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Isolation and characterization of genes encoding lipid transfer proteins in *Linum usitatissimum*

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Abstract

Very little is known about lipid transfer proteins from flax (*Linum usitatissimum* L.). In the present work, three genes encoding a lipid transfer protein (LTP) were isolated from flax, two of which encoded Type-1 and one Type-2 LTPs with molecular masses of about 9 and 7 kDa, respectively. The analysis of deduced amino acid sequence reveals that only Type 2 of the *L. usitatissimum* leaf specific LTP (LuLTP_Ls) had an N terminal signal peptide consisting of 23 amino acids. The phylogenetic analyses of LuLTP_Ls suggest their closest relatedness with respective proteins from *Dimocarpus longan* and *Vitis vinifera*. The gene expression analysis shows that LTP Type 1 genes, which include *LuLTP_Ls*1 and *LuLTP_Ls*3, were progressively expressed during leaf development, whereas *LuLTP Ls*4 (Type 2) was expressed only at initial and terminal senescence stages of cotyledons. The results suggest that both types of *LuLTP_Ls* were differentially yet significantly expressed in cotyledons implicating their function in transport and scavenging lipidic skeletons for the benefit of other developing parts of the plant.

Additional key words: flax, gene expression, molecular cloning, plant development, RT-qPCR.

Introduction

Leaf senescence is terminal phase leading to cellular disassembly and mobilization of released materials to newly developing organs. Leaf senescence is not only associated with a passive degradation of macromolecules but also involves active recycling nutrients to support other developing organs (Quirino *et al*. 2000, Hortensteiner and Feller 2002). Senescence is terminal phase of developmental process that includes highly coordinated changes in gene expression and protein synthesis (Shahri and Tahir 2011). Additionally, underlying signalling and transcriptional networks are up-regulated with initiation of senescence. These signalling programs trigger remobilization of macromolecule breakdown products to sink locations. This macro-molecular transport is mediated *via* specific transporters, and lipid transfer proteins (LTPs) are involved in signalling and transport of lipidic macromolecules (Edqvist and Farbos 2002).

The LTPs are small basic proteins widely distributed in the plant kingdom and are encoded by a multigene family. Non-specific LTPs (ns-LTPs) are ubiquitous and have been reported in various plant species such as rice, peach, cumin, and coffee (Liu *et al.* 2002, Pasquato *et al*. 2006, Zaman and Abbasi 2009, Zottich *et al*. 2011). The Ns-LTPs are classified into two subfamilies as ns-LTP1 (9 kDa) and ns-LTP2 (7 kDa) according to their molecular masses and eight conserved cysteine residues (Kader 1996, Liu *et al*. 2002). Structurally, LTPs are composed of helices and a long flexible loop at the C terminal end (Lee *et al*. 1998, Han *et al*. 2001). The four α-helices enclose a hydrophobic cavity which can specifically accommodate binding lipids and other hydrophobic ligands (Kader 1996, Lascombe *et al*. 2008). The Ns-LTPs lack a tryptophan residue and consist of conserved C-Xn-C-Xn-CC-Xn-CXC-Xn-C-C cysteine motifs. Plant nsLTPs show different binding affinities for monoacylated as well as diacylated hydrophobic molecules (Han *et al*. 2001, Charvolin *et al*. 1999).

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Abbreviations: EST - expressed sequence tag; *GAL 1* - galactose inducible 1; His - histidine; LuLTP_Ls - *Linum usitatissimum* lipid transfer protein; ORF - open reading frame; RT-PCR - reverse transacriptase - polymerase chain reaction.
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In plants, ns-LTPs are major lipid binding proteins and have been implicated in diverse biological processes like cutin synthesis, wax assembly, seed storage lipid mobilization, and protection against bacterial, fungal, and viral pathogens (Buhot *et al*. 2001, Salcedo *et al*. 2007). During plant development, the members of ns-LTP gene families exhibit a wide range of expression profiles. Rice LTPs show organ specific expression, *e.g*., the expression of Type 1 LTP is higher in flowers and stems, whereas that of Type 2 LTPs is higher in leaves and stems (Kim *et al*. 2006). The LTPs are highly expressed during developmental processes of shoots (Kader 1996).

In our laboratory, five members of *LTP* genes were isolated and characterized from flax, and they are specific to heart, globular, and torpedo stages of developing embryo (unpublished). Interestingly, these *LTPs* were found to be different from that of leaf specific *LTP* members. In the present study, we report cloning and characterization of the leaf specific *LTPs* from flax.

Materials and methods

Seeds of flax (*Linum usitatissimum* L.) cv. NL-260 were obtained from Dr. P.B. Ghorpade, the Nagpur Agricultural College, Nagpur, India. The genomic DNA was extracted using a *DNeasy* kit (*Quiagen*, Maryland, USA), and quantified spectrophotometrically (*Lambda EZ201*, *Perkin Elmer*, Waltham, USA) at 260 nm. Quality of DNA was checked by 0.8 % (m/v) agarose gel electrophoresis, and the DNA was stained with ethidium bromide.

The *Escherichia coli* strain (XL-1 blue) and the *pGEMT®-Easy* vector were obtained from *Invitrogen* (Madison, USA) for DNA cloning procedure. Sub-cloning *LuLTP1* was carried out in the pYES2/CT vector. Yeast (*Saccharomyces cerevisiae*) cells were grown in a standard yeast extract peptone dextrose medium at 30 ºC and the strain INVSC1 (a genotype MATa his3Δ1 leu2 trp1-289 ura3-52/MATα his3Δ1 leu2 trp1-289 ura3-52) was used for recombinant protein expression (*Invitrogen,* Madison, USA).

For RNA isolation, flax cotyledons were collected at 0, 4, 12, 20, 24, 32 d after germination and stored in -80 ºC prior to use. The total RNA was obtained using a *Spectrum*™ plant total RNA kit (*Sigma*, Louis, USA). A cDNA was obtained from the RNA with a *Superscript®* first-strand cDNA synthesis kit (*Invitrogen*, Carlsbad, USA). Quality of RNA and cDNA preparations was evaluated by polymerase chain reaction (PCR) amplification with specific 18S ribosomal RNA (rRNA) gene primers as internal standards (data not shown).

The sequences of flax LTPs were obtained from the flax EST database developed at the National Research Council Canada, the Plant Biotechnology Institute. To obtain full length coding sequences of these genes, primer pairs flanking the entire open reading frame (ORF) were designed using the software *Primer Premier 5*. For directional cloning, KpnI and XbaI restriction sites were engineered at the 5' and 3' ends, respectively, nucleotide sequences of forward and reverse primers are illustrated in Table 1. Amplifications by PCR were performed with the flax genomic DNA as template. The amplified products were resolved by gel electrophoresis, and expected size amplicons were eluted with a *Purelink*™ gel extraction kit (*Invitrogen*, Lohne, Germany) and ligated into *pGEMT* (*Promega*, Madison, USA). The chemically competent cells of *E. coli* were transformed with pGEMT-LTPs on Lurrie-Broth plates containing isopropyl β-D-1-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-gal) and ampicilin. The positive transformants were confirmed by colony PCR using the same primers. Plasmids from the positive colonies were isolated using spin columns (*Axygen*, Union City, USA). Inserts were reconfirmed by bidirectional sequencing using T7 and SP6 promoter primers (*Eurofins*).

The flax *LTP* genes (*pGEMT-LTPs*) were sub-cloned into the pYES2/CT yeast expression vector (*Invitrogen*, Carlsbad, USA). The pGEMT-LTPs were digested with KpnI and XbaI restriction enzymes, and the genes were ligated between the identical restriction sites of pYES2/CT resulting into a plasmid referred to as pYES-LTPs. The INVSC1 strain of *S. cerevisiae* was transformed with the pYES-LTP plasmid by the lithium acetate method, and positive transformants were selected using an uracil lacking synthetic complete medium. "In frame" cloning was confirmed by re-sequencing from both ends.

Protein content was estimated by the method of Lowry *et al*. (1951) from a calibration curve using bovine serum albumin. The recombinant protein was purified on a nickel column using a *PrepEasy* histidine tagged protein purification high specificity kit (*USB Corporation*, Cleveland, USA).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the fractions obtained from the nickel column was carried out according to Laemmli (1970). Molecular masses of fractions were estimated with molecular mass protein markers (*Invitrogen*, Carlsbad, USA). The protein bands were visualized by Coomassie brilliant blue staining.

Bacterial strains *Staphylococcus aureus, E. coli, E. faecalis*, *Proteus sp.*, and *Bacillus sp.* were used to determine antibacterial activity. These bacterial cultures were gifted by the *Anuradha Jape* incharge microbial culture facility, Y.M. College, Pune, India. Stock cultures were maintained on nutrient agar slants at 4 °C and periodically sub-cultured. Fungal cultures were procured from the National Collection of Industrial Microorganisms (NCIM), National Chemical laboratory, Pune, India. The procured cultures included *Aspergillus niger* (*NCIM 596*), *Penicillium notatum* (*NCIM 1227*),

A. tenus (*NCIM 1220*), *Fusarium moniliforum* (*NCIM 1099*), *Mucor heimalis* (*NCIM 873*), *A. rhizogenus* (*NCIM 5140*), *A. tumifacience* (*NCIM 2145*), and *Rhizopus leguminosorum* (*NCIM 2005*). The stock cultures were maintained on potato dextrose agar slants at 4 ºC and sub-cultured every three months.

Recombinant LTP proteins were expressed by subcloning the coding sequences into the pYES2/CT expression vector under the control of *GAL1* inducible promoter. The yeast cells transformed with an empty vector served as negative control. Antimicrobial activity of ns-LTP was evaluated on 13 bacterial and fungal strains. On solid media, 20, 30, and 40 mm^3 of recombinant LuLTP in 50, 75, and 100 mM concentrations were loaded onto 0.5-cm *Whatman No. 1* filter paper disks and placed on nutrient agar and potato dextrose agar media in Petri plates. Inhibition of growth of a bacterial lawn indicated anti-microbial activity of the recombinant proteins.

Quantitative expression of *LuLTP_Ls* genes were carried out by real time qPCR (*Applied Biosystems*, Foster City, USA), using a *SYBR Green* kit (*Takara*, Tokyo, Japan). According to the sequence of LuLTP_Ls, two specific primers [Type 1 (KC984526) F: 5'-GGC CGCTTGCCTTACCTATT-3', R: 5'-GGTGGTCTT GGCCATGTTG-3', (KC984528) F: 5'-GCGGGATCA AGTCGCTTAAC-3', R: 5'-TACCCCGGACACCAC TAGCA-3'; and Type 2 (KC984529) F: 5'-GCGGGA TCAAGTCGCTTAAC-3', R: 5'-TACCCCGGACAC CACTAGCA-3'] were designed using the *Primer Express 3* software (*Applied Biosystems*). The flax leaf cDNA was normalized with an eukaryotic transcription initiation factor 5A housekeeping primer (F: 5'-TGC CACATGTGAACCGTACT-3' and R: 5'-CTTTACCCT CAGCAAATCCG-3'). Expression analysis was carried out with a minimum of three biological and technical replicates. Amplificationwas carried out in a total volume of 20 mm^3 containing 0.8 mm^3 of each primer, 2 mm^3 of cDNA, 10 mm^3 of *SYBR Green*, 0.4 mm^3 of Master mix and 6 mm^3 of sterile distilled water. Cycling conditions were: an initial denaturation at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Melt curve analysis was carried out after every run to confirm the specificity of amplification of the target gene (95°C for 15 s, 60 °C for 1 min, and 95 °C for 15 s). The results were analyzed by using the *StepOne* software *v. 2.2.2* and the *DataAssist v. 3.0* software with the ΔΔ Ct method for normalization. Statistical analysis was carried out by using the *Graphpad Prism v. 5.0* software (San Diego, CA, USA). The data were expressed as mean ± SE and one way analysis of variance (*ANOVA*) followed by Bonferroni's test considering day 0 of developing cotyledons as unit.

Sequence homologies of LuLTPs were assessed with the *NCBI BLAST* program (Altschul *et al*. 1990). The ORFs were determined by using the *ORF Finder* tool at *NCBI*. Sequence alignment of obtained sequences of LTPs was done with full length amino acid sequences from other plant LTPs to study the characteristics of conserved regions of LTPs. An isoelectric point (pI) and a mature peptide molecular mass value was calculated by the *Peptide Mass* programme (http://web.expasy.org/cgi-bin/peptide_mass). The *Signal P* web server (http://www.cbs.dtu.dk/services/signalP) was used to predict the signal peptide cleavage site (Thomas *et al*. 2011). A phylogeny tree was constructed using the deduced amino acid sequences of LuLTP_Ls with the other LTP protein sequences which were retrieved from the *GenBank* database. The phylogenetic tree was constructed using the *MEGA 4* (Tamura *et al*. 2007) software with using *PAM 250* matrices, a gap opening penalty of 0.05, and bootstrap neighbour-joining method.

The consensus full length sequences of LuLTP_Ls were submitted to the *NCBI* database. The submitted sequences have accession numbers: KC984526, KC984528, and KC984529.

Table.1. Primer pairs used for full length amplification of flax lipid transfer protein genes. Appropriate restriction sites are shown in *italics*.

Gene name	Forward	Reverse
LuLTP_Ls1 (KC984526)	ATCT*GGATC*CTTTATGGCAGCTGCA	GTCC*AGATCT*GGGTCACTGGATACT
LuLTP_Ls3 (KC984528)	GCCG*GGATCC*GAAATGGCCTCTACT	CCGC*AGATCT*CGCTCACTTCACCCT
LuLTP_Ls4 (KC984529)	ATTC*GGATCC*TTTATGGCCAAACAC	CACG*AGATCT*GCGTCAACATTTGAA

Results

Using specifically designed primers to amplify leaf specific *LuLTP_Ls* loci, four putative amplicons were obtained from the flax genomic DNA, cloned, and sequenced. Three clones belonged to Type 1 LTPs and one was Type 2 LTP. The sequences of Type 1 *LuLTP_Ls*, KC984526 and KC984528, showed a 37 % sequence similarity and revealed all conserved cysteine residues.

The deduced amino acid sequences of all LuLTP_Ls showed a close similarity with other reported plant ns-LTPs. The *CLUSTAL X* alignment of the flax LuLTP_Ls amino acid sequences with the sequences of LTPs from other plant species revealed the conserved cystine, leucine, and proline residues as found in other

species (data not shown). The signal peptide prediction showed that LuLTP_Ls Type 2 had a precursor containing a signal peptide at the N terminal region measuring 24 residues with a cleavage site between residues 23 and 24. This signal peptide was absent in the LuLTP_Ls Type 1 sequences. The LuLTP_Ls sequences did not contain the H/KDEL (an endoplasmic reticulum retention signal) site indicating that LuLTPs are likely to enter in the secretory pathway. Fig. 1 represents the nucleotide sequence of *LuLTP_Ls* which revealed eight conserved cysteine residues. It is clear that *LuLTP_Ls* consists of a full length sequence with typical features similar to other LTPs. Two consensus polypeptide regions T/s-X-X-D-R/K and P-Y-X-I-S were present only in Type 1 LuLTP_Ls.

Phylogenetic analysis was carried out with the deduced amino acid sequences and other plant sequences retrieved from the *BLAST* search tool. The phylogenetic tree was generated with both types of LTP groups, 12 sequences from the Type 1 and 11 sequences from Type 2 LTP families (Fig. 2). The LuLTP_Ls of the Type 1 and Type 2 groups shared a similar clade with *Dimocarpus longan* and *Vitis vinifera*, respectively.

```
  1 atggcagctgcattgaagttggtttctatcttgctagtctgcgcc
    M  A  A  A  L  K  L  V  S  I  L  L  V  C  A
 46 ttggtggctgcacccattacggtcagcgggttgacctgcgggcaa
    L  V  A  A  P  I  T  V  S  G  L  T  C  G  Q
 91 gtgagcagcgggatggccgcttgccttacctatttgaccggtcga
    V  S  S  G  M  A  A  C  L  T  Y  L  T  G  R
136 gcaccagtcacccctgcttgctgcaacgggatgaggggactcctc
    A  P  V  T  P  A  C  C  N  G  M  R  G  L  L
181 aacatggccaagaccaccgctgaccgccgcctggcttgcacctgc
    N  M  A  K  T  T  A  D  R  R  L  A  C  T  C
226 ttgaaaaccgccgccggcaacgtccctgggttgaatccggcaatc
    L  K  T  A  A  G  N  V  P  G  L  N  P  A  I
271 gctgctggtctcccaggaaagtgcggtgtcaagattccgtataag
    A  A  G  L  P  G  K  C  G  V  K  I  P  Y  K
316 atcagcacctccaccaactgcaacacgtacgtataa 351
    I  S  T  S  T  N  C  N  T  Y  V  *
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Fig. 1. Sequence analysis of *LuLTP_Ls* shows the full length sequence with start and stop codon with eight conserved cysteine residues marked by *underlines* and *boxes* represent the conserved polypeptide regions.

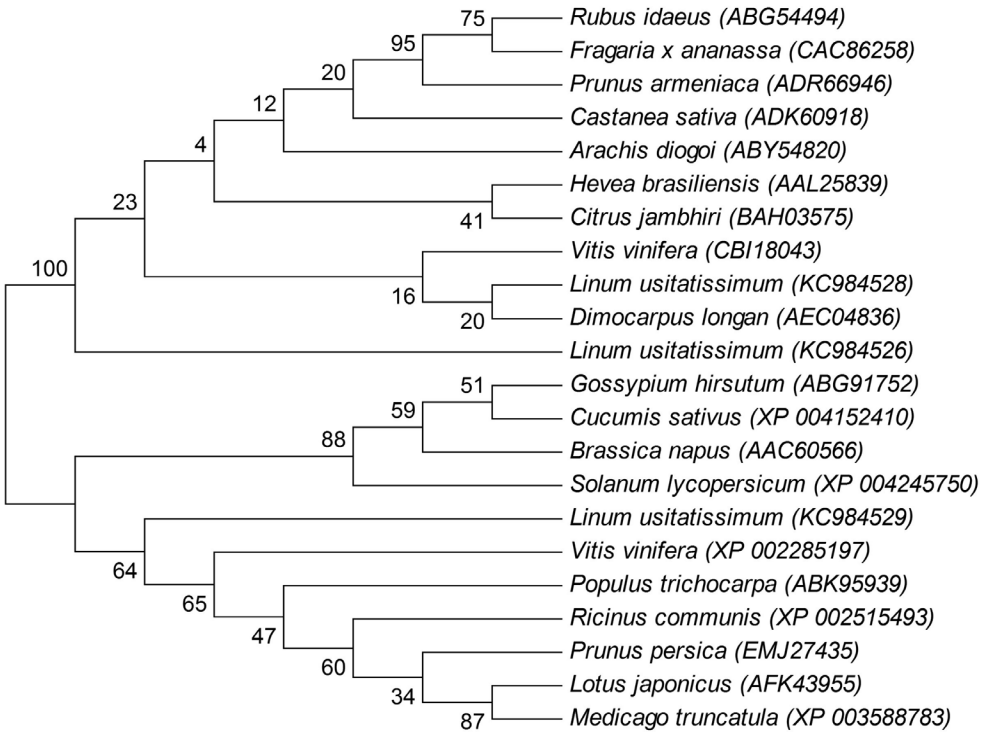

Fig. 2. Phylogenetic analysis of flax Type 1 and Type 2 (LuLTP_Ls) with 23 other plant LTPs based on generated sequence alignment. The phylogenetic tree was constructed on the basis of the boot-strap neighbor-joining method using the *Clustal W* program. The plant LTP sequences used for generating the phylogenetic tree and their GenBank accession numbers in parenthesses.

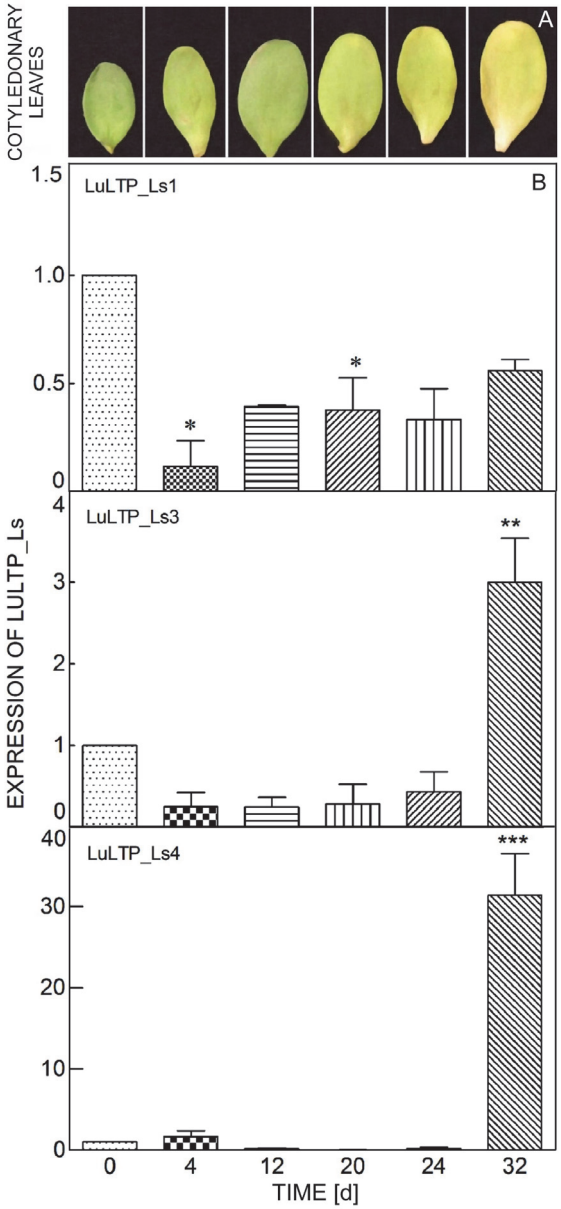

Fig. 3. *A* - Flax cotyledon development stages (0, 4, 12, 20, 24 and 32 days), *B* - Gene expression study as demonstrated by qPCR and showing the respective expression of *LuLTP_Ls*1, *LuLTP_Ls*3 and *LuLTP_Ls*4 in developing stages of cotyledonary leaves, *asterisk* indicates the significance of differences.

Gene expression analyzed by RT-qPCR revealed differential expression patterns of Type 1 and Type 2 *LuLTP_Ls* in different stages of leaf development in flax (Fig. 3*A*). There were significant increases in expression of *LuLTP_Ls*1 ($P < 0.008$) and *LuLTP_Ls*3 ($P < 0.0001$) during leaf development, and the highest was at senescence (Fig. 3*B*). The Type 2 *LuLTP_Ls*4 gene was up-regulated by ~30 fold at senescence in cotyledons. Significant ($P < 0.0002$) expression of the *LuLTP_Ls*4 gene was observed at the initial stage of senescence (day 4), then dropped to an undetectable level (day 24) and increased again at the terminal phase of senescence (day 32).

The *LuLTP_Ls* genes were transformed in the pYES2 vector for yeast expression under the control of the galactose inducible *Gal 1* promoter. The recombinant proteins accumulated in the yeast with time and the His-tag purified recombinant LuLTP_Ls proteins showed expected molecular masses of 9 kDa for LTP1 and 7 kDa for LTP2, respectively. However, actual molecular masses calculated using the *Expasy* tool (http://web.expasy.org/compute_pi/) based on the deduced amino acid sequences were turned out to be 9.3 and 8.9 kDa, respectively.

Antimicrobial activity of recombinant LuLTP_Ls was tested against eight fungal and five bacterial species by the disc diffusion method. The protein did not inhibit any of the fungal species. Among the five bacterial strains tested, both *LuLTP_Ls*1 and *LuLTP_Ls*4 showed antibacterial activity against *Staphylococcus aureus*. Clear inhibition zones were found after 24 h (Fig. 4). Growth inhibition of *Staphylococcus aureus* was observed in a dose depended manner. The *LuLTP_Ls*1 (Type 1) showed a higher antibacterial activity than the *LuLTP_Ls*4 (Type 2) at equal concentrations.

Discussion

Lipid transfer proteins are thought to participate in regulation of intracellular fatty acid pools, in membrane biogenesis, and in cutin formation. In this study, we isolated three flax *LTP* genes that are expressed specifically in leaves and encode LTPs Type 1 and Type 2. Sequence analysis reveals that the LTP Type 1 polypeptide was of 116 amino acids, whereas Type 2 polypeptides of 129 amino acids with an N terminal signal peptide (23 residues). The phylogenetic trees constructed with the deduced amino acid sequences of LuLTP_Ls showed a significant sequence homology with ns-LTPs from *Dimocarpus longan* and *Vitis venifera*. The cloned sequences revealed two consensus polypeptides T/s-X-X-D-R/K and P-Y-X-I-S that are present only in the Type 1 subfamily and play an important role in lipid binding (Douliez *et al*. 2000).

Plant *ns-LTPs* are evolved into small multigene families and are differentially expressed in different tissues. For example, in tobacco, *LTPs* are highly expressed in leaf epidermal cells as well as in shoot apical meristem (Fleming *et al*. 1992). The analysis by RT-qPCR shows that the expression of *LuLTP_Ls1* and *LuLTP_Ls3* progressively increased at various stages from emergence to senescence. This feature suggests their role in an active mobilization of stored lipids. Lipid transfer occurs in leaves and other green tissues involving

substantial changes between the endoplasmic reticulum and the chloroplast membrane (Browse *et al*. 1986). In general, cotyledons act as depot of stored food. Soon after emergence, cotyledons become photosynthetically

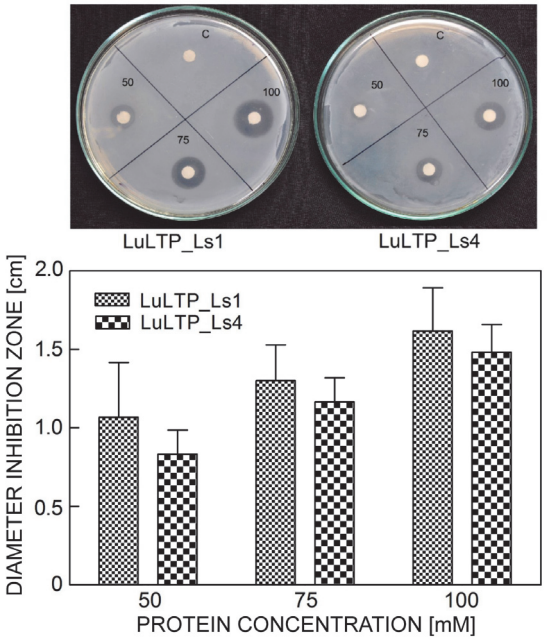

Fig. 4. LuLTP_Ls1 and LuLTP_Ls4 showing growth inhibition of *Staphalococcus aureus.*

active and become functionally similar to true leaves. Senescence is the terminal stage of leaf development and involves degradation of macromolecules and an accelerated mobilization of nutrients toward the growing parts of a plant (Nooden *et al.* 1989). Very little information is available about how the macromolecules are recycled in plants. It is reported that after depletion of a storage material, endosperm cells undergo programmed cell death (Schmit *et al*. 1999). Eklund and Edquist (2003) have studied expression of LTPs and also noted that endosperm cells undergo programmed cell death. Many complex lipid structures, like parts of cellular membranes, release during cellular destruction. To reuse them require an active transport mechanism. Interestingly, LuLTP_Ls4 was highly expressed at senescence (Fig. 3*B*), which strongly suggests its role in scavenging activity and participation in recycling. Lipid transfer proteins are known to express in drying leaves of tobacco (Cameron *et al*. 2006). Gene expression analysis using Northern blot analysis of tobacco drying leaves shows a 6-fold increase in *LTP* gene transcripts involved in cuticle deposition (Cameron *et al*. 2006). Drying tobacco leaves shows parallel physiological changes to that of senescence, which therefore corroborates present observations. Expression of *LTP* has also shown a significant increase in *Euphorbia lagascae* cotyledons undergoing senescence (Eklund and Edqvis 2003). Together, these results indicate that, different *LuLTP_Ls* are highly expressed in senescent cotyledons with possible involvement in transfer and recycling functions.

Additionally, LTPs have also been suggested to play roles in plant defence and have antimicrobial properties *in vitro* (Terras *et al.* 1992, Molina *et al*. 1993, Segura *et al*. 1993). Barley leaf LTPs are reported to possess antimicrobial properties which are up-regulated in response to challenge by a pathogen (Molina and Garcia-Olmedo 1993). Furnishing support for this hypothesis: LTPs from maize leaves were also shown to inhibit bacterial and fungal growth (Molina *et al*. 1993). Antimicrobial activities of LTPs are the first function shown in barley, maize, and spinach (Terras *et al*. 1992, Molina *et al*. 1993), although the antifungal activity of LuLTP_Ls is not significant. Consistent with these reports, both the types of LuTP_Ls showed antibacterial activity against *Staphylococcus aureus*, and Type 1 LuLTP_Ls was more effective than Type 2 LuLTP_Ls. However, no antifungal properties could be seen to these recombinant proteins. A similar antibacterial activity was observed previously with LTPs from onion seeds (Cammue *et al*. 1995) and mung been (Wang *et al*. 2004) where activity against gram-positive bacteria was reported. However, the mechanism of antimicrobial activity of LTPs is not clear.

In conclusion, we isolated leaf specific genes encoding LTPs from flax (*LuLTP_Ls*) and the analysis of sequence reveals that the proteins belong to the Type 1 and Type 2 families of LTPs. The expression of *Type 1 LTPs* increased from emergence to senescence, whereas *Type 2* was significantly expressed only at the time of senescence. Thus, the main role of LuLTP_Ls1 and LuLTP_Ls3 proteins appears to be in transport of storage lipids, whereas LuLTP_Ls4 is linked with scavenging precursors released from a breakdown process in senescent leaves and transporting them towards other sink locations.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J.: Basic local alignment search tool. - J. mol. Biol. **215**: 403-410, 1990.

Browse, J., Warwick, N., Somerville, C.R., Slack, C.R.: Fluxes through the prokaryotic and eukaryotic pathways of lipid synthesis in the '16:3' plant *Arabidopsis thaliana*. - Biochem. J. **235**: 25-31, 1986.

Buhot, N., Douliez, J.P., Jacquemard, A., Marion, D., Tran, V., Maume, B.F., Milat, M.L., Ponchet, M., Mikes, V., Kader, J.C., Blein, J.P.: A lipid transfer protein binds to a receptor involved in the control of plant defence responses. - FEBS Lett. **509**: 27-30, 2001.

Cameron**,** K.D., Teece**,** M.A., Smart, L.B.: 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**: 176-183, 2006.

Cammue, B. P. A., Thevissen, K., Hendriks, M., Eggermont, K., Goderis, I. J., Proost, P., Broekaert, W. F.: A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins. - Plant. Physiol. **109**: 445-455, 1995.

Charvolin, D., Douliez, J., Marion, D., Cohen-Addad, C., Pebay-Peyroula, E: The crystal structure of a wheat nonspecific lipid transfer protein (ns-LTP1) complexed with two molecules of phospholipid at 2.1 Å resolution. - Eur. J. Biochem. **264**: 562-568, 1999.

Douliez, J.P., Michon, T., Elmorjani, K., Marion, D.: 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, 2000.

Edqvist, J., Farbos, I.: Characterization of germination-specific lipid transfer proteins from *Euphorbia lagascae*. - Planta **215**: 41-50, 2002.

Eklund, D.M., Edqvis, J.: Localization of nonspecific lipid transfer proteins correlate with programmed cell death responses during endosperm degradation in *Euphorbia lagascae* seedlings. - J. Plant. Physiol. **132**: 1249-1259, 2003.

Fleming, A.J., Mandel, T., Hofman, S., Sterk, P., De Vries, S.C., Kuhlemeier, C.: Expression pattern of a tobacco lipid transfer protein gene within the shoot apex. - Plant. J. **2**: 855-862, 1992.

Han, G.W., Lee, J.Y., Song, H.K., Chang, C., Min, K., Moon, J., Shin, D.H., Kopka, M.L., Sawaya, M.R., Yuan, H.S.: Structure basis of non-specific lipid binding in maize lipid–transfer protein complexes revealed by high-resolution X-ray crystallography. - J. mol. Biol. **308**: 263-278, 2001.

Hortensteiner, S., Feller, U.: Nitrogen metabolism and remobilization during senescence. - J. exp. Bot. **53**: 927-937, 2002.

Kader, J.C.: Lipid-transfer protein in plants. - Annu. Rev. Plant Physiol. Plant. mol. Biol. **47**: 627-654, 1996.

Kim, T.H., Kim, M.C., Park, J.H., Han, S.S., Kim, B.R., Moon, B.Y.,Cho, S.H.: 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**: 371-375, 2006.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Lascombe, M.B., Bakan, B., Buhot, N., Marion, D., Blein, J.P., Larue, V., Lamb, C., Prange, T.: The structure of "defective in induced resistance" protein of *Arabidopsis thaliana*, DIR1, reveals a new type of lipid transfer protein. - Protein. Sci. **17**: 1522-1530, 2008.

Lee, J.Y., Min, K., Cha, H., Shin, D.H., Hwang, K.Y., Suh, S.W.: Rice nonspecific lipid transfer protein: the 1.6 Å crystal structure in the unliganded state reveals a small hydrophobic cavity. - J. mol. Biol. **276**: 437-448, 1998.

Liu, Y.J., Samuel, D., Lin, C.H., Liu, P.C.: Purification and characterization of a novel 7-kDa non-specific lipid transfer protein-2 from rice (*Oryza sativa*). - Biochem. biophys. Res. Commun. **294**: 535-540, 2002.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

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

Molina, A., Segura, A., Garcia-Olmedo, F.: Lipid transfer proteins (nsLTPs) from barley and maize leaves are potent inhibitors of bacterial and fungal plant pathogens. - FEBS. Lett. **316**:119-22, 1993.

Nooden, L.D., Guiamet, J.J.: Regulation of assimilation and senescence by the fruit in monocarpic plants. - Physiol. Plant. **77**: 267-274, 1989.

Pasquato, N., Berni, R., Folli, C., Folloni, S., Cianci, M., Pantano, S., Zanotti, G.: Crystal structure of peach Pru p 3, the prototypic member of the family of plant non-specifc lipid transfer protein pan-allergens. - J. mol. Biol. **356**: 684-694, 2006.

Petersen, T.N., Brunak, S., Von Heijne, G., Nielsen, H.: Signal P 4.0: discriminating signal peptides from transmembrane regions. - Natur. Methods **8**: 785-786, 2011.

Quirino, B.F., Noh, Y.S., Himelblau, E., Amasino, R.M.: Molecular aspects of leaf senescence. - Trends Plant. Sci. **5**: 278-282, 2000.

Salcedo, G., Sanchez-Monge, R., Barber, D., Diaz-Perales, A.: Plant nonspecific lipid transfer proteins: an interface between plant defence and human allergy. - Biochim. biophys. Acta **1771**: 781-791, 2007.

Schmit, M., Simpson, D., Gietl, C.: Programmed cell death in castor bean endosperm is associated with the accumulation and release of a cysteine endopeptidase from ricinosomes. - Proc. nat. Acad. Sci. USA **96**: 14159-14164, 1999.

Segura, A., Moreno, M., Garcia-Olmedo, F.: Purification and antipathogenic activity of lipid transfer proteins (LTPs) from the leaves of *Arabidopsis* and spinach. - FEBS Lett. **332**: 243-246, 1993.

Shahri, W., Tahir, I.: Physiological and biochemical changes associated with flower development and senescence in *Consolida ajacis* Nieuwl cv.Violet blue. - Front. Agr. China **5**: 201-208, 2011.

Tamura, K., Dudley, J., Nei, M., Kumar, S.: MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. - Mol. Biol. Evol. **24**: 1596-1599, 2007.

Terras, F.R.G., Goderis, I.J., Van Leuven, F., Van der Leyden, J., Camimue, B.P.A., Broekaert, W.F.: *In vitro* antifungal activity of a radish (*Raphanus sativus* L.) seed protein homologous to nonspecific lipid transfer proteins. - Plant. Physiol. **100**: 1055-1058, 1992.

Wang, S.Y., Wu, J.H., Ng, T.B., Ye, X.Y., Rao, P.F.: A non-specific lipid transfer protein with antifungal and antibacterial activities from the mung bean. - Peptides **25**: 1235-1242, 2004.

Zaman, U., Abbasi, A.: Isolation, purification and characterization of a nonspecific lipid transfer protein from *Cuminum cyminum*. - Phytochemistry **70**: 979-987, 2009.

Zottich, U., Da, Cunha. M., Carvalho, A.O., Dias, G.B., Silva, N.C., Santos, I.S., do Nacimento, V.V., Miguel E.C., Machado, O.L., Gomes, V.M.: Purification, biochemical characterization and antifungal activity of a new lipid transfer protein (LTP) from *Coffea canephora* seeds with α-amylase inhibitor properties. - Biochim. biophys. Acta **1810**: 375-383, 2011.