General and Applied Plant Physiology – 2010, Volume 36 (3–4), pp. 212–221
©2010 ISSN 1312-8183 Published by the Institute of Plant Physiology – Bulgarian Academy of Sciences
Available online at http://www.bio21.bas.bg/ipp/

CLONING AND CHARACTERIZATION OF TWO STRESS INDUCIBLE GENES FROM THE MANGROVE SPECIES *AVICENNIA MARINA* VIERCH. FORSK

Sadhasivam V.[1], S. R. Prashanth[2], K. R. Sivaprakash[1], A. Venkat[3], A. Parida[1*]

[1]M.S. Swaminathan Research Foundation, Third Cross Street, Taramani Institutional Area, Chennai, India, [2] Baba Ataomic Research Centre, Mumbai, [3] Vellore Institute of Technology, Vellore, India.

Received: *4 February 2010* Accepted: *13 September 2010*

Summary. Salinity stress is one of the major abiotic stresses that affect crop productivity. Halophytes are plants which are able to withstand and grow under high soil saline conditions. Mangroves are a plant community which are halophytes and survive well under high saline conditions. Induction of abiotic stress tolerance genes is one of the important strategies of these halophytes to combat salinity stress. *SOD* (superoxide dismutase) and *LTP* (lipid transfer protein) play a key role in abiotic stress tolerance in plants. In the present study, we report the mRNA accumulation patterns of *Sod2* and *LTP1* encoding MnSod and LTP respectively under salinity stress in the mangrove species *Avicennia marina*.

***Key words*:** salinity stress; manganese superoxide dismutase; lipid transfer protein.

INTRODUCTION

Plants are exposed to several abiotic stresses such as high salinity, drought, extreme light and temperature under natural conditions which thereby affect their growth and development (Jithesh et al., 2006). Salinity stress is of particular importance since it is a threat which confronts the modern agricultural productivity. Salinity stress affects seed germination, seedling growth and vigour, vegetative growth, flowering and fruit set in plants (Sairam and Tyagi, 2004). The series of events that happen when a plant is subjected to salinity stress are physiological water deficit, abscisic acid regulated stomatal closure in leaves, limited CO_2 availability, over-reduction of electron transport chain and finally, generation of reactive oxygen species (ROS), (Jithesh et al., 2006). This condition, termed photo-oxidative stress, underlies also other plant stress responses like drought, temperature and light stress (Jithesh et al., 2006).

Halophytes are plants of salty environments, capable of thriving and growing under high concentrations of NaCl (Hellebust, 1976; Flowers et al.,

*Corresponding author: sequencing@mssrf.res.in

1986). Amongst, halophytes, mangroves form an important constituent. These are woody trees and shrubs present in the tropical and sub-tropical regions of the world. Mangroves are ideal candidates for salt stress research because of their capacity to maintain active leaves under conditions that severely reduce photosynthetic capacity through photoinhibition (Cheesman et al., 1997). *LTP* and *MnSod* have been shown to play key roles in conferring tolerance to salt stress in plant systems (Jung et al., 2003; Wang et al., 2004; Parida et al., 2004). In the present study, we have reported the cloning, characterization and mRNA accumulation pattern of two genes involved in stress response: lipid transfer protein (*LTP*) and manganese superoxide dismutase (*MnSod*) (*LTP1* and *SOD2*) from the mangrove plant species *A. marina* during salinity stress.

MATERIALS AND METHODS

Construction of c-DNA Library and Sequencing of Expressed Sequence Tags (ESTs).

Library was constructed and the ESTs were sequenced according to Parani et al. (2002). One-year-old wild plants of *A. marina* were collected and treated with 0.5 M NaCl for 48 h. Total RNA from the leaf tissue was isolated following the GITC method (Chomzynski and Sacchi, 1987) with minor modifications (Parani et al., 1999). Poly $(A)^+$ RNA was purified using an oligo-(dT) cellulose column and used as a template for cDNA synthesis. The SuperScript™ Lambda System for cDNA Synthesis and λ Cloning (Life Technologies, USA) was used for cDNA synthesis. First strand cDNA synthesis was primed with *Not*I-primer adapter, and the double stranded cDNA was directionally cloned in a plasmid vector (pSPORT 1) using *Sal*I adapter ligated at the 5' end. The *Sal*I adapter ligated cDNAs were size fractionated through a SizeSep™-400 Sepharose CL-4B spun column (pharmacia Biotech, USA) before cloning in the plasmid vector. The ligated cDNAs were transformed in the DH5α strain of *Escherichia coli*. Several clones from *A. marina* cDNA library were randomly selected, and the insert size in each clone was determined by PCR using the universal M13 forward and reverses primers. The plasmid DNA from the cDNA clones having cDNA above 600 bp size were isolated by the alkaline lysis method (Birnboim & Doly, 1979). The 5' end of the cDNAs were subjected to single-read sequencing using M13 reverse primer and Big-Dye™ Terminators in an automated sequencing machine (ABI310, Applied Biosystems, USA). The DNA sequences were clipped for removing vector and adapter sequences and manually edited for sequencing errors. The edited DNA sequences were used for searching nucleotide and protein homology to the existing genes in the database at www.ncbi.nlm.nih.gov using BLASTN and BLASTX algorithms, respectively. While the clones identified to be partial or having homology with unknown proteins were reserved for future studies, the clones having potentially full-length genes were completely sequenced from both strands and further characterized (Parani et al., 2002). This study reports the characterization of a full-length cDNA that codes the lipid transfer protein in *A. marina*.

Salt stress treatment.

Seeds of *A. marina* collected from the Pichavaram mangrove forest, Tamilnadu, India, were grown in sand-filled trays in a greenhouse at 35°±2°C under a 12 h/12 h (light/dark) photoperiod for one month with daily watering (Mehta et al., 2005). The seedlings were then removed from the soil and grown in half strength Murashige and Skoog inorganic salt medium under control light conditions of 100µmol m^{-2} s^{-1} with a photoperiod of 16 h/8 h (light/dark) for 3 days. Salt stress treatment was applied by adding 0.5 M NaCl to the freshly prepared half strength MS nutrient to the acclimatized *A. marina* seedlings. Leaves were harvested from the treated seedlings at time intervals of 0, 12, 24, 36 and 48 h after NaCl treatment.

RNA isolation and northern hybridization.

Total RNA was extracted from the leaves of *A. marina* seedlings using the LiCl method (Abbas Alemzadeh et al., 2005) at different time points after treatment with NaCl. RNA (20µg) was resolved on a 1.4% MOPS-formaldehyde gel, capillary-transferred (Sambrook et al., 1989) to Hybond N+ membrane (Amersham Inc., USA) and fixed by UV cross-linking according to manufacturer's instructions (Hoefer, Germany). Blots were probed using 3'UTR (untranslated region) of *LTP1* and *SOD2*. The 3' UTR probe was amplified using specific primers (*LTP1*: Forward 5'- GTGGCGTTAACATTCCCTACA-3', Reverse 5'-GCAAAAGGAGCTAGCG-TCCA-3' and *SOD2:* Forward 5'-GAG-GTTCTGGTAACTGTGA-3',Reverse 5'-AAGTTATTATATATAGATATAATG -3'). The amplified product was gel eluted and labelled with ^{32}P by random priming (Rediprime, Amersham Biosciences). The probe was purified using a ProbeQuant G-50 column (Amersham Biosciences) and hybridization was carried out at 63°C overnight using Perfect Hyb PLUS hybridization buffer (Sigma, USA). Blots were washed sequentially using 1X and 0.5X SSC containing 0.1% SDS (w/v) at 63°C. Northern hybridization was repeated twice with two different blots.

Amino acid sequence analysis.

The phylogenetic analysis was done using the Phylogenetic tree prediction online software Treetop (http://www.genebee.msu.su/services/phtree_reduced.html). The details of the protein sequences used such as plant species and GenBank protein accession number are presented in Table 1.

RESULTS

Full length cDNAs encoding LTP and MnSod (*LTP1* and *SOD2*) were isolated as mentioned previously (Parani et al., 2002). Both genes were completely sequenced. The cDNAs encoding *LTP* (GenBank cDNA accession number AF331710.1) and *MnSod* (GenBank cDNA accession number AY137205.1) were of 588 and 925 bp, respectively. A sequence alignment study was done to compare the aminocid sequence of SOD2 with MnSOD sequences from 13 other plant species (Fig. 1a) and the same for LTP (Fig. 1b). It was reported earlier that the active sites of the MnSod protein was conserved very stringently even across taxonomically different species (Fink and Scandalios, 2002). Our study confirmed this observation and all the

Table 1. SOD and LTP-encoding sequences used for phylogenetic analysis.

SOD	Species	Accession number
CsMnSOD	*Camellia sinensis*	AAT68778.2
CaMnSOD	*Capsicum annuum*	AAB88870.1
NtMnSOD	*Nicotiana tabacum*	BAC75399.1
GmMnSOD	*Glycine max*	ABQ52658.1
Ta1MnSOD	*Tamarix androssowii*	AAS77885.2
ZaMnSOD	*Zantedeschia aethiopica*	AAC63379.1
TaMnSOD	*Triticum aestivum*	AAC62115.1
ZmMnSOD	*Zea mays*	AAA72022.2
RsMnSOD	*Raphanus sativus*	AAL07333.1
AtMnSOD	*Arabidopsis thaliana*	AAL66910.1
ThMnSOD	*Thellungiella halophila*	ABQ81865.1
AeMnSOD	*Acanthus ebracteatus*	ABK32075.1
AmMnSOD	*Avicennia marina*	AAN15216.1
EeMnSOD	*Euphorbia esula*	AAF65768.1
LTP	**Species**	**Accession number**
AmLTP	*Avicennia marina*	AAK01293
SiLTP	*Sesamum indicum*	ABQ53934.1
SmLTP	*Salvia miltiorrhiza*	ABP01769.1
TaLTP	*Triticum aestivum*	AAK20395.1
LpLTP	*Lycopersicon pennellii*	AAB07487.1
LcLTP	*Lycopersicon chilense*	AAZ22829.1
NtLTP	*Nicotiana tabacum*	BAA03044.1
StLTP	*Solanum tuberosum*	AAM82606.1
CaLTP	*Capsicum annum*	ACB05670.1
VvLTP	*Vitis vinifera*	AAO33394.1
VaLTP	*Vitis aestivalis*	AAQ96338.1
GhLTP	*Gossypium hirsutum*	ACI26696.1
SoLTP	*Spinacia oleracea*	AAA34032.1
CsLTP	Citrus sinensis	AAM21292.1

major functional domains of the protein sequence were conserved in SOD2 as in the sequences from other species (suppl. Fig 1). A dendrogram representing the relationship of fourteen MnSOD amino acid sequences was constructed. The SOD2 sequence grouped with the MnSOD sequence of *Acanthus ebracteatus,* which is a halophyte species (Fig. 1a). However, the MnSOD sequence of *Thellungiella halophilla* which is also a halophyte, did not group with *Avicennia marina*. It grouped with the sequences of *Arabidopsis thaliana* and *Raphanus sativus*. The

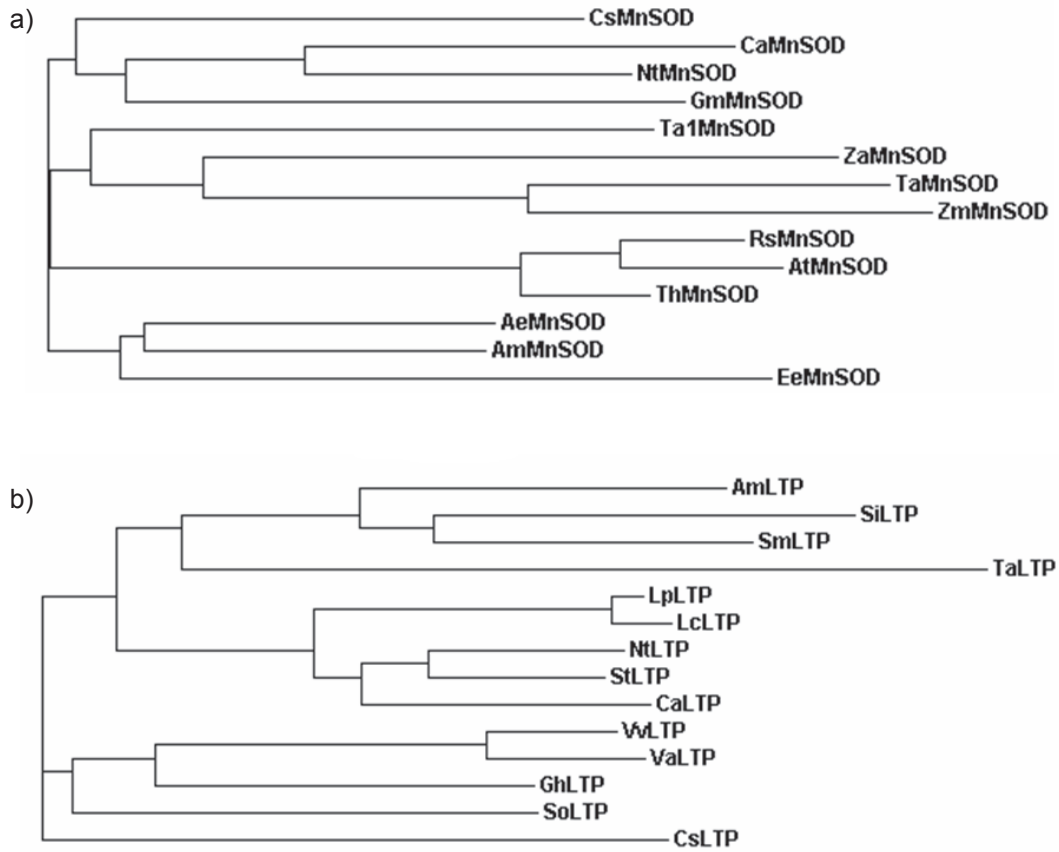


Fig. 1. Phylogenetic tree showing the relationship between MnSOD sequences (a) and LTP sequences (b) with different plant species.

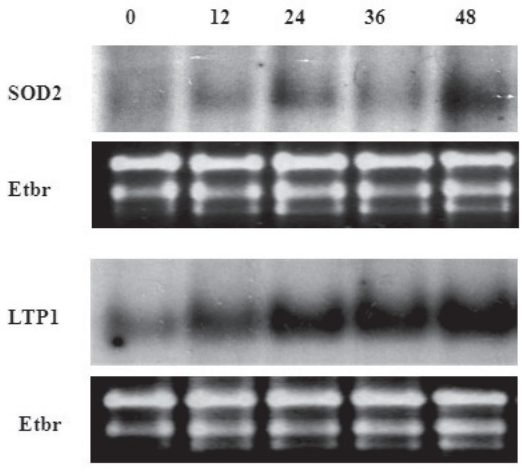


Fig 2. Effect of salt stress on the mRNA levels of *SOD2* and *LTP1*. after 0, 12, 24, 36, 48 h of exposure to 500mM NaCl. For RNA control loading, the gel was stained with ethidium bromide (Etbr).

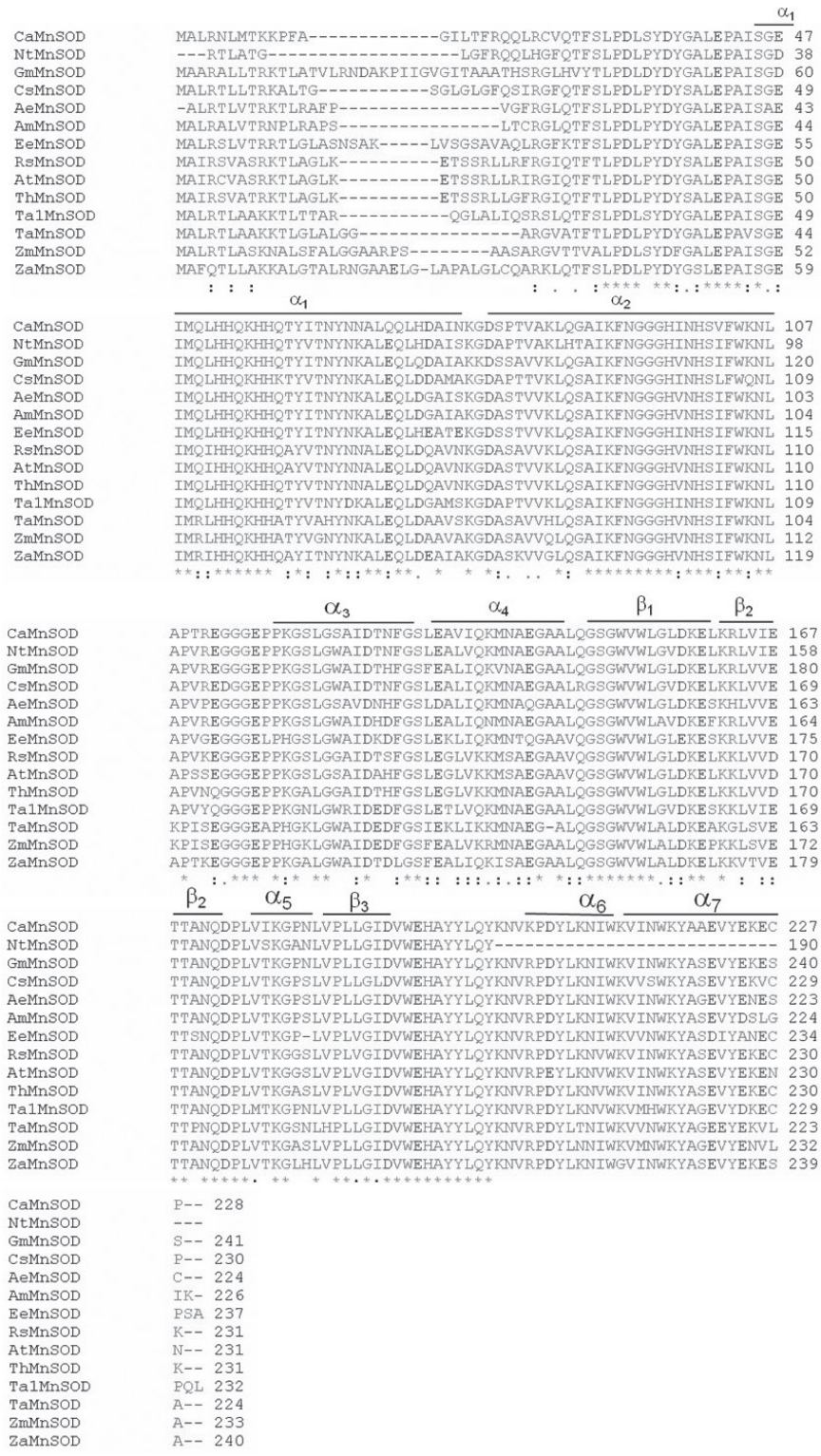
multiple sequence alignment of LTP1 with 13 other plant species revealed the presence of eight conserved cysteine residues (suppl Fig. 2) that are specific for the nonspecific LTPs which is shown to form four disulphide bridges important for structure and function (Takishima et al. 1986). LTP1 formed a group with *Sesamum indicum* and *Salvia miltiorrhiza* in the dendrogram constructed with LTP aminoacid sequences of 14 different plant species (Fig. 1b).

Under 500mM salt stress conditions both *SOD2* and *LTP1* mRNA transcripts accumulation increased with time of exposure to NaCl stress and attained maximal accumulation at 48 h of salt stress (Fig. 2).

DISCUSSION

LTP and *MnSod* have been shown to play key roles in conferring tolerance to salt stress in plant systems (Jung et al., 2003; Wang et al., 2004; Parida et al., 2004). MnSod activity has been known to increase under salinity stress in halophytes (Wang et al., 2004; Parida et al., 2004). An increase in *MnSod* and *Fesod* activities has been documented to be an important stress response in halophytes since these two enzymes are localized in the mitochondria and chloroplasts, respectively which are the sites of generation of superoxide radicals during stress conditions due to electron transport chain (Jithesh et al., 2006). It has been observed in a number of halophytes that salt stress results in an increase in the activities of *MnSod* and *FeSod*, but not in *Cu/ZnSod* (Wang et al., 2004; Parida et al., 2004). In the halophyte *A. marina*, cytosolic *Cu/ZnSod* is not induced by salt stress (Jithesh et al., 2006). However, in the present study, we were able to observe that *MnSod* is induced by salinity stress in *A. marina*. This is entirely in agreement with the observations made by Wang et al. (2004) and Parida et al. (2004). Cherian et al. (1999) observed an increase in SOD activity in the leaves of *A. marina* under salinity stress. The results of the present study suggest that the increase in *MnSod* transcript levels in *A. marina* may be significantly contribute to the increase in the *SOD* activity in leaves during salinity stress in *A. marina* (Cherian et al, 1999) considering that transcript levels of cytosolic *Cu/ZnSod* were not induced in *A.marina* under salinity stress (Jithesh et al 2006). However, further studies are underway to validate this observation. Although there are reports showing involvement of *LTP* in abiotic stress (Jung et al., 2003), to our knowledge the present study happens to be the first study on the effect of salinity stress on *LTP* transcription in halophytes. Our results showed that *LTP1* transcript synthesis was induced under salinity stress thereby underlying the importance of *LTP* in the abiotic stress response of *A. marina*. Liu and Lin (2003) reported the induction of *LTP* in *Vigna radiata* under both salinity and dehydration stress. Choi et al. (2008) observed the induction of two isofroms of *LTPs* (*SiLTP2* and *SiLTP4*) under NaCl, mannitol and ABA application in *Sesamum indicum*. However, the precise function of LTP is still largely unknown (Bakan et al., 2007). It has been suggested that *LTPs* play a key role in cuticle biosynthesis during abiotic stress (Sterk et al., 1991) by maintaining water balance and membrane damage repair by increasing the cuticle thickness (Jung et al., 2003). The halophyte *A. marina* studied in the present report has been reported to have a thick cuticle (Datta et al., 2005). Whether the increased synthesis of *LTP* mRNA during salinity stress might contribute to maintaining the cuticle thickness as observed by Jung et al. (2003) or *LTP* could play some other role under abiotic stress conditions in mangroves would have to be further investigated.

Acknowledgements: We acknowledge Department of Biotechnology, Government of India for research support.



Supplementary Fig. 1. Comparison of the MnSod amino acid sequences from different plant species. The plant species and the accession numbers are listed in Table 1. α1, α2, α3, α4, α5, α6, α7, β1, β2 and β3 are the functional domains, conserved across all the 14 species studied.

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                                                C       C               CC
SiLTP          MASMIKALCVALVGAVLVIAVVPP--AEAALGCGSVISYLGSCRPYVTDKGP---LGGCC 55
SmLTP          ----MKLIGALLIAAALIAAVAPP--SEAAIGCGAVVSYLNPCLPYVTNKGP---LGGCC 51
AmLTP          MEGMNKSMCIIVVVAVLAAWVVPH--GEAAISCGTVASKLAPCIPYVTNRGP---LGGCC 55
VvLTP          --MGSSGAVKLACVMVICMVVAAPAVVEATVTCGQVASALSPCISYLQKGGA--VPAGCC 56
VaLTP          --MGSSGAVKLACVMVICMVMAAPAAVEAAITCGQVASALSPCISYLQKGGA--VPPACC 56
GhLTP          --MASSMSLKLACVAVLCMVVGAP-LAQGAVTCGQVTSSLAPCIGYLTGNGAGGVPPGCC 57
SoLTP          --MASSAVIKLACAVLLCIVVAAP-YAEAGITCGMVSSKLAPCIGYLK-GGP--LGGGCC 54
CsLTP          -----MAALKLVSALVLCMLVTGP-LSAQAITCGQVSGSLAPCIGFLRSGGP--IPMPCC 52
LpLTP          ----MEMVNKIACFVLLCMVVVAP--HAEALTCGQVTSTLAPCLPYLMNRGP---LGGCC 51
LcLTP          ----MEMVNKIACFVLLCMVVVAP--HAEALTCGQVTSTLAPCLPYLMNRGP---LGGCC 51
NtLTP          ----MEMVGKIACFVVLCMVVVAP--HAEALSCGQVQSGLAPCLPYLQGRGP---LGSCC 51
StLTP          ----MEMFGKIACFVLLCMVVVAP--HAEALSCGQVTSGLAPCLPYLQGRGP---IGGCC 51
CaLTP          ------MVGKIACVVLLCMVVVAP--HAEALTCGQVQSRMTPCLPYLTGSGP---LGRCC 49
TaLTP          --MARTAATKLVLVALVAAMILAA--SDAAISCGQVSSALTPCVAYAKGSGTS-PSGACC 55
                             :   :        : ** * . : .*  :    *.      **

                                C C                       C               C
SiLTP          SGVKGLYKAAKTTADRQATCSCLKTLASTYKGVNLSKAAGLPQQCGVNIPYKISPSTDCS 115
SmLTP          GGIKGLYGAAKTTPDRQSVCNCLKTLASSYKGVNLGKAAGLPGQCGVSIPYKISPSTDCS 111
AmLTP          GGVKSLYGLARTTPDRQSVCGCLKSLASSYN-VNLGKAAGLPGQCGVNIPYKISPSTDCS 114
VvLTP          SGIKSLNSAAKTTGDRQAACKCLKTFSSSVSGINYGLASGLPGKCGVSVPYKISPSTDCS 116
VaLTP          SGIKSLNSSAKTTADRQAACKCLKNFSSTVSGINLSLASGLPGKCGVSVPYKISPSTDCT 116
GhLTP          GGIKSLNSAAQTTPDRQAACKCIKSAAAGISGINYGIASGLPGKCGVNIPYKISPSTDCN 117
SoLTP          GGIKALNAAAATTPDRKTACNCLKSAANAIKGINYGKAAGLPGMCGVHIPYAISPSTNCN 114
CsLTP          NGVRSLNAAARTTPDRQTACNCLKQAAGSIPNLNLNNAAGLPGACGVSIPYKISTSTDCS 112
LpLTP          GGVKGLLGQAQTTVDRQTACTCLKSAASSFTGLDLGKAASLPSTCSVNIPYKISPSTDCS 111
LcLTP          GGVKGLLGQAQTTVDRQAACACLKSAASSFTDLDLGKAASLPSTCNVNIPYKISPSTDCS 111
NtLTP          GGVKGLLGAAKSLSDRKTACTCLKSAANAIKGIDMGKAAGLPGACGVNIPYKISPSTDCS 111
StLTP          GGIKGLLGAAKTPADRKTACTCLKSAASAIKGINVGKAAGIPRVCGVNIPYKISPSTDCS 111
CaLTP          GGVKGLLGAAKTPADRKTVCTCLKSAAGSIGGINVRKAAGLPNMCGVNIPYQISPSTDCT 109
TaLTP          SGVRKLAGLARSTADKQATCRCLKSVA---GGLNPNKAAGIPSKCGVSVPYTISASVDCS 112
               .*:: *   * :  *:::.* *:*  :     ::   *:.:*  *.* :** **.*.:*.

SiLTP          KVT 118
SmLTP          KVK 114
AmLTP          KVH 117
VvLTP          KVT 119
VaLTP          KVT 119
GhLTP          SVK 120
SoLTP          AVH 117
CsLTP          KVR 115
LpLTP          KVQ 114
LcLTP          KVQ 114
NtLTP          KVQ 114
StLTP          KVR 114
CaLTP          KVQ 112
TaLTP          KIH 115
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Supplementary Fig. 2. Comparison of the LTP amino acid sequences from different plant species. The plant species and the accession numbers are listed in Table 1. The C represents the eight conserved cysteine residues.

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