttgaagatgaagaagggtgct
                                     M K K G A      5
61     gcagctgtctggtgggtggcgatagtggttgctggccgtggctgaggtgcatgag
      A A V W W V V A I V V V L A V A E V H E      25
121    acggggggcgcccttgcaacccgtagagctgtcgccctgcttgacgcatatcaggg
      T G A A P C N P V E L S P C L A A I S G      45
181    tcgcagccgctcgscggagtgctgcagcaagctgaaggagcaggtgcccctgcttctgt
      S Q P P S A E C C S K L K E Q V P C F C      65
241    gactacgtgaagaacccggtttcaagccgtatgtcgactctcctaatgccaagaaggtt
      D Y V K N P V F K P Y V D S P N A K K V      85
301    gctgcaacctgcggcgtgccttccccacatgttgaaggttgagaaagagtgacatgg
      A A T C G V A F P T C -      97
361    actaagttgcatgtacccagaaaaagaataaattgattgacaggacgaattgatgggtg
421    tttgtgttttatgtaaaaccaccttcacattcgtgtgtaataaaaatggatatttaag
481    aaagtattaaatgcatctttatcccttaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
541    aaaaaaaaaaaaaaaaaaaaaa

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SiLTP2

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1      gcacgagggcctcgccggctccgggcccgggcttaatttgacaagagacattttcttt
61     acttactatcaatctcaagtcattccaatggagaaggcaacgtgcttggtcctggccgtc
                                     M E K A T C L V L A V      11
121    accatggtggtggttgacttgaccacccagggcgagggcagccatcatgcccact
      T M V V V A L A P P Q A E A A I S C G T      31
181    gtggccaacgacctgtcccggtgcatcaactacgtgatgtatggtggggcggggaccca
      V A N D L S P C I N Y V M Y G G A G T P      51
241    ccggtaccatgctgcaccgggataaagacctctacaatcaggcaagcagcaccgtgac
      P V P C C T G I K T L Y N Q A S S T A D      71
301    cggcaggctgtttgtgggtgcttgaatctgttgccggccaagccagccctgccatcatt
      R Q A V C G C L K S V A G Q A S P A I I      91
361    ggcacagccgcccacttccctggcaaatgtggcgtctctattccttacgagatcagcccc
      G T A A A L P G K C G V S I P Y E I S P      111
421    tccacgattgctccaaggttcattaaagttgacggagctgctgtcagttttccctccta
      S T D C S K V H -      119
481    agctacgatggatgcatgtaacctgcaatctattaattattattatgctagtagcgag
541    tttgtgttgtagtaggagtgctgaaggagaaattagtaatttgcaatgtgatgatgat
601    ccatgtccatcacctcatcatattatataatgtgtgctactctgatttcctttattaa
661    tatgaattaaatcaaaaaaaaaaaaaaaaaaaaaa

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SiLTP3

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1      gcaccaggacggagcagcgcgctagaacttggtttttcttacatgtcaagcttcgatt
61     gcaatggctggaacatcaagccaatgtttgtcgtcctcatcgcgctgctgcatcatg
      M A G T I K P M F V V L I A V V S I M      19
121    tcggtcgttccagagggtgaggtgcataggttcggcactgtagtttcatatctgaat
      S V V P E G E A A I G C G T V V S Y L N      39
181    tcatgctttccatacgtgaccggaactggtgcgttggtggatgctgtaagggttaag
      S C F P Y V T G T G A L G G C C K G I K      59
241    gatctctatgcggcgcccaaacacagccgaccgacagagcgtgtgagactgcccga
      D L Y A A A Q T T A D R Q S V C D C L K      79
301    gatcttgctgctcgaccactggcgtcgacctcgscaggctgctggactccccagccag
      D L A A S T T G V D L G K A A G L P S Q      99
361    tgtggtcttaacatccctacaagatcattccttcaactgattgctcaagggtgcaatga
      C G L N I P Y K I I P S T D C S K V Q -      118
421    gctttgttgaagaagagatctatagtttcatgaacctccgctacattttatcataaaat
481    attaaacaaaataaagagttttagttcgctgctgctggagacttgatctatttagacgt
541    atcattattattgtgatgcaacggtaacctactttcatgtactcaaaaaaaaaaaaaaaaaa

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Fig. 3. Nucleotide and deduced amino acid sequences of five sesame LTP isoforms, *SiLTP1*, *SiLTP2*, *SiLTP3*, *SiLTP4*, and *SiLTP5*. “-” indicates the stop codon.

SiLTP4

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1   gcacgaggagaacactgagctctttgttctcctgtcatttgagatcttttaactacaatg
                                     M      1
61  gctggaattatcaggccaa:gtgcatttctcctcattgcccgctcttgccatggcagtt
    A G I I R P M C I L L I A A V L A M A V      21
121 gccctcgcggtgaggcagcaataggctgcggcacggtggtttcatacctgaatccatgc
    A P R G E A A I G C G T V V S Y L N P C      41
181 ctgccatatgtaaccaaaaaggccctctaggcagttgttgcggtggagttaaagggtctg
    L P Y V T N K G P L G S C C G G V K G L      61
241 tatggagcagccagactgcacaagatcgtaaaagcgtgtgcagctgcttgaatctctc
    Y G A A Q T A Q D R Q S V C S C L K S L      81
301 gctggtctatacaaaagatgtcgacctgaacaaggccgctggactccctggacaatgtggc
    A G S Y K D V D L N K A A G L P G Q C G      101
361 gtcaacattccctacaagattagcccttccactgattgctcaaggtgaattgaggaggt
    V N I P Y K I S P S T D C S K V N -      118
    atgttcagaaaaatggccttctacatatataagttttagcagatataaaataaagggttg
    gtccatgagctgacccaaccagggtactccagagttttctttccatgatggactgat
    gtgaatcaatagccaatgatgaataataccaactttctataaaaaaaaaaaca

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SiLTP5

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1   gcacgaggcaactatccagcaaaaagcagcgacgaggttttcacgcagaattaagtta
61  gtacgtaccatggcttccatgatcaaggcattgtgcgttgcgcttgttggcgcgctgttg
    M A S M I K A L C V A L V G A V L      17
121 gtgatagcagttgtgccccggctgaggccgattaggctgcggcagcgtgatattctat
    V I A V V P P A E A A L G C G S V I S Y      37
181 ctgggttcattgcccccctatgtgaccgacaaaaggtcctttagg:ggttgctgcagtgagg
    L G S C R P Y V T D K G P L G G C C S G      57
241 gtgaaggagctctataaggctgcaaaaaccaccgagatcgatcagccacttgcagctgc
    V K G L Y K A A K T T A D R Q A T C S C      77
301 ctcaaaactcttgccagtacctacaagggtgtcaatcttagcaaggcagccgagcttccct
    L K T L A S T Y K G V N L S K A A G L P      97
361 cagcaatgtggtgttaacattccctataaaatcagcccttcaccgattgctcaaaagtg
    Q Q C G V N I P Y K I S P S T D C S K V      117
421 acg:gaatttaatggaggaaaattgaaggcttgtgcatgtatga:gatgcagcagcagca
    T -      118
481 acc:ttatgatatgttttggataataataataatgatatgagtaataaaaaa

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Fig. 3. (continued).

SiLTP1::smGFP and SiLTP2::smGFP were detected in the protoplast incubation medium (M), suggesting that recombinant SiLTP1::smGFP and SiLTP2::smGFP were secreted from the cells. In addition, recombinant SiLTP1::smGFP was mainly detected in the insoluble fraction (D), whereas recombinant SiLTP2::smGFP was observed in the cytoplasmic soluble fraction (S), suggesting that the secretory pathways for SiLTP1 and SiLTP2 are probably different. The smaller size band detected in the total or soluble fraction may be due to proteolytic processing of the recombinant proteins in the cell (Fig. 7).

4. Discussion

Plant LTPs are abundantly expressed in various aerial tissues, except roots, and are involved in multiple biological functions including cutin formation, embryogenesis, defense responses against pathogens, symbiosis between *Rhizobium* and symbiotic bacteria, and adaptation of plants to various

environmental conditions [17]. In this study, we demonstrated that LTPs are abundantly expressed in developing sesame seeds. Fourteen contigs and seven singletons encoding LTP were isolated from developing sesame ESTs (Table 1). According to Grobois et al. [13], LTPs purified from maize seeds comprised up to 4% of total soluble proteins. Recently, 76 members of the LTP family have been annotated in *Arabidopsis* [1]. The rice LTP gene family comprises at least 17 genes that can be categorized into three subfamilies according to the LTP protein structure [19,39]. This finding also supports that ESTs encoding LTPs comprised up to 7% of total rose petal ESTs [7].

LTPs were purified from plant sources including seeds and leaves based on molecular size (7–10 kDa) and isoelectric point (around 9). The purified LTPs were very stable and maintained their activity after months of storage at 4 °C [17]. LTPs have previously been characterized as a family of proteins capable of transferring lipid molecules such as phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine

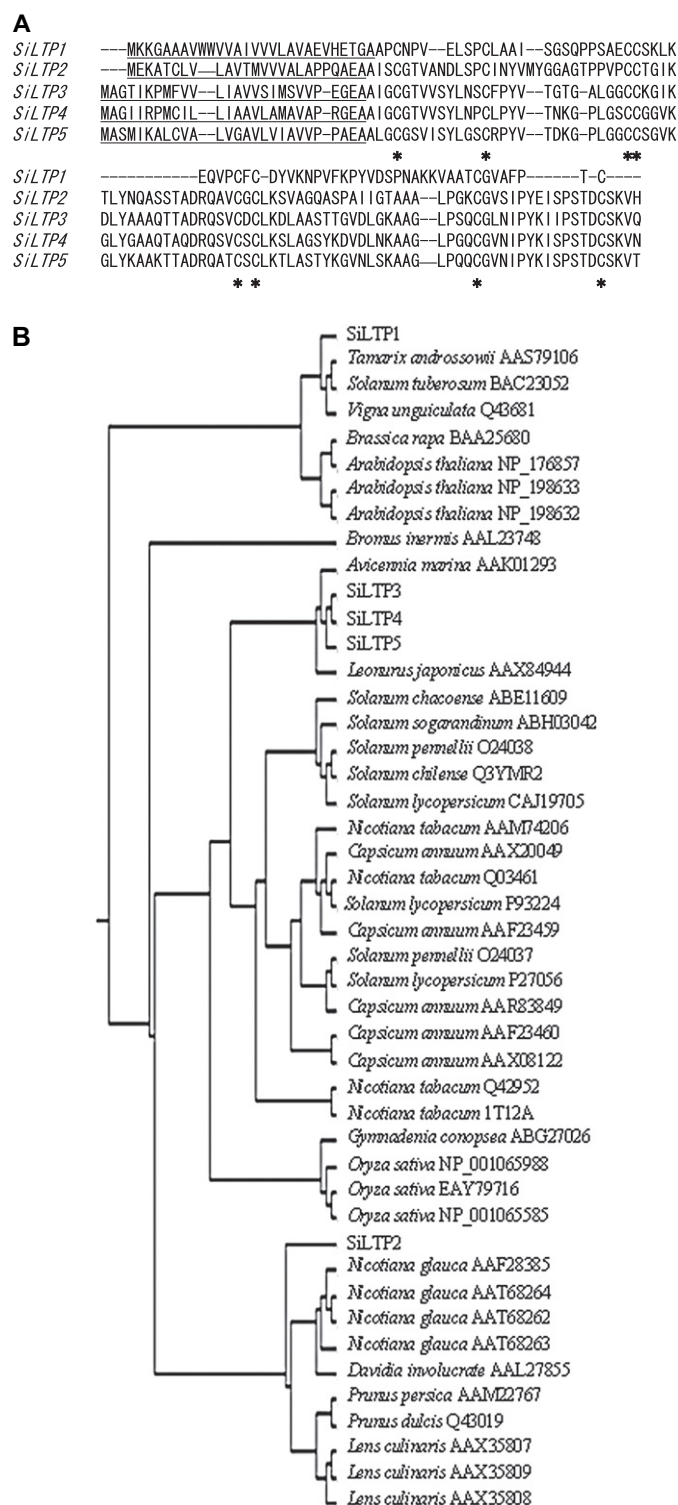


Fig. 4. Comparison of deduced amino acid sequences of five sesame LTP isoforms (A) and phylogenetic tree of sesame and other plant LTPs (B). (A) Gaps indicated by dashes were introduced to obtain optimal alignment. Putative signal sequences are underlined and the eight conserved cysteine residues are marked by asterisks. (B) The nucleotide sequences of *SiLTP1*, *SiLTP2*, *SiLTP3*, *SiLTP4*, and *SiLTP5* cDNAs were registered in GenBank™/EBI Data Bank under accession numbers, EF581314, EF581315, EF581316, EF581317, and EF581318, respectively.

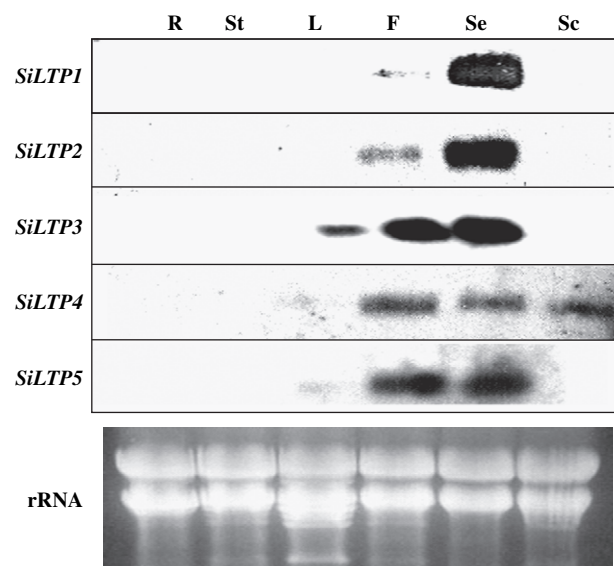


Fig. 5. Northern blot analysis of five sesame LTP isoforms in various tissues of sesame. Twenty μ g of total RNAs were isolated from roots (R), stems (St), leaves (L), flowers (F), seeds (Se), and seed coats (Sc) of sesame. Ethidium bromide staining of the gel (rRNA, ribosomal RNA) prior to transfer indicates RNA loading.

(PE), phosphatidylglycerol (PG), and galactolipids, as well as fatty acids and acyl-CoAs, *in vitro* and *in vivo*. For example, several LTPs from oilseed rape were identified as having both the ability to transfer PC and to bind oleoyl-CoA. Castor bean LTPs contain binding capacities for oleic acid and oleoyl-CoA [17]. As described in Figs. 1 and 2, sesame seed LTPs are typical plant LTPs that are able to both transfer phospholipids and bind acyl chains.

The expression patterns of LTP genes in various plants are reported to be temporally and spatially controlled [12,17]. Transcripts of the *SiLTP1* and *SiLTP2* isoforms, the most abundant ESTs, were predominantly expressed in developing

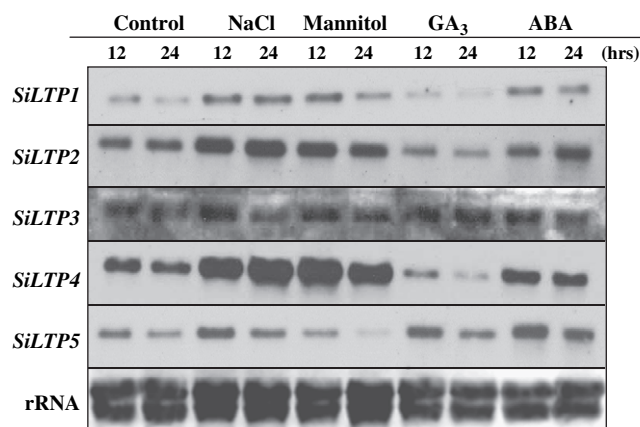


Fig. 6. Northern blot analysis of five sesame LTP isoforms after exogenous application of abiotic stresses and plant hormones. Six-day-old sesame seedlings were incubated with 300 mM NaCl, 300 mM mannitol, 10 μ M GA_3 or 10 μ M ABA for 12 h or 24 h. Ethidium bromide staining of the gel (rRNA, ribosomal RNA) prior to transfer indicates RNA loading.

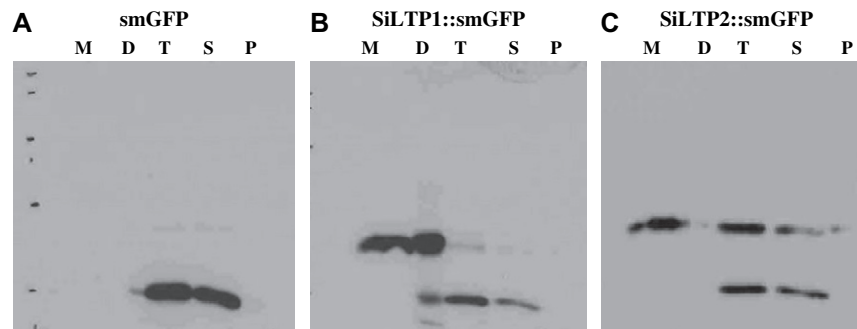


Fig. 7. Subcellular localization of recombinant SiLTP1::GFP and SiLTP2::GFP in *Arabidopsis* protoplasts. The recombinant SiLTP1::GFP and SiLTP2::GFP constructs were introduced into *Arabidopsis* protoplasts. Protein extracts were harvested from the protoplast incubation medium (M), the insoluble fraction (D) containing large organelles such as mitochondria, the soluble supernatant fraction (T) containing the microsomal and cytoplasmic fractions, the microsomal fraction (P), and the cytoplasmic fraction (S). The extracted proteins were analyzed by Western blot using anti-GFP antibody. Whole-cell extracts were assayed by Western blot using anti-GFP (Clontech, Palo Alto, CA) and the ECL detection system (Amersham). Molecular mass standards were purchased from Gibco BRL.

sesame seeds (Table 1 and Fig. 5). When recombinant SiLTP1::smGFP and SiLTP2::smGFP proteins were transiently expressed in *Arabidopsis* protoplasts, fluorescent signals derived from smGFP were detected at the plant cell surface, which is consistent with previous reports [4,36]. Indeed, Western blot analysis demonstrated that the SiLTP2::smGFP protein was in the soluble fraction and that the SiLTP1::smGFP protein was in the insoluble fraction containing large organelles (Fig. 7). In the classical secretory pathway, proteins containing a signal peptide are translocated from the cytoplasm into the ER lumen. From the ER, the proteins are transported to the Golgi apparatus and finally to the plasma membrane or extracellular space [10,40]. However, some extracellular proteins leave the cell by non-classical secretory pathways such as through proteinaceous membrane channels [22] and evaginations of the plasma membrane [8]. Proteins such as basic fibroblast growth factor [24], interleukin-1 [32], and the lectin L-14-1 [8] are synthesized without a signal peptide and remain mainly in the cytoplasm until they are released from the cell. Therefore, SiLTP1::smGFP protein, which associated with large organelles such as ER, is probably secreted by the classical secretory pathway. Although little is known about the non-classical secretory pathways in plant cells, the SiLTP2::smGFP protein, detected in the soluble fraction, is probably secreted by a non-classical secretory pathway. Secretion of these two sesame LTP isoforms should be further confirmed by brefeldinA or monensin inhibitors, which are known to inhibit protein transport through the classical secretory pathway.

The *SiLTP3* and *SiLTP5* isoforms were abundantly expressed in flower tissues, as well as developing seeds (Fig. 5). LTPs, which are abundant in floral organs, petal corolla, carpel, and anther, are thought to participate in the deposition of lipophilic materials [7,11,20]. Park et al. [29] reported that LTPs are necessary for pollen-tube adhesion of lily flower. According to Jeroen et al. [15], tobacco LTP isolated from tobacco stigma exudates had cell wall-loosening activity and enhanced cell wall extension. The biological roles of *SiLTP3* and *SiLTP5* isoforms should be further elucidated in sesame flowers.

LTP transcripts from several plant sources are also responsive to environmental stresses such as cold, drought, salt, pathogens, and ABA [3,14,19,21,25,26,28,31,37,39,41]. By subtractive hybridization between drought-tolerant and -sensitive sunflowers, a non-specific LTP gene was isolated and its transcripts were induced by water-stress and ABA [27]. LTP mRNA expression and cuticular wax were increased by periodic dehydration stress in tree tobacco (*Nicotiana glauca*) leaves, suggesting that LTP is involved in cuticle deposition. *SiLTP2* and *SiLTP4* isoforms exhibited significantly inducible expression patterns with exogenous application of 300 mM NaCl and 300 mM mannitol. Exogenous ABA, which has been shown to mediate plant tolerance to water or cold stresses, also significantly induced the *SiLTP2* and *SiLTP4* isoforms (Fig. 6). Although the biological roles of the *SiLTP2* and *SiLTP4* isoforms in response to salt and osmotic stresses remain to be elucidated, these isoforms may play an important role in plant acclimation to water stress during seed development.

In conclusion, we identified 21 LTP isoforms that are abundant in developing sesame seed ESTs. Full-length cDNAs of five abundant LTP isoforms were determined. LTPs partially purified from developing sesame seeds can bind fatty acids and transfer phospholipids. SiLTP1 and SiLTP2 were secreted out of cells. Although the biological roles of sesame LTPs are not yet known, the abundant SiLTP1 and SiLTP2 are probably involved in early embryogenesis by participating in the formation of a protective layer around the young embryo. *SiLTP2* and *SiLTP4* isoforms, which are induced by desiccation conditions, may be involved in seed adaptation to water shortage during seed development.

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References

- [1] F. Besson, A.J.K. Koo, S. Ruuska, J. Schwender, M. Pollard, J.J. Thelen, T. Paddock, J.J. Saslas, L. Savage, A. Milcamps, V.B. Mhaske, Y. Cho, J.B. Ohlrogge, *Arabidopsis* gene involved in acyl lipid metabolism. A 2003 genes of the candidates, a study of the distribution of expressed sequence tags in organs, and a web-based database, *Plant Physiol.* 132 (2003) 681–697.
- [2] M.M. Bradford, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding, *Anal. Biochem.* 72 (1976) 248–254.
- [3] K.D. Cameron, M.A. Teece, L.B. Smart, Increased accumulation of cuticular wax and expression of lipid transfer protein in response to periodic drying events in leaves of tree tobacco, *Plant Physiol.* 140 (2006) 176–183.
- [4] S. Canevascini, D. Caderas, T. Mandel, A.J. Fleming, I. Dupuis, C. Kuhlemeier, Tissue-specific expression and promoter analysis of the tobacco *ltp1* gene, *Plant Physiol.* 112 (1996) 513–524.
- [5] Carlsson, A.S., Chanana, N.P., Gudu, S., Suh, M.C., Were, B.A., Sesame, in: Kole, C., Hall, T.C. (Eds.), *A Compendium of Transgenic Crop Plants*, vol. 2, Wiley-Blackwell, (In Press).
- [6] A.O. Carvalho, C.E.S. Teodoro, M. Da Cunha, A.L. Okorokova-Façanha, L.A. Okorokova, K.V.S. Fernandes, V.M. Gomes, Intracellular localization of a lipid transfer protein in *Vigna unguiculata* seeds, *Physiol. Plant.* 122 (2004) 328–336.
- [7] S. Channeliere, S. Riviere, G. Scalliet, J. Szecsi, F. Jullien, C. Dolle, P. Vergne, C. Dumas, M. Bendahmane, P. Hugueney, J.M. Cork, Analysis of gene expression in rose petals using expressed sequence tags, *FEBS Lett.* 515 (2002) 35–38.
- [8] D.N.W. Cooper, S.H. Barondes, Evidence for export of a muscle lectin from cytosol to extracellular matrix and for a novel secretory mechanism, *J. Cell Biol.* 110 (1990) 1681–1691.
- [9] S.J. Davis, R.D. Vierstra, Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants, *Plant Mol. Biol.* 36 (1998) 521–528.
- [10] N.A. Eckardt, Green light for traffic in the early secretory pathway, *Plant Cell.* 12 (2000) 2009–2011.
- [11] G.D. Foster, S.W. Robinson, R.P. Blundell, M.R. Roberts, R. Hodge, J. Draper, R.J. Scott, A *Brassica napus* mRNA encoding a protein homologous to phospholipid transfer proteins, is expressed specifically in the tapetum and developing microspores, *Plant Sci.* 84 (1992) 184–192.
- [12] K. Gausing, Lipid transfer protein genes specifically expressed in barley leaves and coleotiles, *Planta* 192 (1994) 574–580.
- [13] M. Grobois, F. Guerbette, J.C. Kader, Changes in level and activity of phospholipids transfer protein during maturation and germination of maize seeds, *Plant Physiol.* 90 (1989) 1560–1564.
- [14] E. Guiderdoni, M.J. Cordero, F. Vignols, J.M. Garcia-Garrido, M. Lescot, D. Tharreau, D. Meynard, N. Ferriere, J.L. Notteghem, M. Delseny, Inducibility by pathogen attack and developmental regulation of the rice *Ltp1* gene, *Plant Mol. Biol.* 49 (2002) 683–699.
- [15] N. Jeroen, R. Feron, B.A.H. Huisman, A. Fasolino, C.W. Hilbers, J. Derksen, C. Mariani, Lipid transfer proteins enhance cell wall extension in tobacco, *Plant Cell.* 17 (2005) 2009–2019.
- [16] J.B. Jin, Y.A. Kim, S.J. Kim, S.H. Lee, D.H. Kim, G.W. Cheong, I. Hwang, A new dynamin-like protein, ADL6, is involved in trafficking from the trans-Golgi network to the central vacuole in *Arabidopsis*, *Plant Cell.* 13 (2001) 1511–1526.
- [17] J.C. Kader, Lipid-transfer proteins in plants, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47 (1996) 627–654.
- [18] D.H. Kim, Y.J. Eu, C.M. Yoo, Y.W. Kim, K.T. Pih, J.B. Jin, S.J. Kim, H. Stenmark, I. Hwang, Trafficking of phosphatidylinositol 3-phosphate from the trans-Golgi network to the lumen of the central vacuole in plant cells, *Plant Cell.* 13 (2001) 287–301.
- [19] T.H. Kim, M.C. Kim, J.H. Park, S.S. Han, B.R. Kim, B.Y. Moon, M.C. Suh, S.H. Cho, Differential expression of rice lipid transfer protein gene (LTP) classes in response to abscisic acid, salt, salicylic acid, and the fungal pathogen *Magnaporthe grisea*, *J. Plant Biol.* 49 (2006) 371–375.
- [20] M. Kotilainen, Y. Helarriutta, P. Elomaa, L. Paulin, T.H. Teeri, A corolla- and carpel-abundant, nonspecific lipid transfer protein gene is expressed in the epidermis and parenchyma of *Geranium hybridum* var Regina (Compositae), *Plant Mol. Biol.* 26 (1994) 971–978.
- [21] A.K. Kristensen, J. Brunstedt, K.K. Nielsen, P. Roepstorff, J.D. Mikkelsen, Characterization of a new antifungal non-specific lipid transfer protein (nsLTP) from sugar beet leaves, *Plant Sci.* 155 (2000) 31–40.
- [22] K. Kuchler, J. Thorner, Membrane translocation of proteins without hydrophobic signal peptides, *Curr. Opin. Cell Biol.* 2 (1990) 617–624.
- [23] A.M. Maldonado, P. Doerner, R.A. Dixon, C.J. Lamb, R.K. Cameron, A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*, *Nature* 419 (2001) 399–403.
- [24] L. Mignatti, T. Morimoto, D.B. Rifkin, Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex, *J. Cell Physiol.* 151 (1992) 81–93.
- [25] A. Molina, I. Diaz, I.K. Vasil, P. Carbonero, F. Garcia-Olmedo, Two cold-inducible genes encoding lipid transfer protein LTP4 from barley show differential response to bacterial pathogens, *Mol. Gen. Genet.* 252 (1996) 162–168.
- [26] A. Molina, F. Garcia-Olmedo, Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2, *Plant J.* 12 (1997) 669–675.
- [27] O. Ouvrard, F. Cellier, K. Ferrare, D. Tousch, T. Lamaze, J.M. Dupuis, F. Casse-Delbart, Identification and expression of water stress- and abscisic acid-regulated genes in a drought-tolerant sunflower genotype, *Plant Mol. Biol.* 31 (1996) 819–829.
- [28] C.J. Park, R. Shin, J.M. Park, G.J. Lee, J.S. You, K.H. Paek, Induction of pepper cDNA encoding a lipid transfer protein during the resistance response to tobacco mosaic virus, *Plant Mol. Biol.* 48 (2002) 243–254.
- [29] S.Y. Park, G.Y. Jauh, J.C. Mollet, K.J. Eckard, E.A. Nothnagel, L.L. Walling, E.M. Lord, A lipid transfer-like protein is necessary for lily pollen tube adhesion to an *in vitro* stylar matrix, *Plant Cell.* 12 (2000) 151–164.
- [30] J. Pyee, P.E. Kolattukudy, The gene for the major cuticular wax-associated protein and three homologous genes from broccoli (*Brassica oleracea*) and their expression patterns, *Plant J.* 7 (1995) 49–59.
- [31] S. Roy-Barman, C. Sautter, B.B. Chattoo, Expression of the lipid transfer protein *Ace-AMP1* in transgenic wheat enhances antifungal activity and defense responses, *Transgenic Res.* 15 (2006) 435–446.
- [32] A. Rubartelli, F. Cozzolino, M. Talio, R. Sitia, A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence, *EMBO J.* 9 (1990) 1503–1510.
- [33] J. Sambrook, E.F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, second ed. Cold Spring Harbor Laboratory Press, Plainview, NY, 1989.
- [34] D.H. Shin, J.Y. Lee, K.Y. Hwang, K.K. Kim, S.W. Suh, High-resolution crystal structure of the non-specific lipid-transfer protein from maize seedlings, *Structure* 3 (1995) 189–199.
- [35] M.C. Suh, M.J. Kim, C.-G. Hur, J.M. Bae, Y.I. Park, C.-H. Chung, C.-W. Kang, J.B. Ohlrogge, Comparative analysis of expressed sequence tags from *Sesamum indicum* and *Arabidopsis thaliana* developing seeds, *Plant Mol. Biol.* 52 (2003) 1107–1123.
- [36] S. Thoma, U. Hecht, A. Kippers, J. Botella, S. De Vries, C.R. Somerville, Tissue-specific expression of a gene encoding a cell wall-localized lipid transfer protein from *Arabidopsis*, *Plant Physiol.* 105 (1994) 35–36.
- [37] M.B. Trevino, M.A. O'Connell, Three drought-responsive members of the non-specific lipid transfer protein gene family in *Lycopersicon*

- pennellii* show different developmental patterns of expression, Plant Physiol. 116 (1998) 1461–1468.
- [38] S. Tsuboi, T. Osafune, I.R. Tsugek, M. Nishimura, M. Yamada, Nonspecific lipid transfer protein in castor bean cotyledons cells: subcellular localization and a possible role in lipid metabolism, J. Biochem. 111 (1992) 500–508.
- [39] F. Vignols, M. Wigger, J.M. Garcia-Garrido, F. Grellet, J.C. Kader, M. Delseny, Rice lipid transfer protein (LTP) genes belong to a complex multigene family and are differentially regulated, Gene 195 (1997) 177–186.
- [40] A. Vitale, J. Denecke, The endoplasmic reticulum: gateway of the secretory pathway, Plant Cell. 11 (1999) 615–628.
- [41] E.M. Yubero-Serrano, E. Moyano, N. Medina-Escobar, J. Munoz-Blanco, J.L. Caballero, Identification of a strawberry gene encoding a non-specific lipid transfer protein that responds to ABA, wounding and cold stress, J. Exp. Bot. 54 (2003) 1865–1877.
- [42] A. Zachowski, F. Guerbet, M. Grosbois, A. Jolliot-Croquin, J.C. Kader, Characterization of acyl binding by a plant lipid-transfer protein, Eur. J. Biochem. 257 (1998) 443–448.