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Three Drought-Responsive Members of the Nonspecific Lipid-Transfer Protein Gene Family in *Lycopersicon pennellii* Show Different Developmental Patterns of Expression[1]

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Genomic clones of two nonspecific lipid-transfer protein genes from a drought-tolerant wild species of tomato (*Lycopersicon pennellii* Corr.) were isolated using as a probe a drought- and abscisic acid (ABA)-induced cDNA clone (pLE16) from cultivated tomato (*Lycopersicon esculentum* Mill.). Both genes (*LpLtp1* and *LpLtp2*) were sequenced and their corresponding mRNAs were characterized; they are both interrupted by a single intron at identical positions and predict basic proteins of 114 amino acid residues. Genomic Southern data indicated that these genes are members of a small gene family in *Lycopersicon* spp. The 3′-untranslated regions from *LpLtp1* and *LpLtp2*, as well as a polymerase chain reaction-amplified 3′-untranslated region from pLE16 (cross-hybridizing to a third gene in *L. pennellii*, namely *LpLtp3*), were used as gene-specific probes to describe expression in *L. pennellii* through northern-blot analyses. All *LpLtp* genes were exclusively expressed in the aerial tissues of the plant and all were drought and ABA inducible. Each gene had a different pattern of expression in fruit, and *LpLtp1* and *LpLtp2*, unlike *LpLtp3*, were both primarily developmentally regulated in leaf tissue. Putative ABA-responsive elements were found in the proximal promoter regions of *LpLtp1* and *LpLtp2*.

Among several responses at the cellular level, drought stress is known to cause specific alterations in the gene expression patterns of plants, commonly mediated by the hormone ABA (Chandler and Robertson, 1994). These changes have been described in cultivated tomato (*Lycopersicon esculentum* Mill.) and other members of the genus, including drought-tolerant wild species such as *Lycopersicon pennellii* Corr. and *Lycopersicon chilense* Dun. (Bray, 1988; Cohen and Bray, 1990; Plant et al., 1991; Chen and Tabaeizadeh, 1992a, 1992b; Kahn et al., 1993). However, the significance of drought-induced genes in the performance of the plant during stress cannot be understood without knowledge of their function. Transcript accumulation of four ABA- and drought-induced cDNAs has been compared between *L. esculentum* and *L. pennellii*, demonstrating similar but not identical patterns of expression in the two species and their interspecific hybrid (Kahn et al., 1993). These studies showed that expression of one of these genes was also spatially regulated. In the present study, DNA sequence evidence demonstrates this gene to be a member of a small gene family encoding nsLTPs.

In general, LTPs have the ability to transfer lipids between membrane vesicles in vitro (Yamada, 1992; Bourgis and Kader, 1997). Unlike specific LTPs, nsLTPs exhibit a broad range of substrate specificity capable of transferring several classes of phospholipids and/or glycolipids (for review, see Helmkamp, 1986; Wirtz and Gadella, 1990). nsLTPs have been described in a variety of plant species, including monocots, dicots, and at least one gymnosperm (for review, see Kader, 1996). A number of possible functions have been proposed for plant nsLTPs, including involvement in epicuticular wax or cuticle biosynthesis (Sterk et al., 1991; Pyee et al., 1994), as well as a pathogen-defense role (Svensson et al., 1986; Molina et al., 1993; Segura et al., 1993; Cammue et al., 1995). Moreover, nsLTP expression can be induced by different forms of abiotic stress: Dunn et al. (1991) demonstrated cold- and drought-stress induction in barley, Torres-Schumann et al. (1992) reported salt-induced expression in tomato, and Ouvard et al. (1996) observed drought-stress induction in sunflower leaves.

nsLTP tissue-specific and developmentally regulated expression has been documented for different organs in a variety of plant species, and the existence of a small family of related genes has also been reported for most of the plant species thus far analyzed (Sterk et al., 1991; Fleming et al., 1992; Pelèse-Siebenbourg et al., 1994; Thoma et al., 1994; Pyee and Kolattukudy, 1995; Molina et al., 1996; Soufleri et al., 1996). However, very rarely have gene-specific probes been used to monitor differential patterns of expression of nsLTPs (Molina et al., 1996). It is therefore possible that different gene family members account for the observed diversity in patterns of expression, each one perhaps performing a different function. In the present study, the characterization of the spatial, developmental, drought-, and ABA-induced expression of three nsLTP gene family members are presented.

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Abbreviations: ABRE, ABA-responsive element; L1 through L5, five different stages in leaf expansion, L1 smallest to L5 largest; nsLTP, nonspecific lipid-transfer protein; RT-PCR, reverse-transcriptase-PCR; UTR, untranslated region.

MATERIALS AND METHODS

Plant Material and Drought-Stress and ABA Treatments

Lycopersicon pennellii Corr. LA716 and *Lycopersicon esculentum* Mill. cv UC82 were grown from seed in the greenhouse and fertilized with Osmocote (Scotts-Sierra, Maysville, OH). Plants were well watered except during drought-stress treatments, when water was withheld until the plants were visibly wilted. The time to wilt varied slightly from experiment to experiment but usually fell within the range of 5 to 7 d, depending on air temperature and size of the plant. Collection of tissue throughout leaf-blade expansion was based on leaf growth stage, from the smallest leaf readily identifiable (L1) to a fully expanded leaf (L5), and three intermediate sizes (L2, L3, and L4). For ABA treatments, the petioles of fully expanded, detached leaves were immersed for 6 h (on the laboratory bench) in either 10^{-3}, 10^{-4}, or 10^{-5} M ABA solutions in 10 mM Mes buffer, pH 5.8; these solutions were prepared from a 0.1 M ABA stock solution in ethanol. Petioles of control leaves were immersed in water or in 10 mM Mes buffer.

Nucleic Acid Isolation and Blot Hybridization

A genomic library of *L. pennellii* in bacteriophage EMBL3 was screened with the cDNA pLE16 using standard methods. Plant tissues were collected directly into liquid N_2 for RNA or DNA extractions. Genomic DNA and total RNA isolations, as well as Southern-blot and northern-blot transfers and hybridizations were performed as previously described (Kahn et al., 1993); poly(A^+) mRNA was purified from total RNA preparations using oligo(dT)-cellulose (Promega). Four identical sets of RNA blots were prepared for every treatment, and each one was hybridized to one of four different probes. Probes consisted of gel-purified DNA fragments oligolabeled with [^{32}P]dCTP. The relative amount of hybridization to the probes was determined using a scanning densitometer. All northern displays were replicated using a second set of independently isolated RNA samples.

DNA Sequencing and Analysis

The genomic insert was subcloned into the plasmid vector pBluescript KS (Stratagene) and overlapping deletions were generated using the ExoIII mung bean nuclease system (Stratagene). Both strands of each insert were sequenced using the Sequenase 2.0 kit (United States Biochemical). RT-PCR and PCR products were cloned into the plasmid vector pGEM-T (Promega) before sequencing. Sequence data were manipulated using the programs SeqAid, DNA Inspector II, DNAnalysis88, and M-fold (http://ibc.wustl.edu/~zuker/rna/.cgi). DNA sequences were searched against DNA databases using the BLAST algorithm (Altschul et al., 1990), and amino acid sequence alignments were performed with ClustalW (1.60) (http://alfredo.wustl.edu/msa/clustal.cgi).

Anchored RT-PCR

One-sided (anchored) RT-PCR assays for cDNA amplification were carried out essentially as described by Ausubel et al. (1996), except for two modifications. First, aside from poly(A^+) RNA, total RNA was used in parallel as a template for cDNA synthesis. Second, a primer consisting of a random 20-mer sequence (GTGAACTTAGGTGACTGACG) followed by a $(dT)_{12}$ tail was used for the RT reaction instead of an oligo$(dT)_{20}$ primer, and the 20-mer sequence alone was used for the PCR reactions. Either normal or wilted *L. pennellii* leaves were used as a source for RNA; *L. esculentum* leaf RNA and *L. pennellii* root RNA were used as negative controls. All nucleotide locations refer to DNA sequences deposited in GenBank with the following accession numbers: *LpLtp1,* U66465; *LpLtp2,* U66466; and pLE16, U81996. Upstream anchored RT-PCR was used to map the transcription initiation points; the primer used for cDNA synthesis and for the first PCR round was complementary to nucleotides 385 to 402 in *LpLtp1*, which are identical to positions 373 to 390 in *LpLtp2*; gene-specific primers were used for the second PCR round (a 20-mer complementary to positions 319–338 in *LpLtp1* and a 19-mer complementary to positions 307–325 in *LpLtp2*). The intron splice sites and the transcription termination points were mapped simultaneously using downstream anchored RT-PCR. A sequence common to both genes was used to prime the first PCR round (nucleotides 231–249 in *LpLtp1* or 219–237 in *LpLtp2*); gene-specific primers for the second PCR round corresponded to positions 315 to 334 in *LpLtp1* and 303 to 321 in *LpLtp2*.

Conserved-Region and Gene-Specific Probe Preparation

DNA fragments partially encompassing the 3′ UTRs were used as gene-specific probes: a 336-bp *Bam*HI/*Dra*I fragment for *LpLtp1* and a 268-bp *Bam*HI/*Ssp*I fragment for *LpLtp2* (Figs. 2 and 3, respectively), both of which were cloned into pBluescript. The gene-specific probe for *LpLtp3* consisted of a 193-bp PCR-amplified fragment from the tomato cDNA clone pLE16 (nucleotides 576–768). A 251-bp *Nla*IV/*Ssp*I fragment from the coding region in *LpLtp1* was cloned into pBluescript and used as a conserved-region probe to detect all members of the *nsLtp* gene family.

RESULTS

Structure of Two Members from the *nsLtp* Gene Family in *L. pennellii*

The cDNA clone pLE16 was isolated from *L. esculentum* based on differential screening for ABA- and drought-induced leaf transcripts (Plant et al., 1991), and was used to screen an *L. pennellii* genomic library. A restriction endonuclease map was obtained for the 15-kb genomic insert in a hybridizing recombinant plaque. Two regions of hybridization to pLE16 were identified within a 9.1-kb *Hin*dIII/*Sal*I fragment (Fig. 1). A comparison of these sequences with DNA databases revealed that each hybridizing region contained a full-length gene with a high degree of sequence similarity to plant *nsLTP* genes. The genes are referred to as *LpLtp1* (accession no. U66465) and *LpLtp2* (accession no. U66466). They are oriented in tandem in the *L. pennellii*

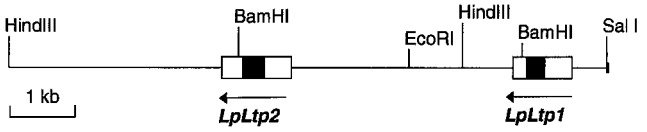

Figure 1. Restriction endonuclease map of a 9.1-kb DNA fragment from the *L. pennellii* genomic clone Pen16. The indicated restriction fragments were subcloned into pBluescript for sequence analysis. The *Sal*I site is provided by the EMBL3 phage vector. The exons (open bars) and introns (filled bars) of *LpLtp1* and *LpLtp2* are shown, with the direction of transcription indicated by the arrows.

genome; the distance between the 3′ end of *LpLtp1* transcript and the 5′ end of *LpLtp2* transcript is 3.4 kb (Fig. 1).

Upstream, anchored RT-PCR was used to map transcript initiation sites. After cloning into pGEM-T, the PCR fragments were sequenced to determine the position of transcript initiation for each gene. The lengths of the 5′ UTRs in *LpLtp1* and *LpLtp2* are 84 and 72 nucleotides, respectively. CAAT and TATA boxes were identified upstream from the transcription initiation site at positions −49 and −35 in *LpLtp1* and positions −47 and −34 in *LpLtp2*, respectively.

Based on the Blast sequence comparisons, an intron was predicted to be present in both genes. Using primers located upstream from the predicted intron position, the intron splice sites and transcription termination points were simultaneously mapped with downstream-anchored RT-PCR. Two bands (350 and 450 bp) were obtained with the *LpLtp2*-specific primer, suggesting that two alternative transcript sizes are produced for this gene. Sequencing of the cloned PCR fragments confirmed the predicted intron positions and identified the transcript termination sites: *LpLtp1*, 3′ UTR is 212 nucleotides; and *LpLtp2*, 3′ UTRs are 201 and 313 nucleotides. Examination of the nucleotide sequence at the intron/exon boundaries revealed the presence in both genes of consensus nucleotides found in the boundaries of class III introns from plants. The exon length (335 and 10 nucleotides) and the intron position are identical in both genes, but the intron in *LpLtp1* is shorter than the one in *LpLtp2* (269 versus 315 nucleotides, respectively). A putative polyadenylation signal was found in the 3′ UTR of each gene.

Possible Regulatory Elements in the 5′-Flanking Regions of *LpLtp1* and *LpLtp2*

Transcripts of *nsLTP* genes accumulate in *L. pennellii* and *L. esculentum* leaves in response to ABA (Kahn et al., 1993). It is not known, however, whether transcript induction by ABA occurs with all or only specific members of the *nsLTP* family. Inspection of the 5′-flanking region of *LpLtp1* and *LpLtp2* revealed the presence of consensus regulatory elements associated with ABA responsiveness (Marcotte et al., 1989; Shen and Ho, 1995). A putative ABRE of this type is found at position −329 (sequence CACGTTTC) in *LpLtp1* and at position −176 (sequence CACGTAAG) in *LpLtp2*. An additional G-box, GAACGTCAG, is found at position −621 in *LpLtp2*. A G-box-type ABRE is required but not sufficient for ABA-induced gene expression, for the ABA-responsive barley gene *HVA22*, a novel coupling element (CE1), in combination with the G-box, is required for ABA responsiveness (Shen and Ho, 1995). Five CE1-like sequences (core consensus CACC) were found in *LpLtp1* at positions −114, −86, −74, −4, and +25, whereas in *LpLtp2* a single CE1-like sequence is present at position −119.

Comparison of LpLTP1 and LpLTP2 with Other Plant nsLTPs

LpLtp1 and *LpLtp2* encode basic proteins of 114 amino acid residues, with calculated molecular masses of 11,545 D for LpLTP1 and 11,718 D for LpLTP2. The predicted polypeptides contain a hydrophobic region at the amino terminus with the characteristics of a signal peptide; according to the rules outlined by von Heijne (1986), cleavage of this putative signal peptide is predicted to occur between positions 24 (Ala) and 25 (Leu) in both gene products. The calculated pIs and molecular masses of the puta-

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                          **^  ******^ **** ** **^^^***^ ^ ***** ** ^ **** ****** *^^
L. pennellii (LpLTP1)     MEMVSKIACFVLLCMVVVAPHAEALTCGQVTAGLAPCLPYLQGRGPLGGCCGGVKGLLGS      60
L. esculentum (TSW12)     ..........................................................N....      60
L. pennellii (LpLTP2)     ....N.........................ST........MN.................Q      60
L. esculentum (LE16)      ...FG......VF..........S.S..E..S.........E..................A      60
N. tabacum (TobLTP)       ....G......V.............S....QS................S..........A      60
N. tabacum (NTLTP1)       ..IAG......V.....A..C...I......SN.....A..RNT....R......A.VN.      60

                          *^^  **^ ********* ^  *^  ^*** ^*  * ************** **
L. pennellii (LpLTP1)     AKTTADRKTACTCLKSAANAIKGIDLNKAAGIPSVCKVNIPYKISPSTDCSTVQ           114
L. esculentum (TSW12)     ..........................................................      114
L. pennellii (LpLTP2)     .Q..V..Q..........SSFT.L..G...SL..T.S..............K..      114
L. esculentum (LE16)      ...PE.............S.....TG....L.G..G...............      114
N. tabacum (TobLTP)       ..SLS.....................MG....L.GA.G.............K..      114
N. tabacum (NTLTP1)       .R..E..QI.........G..S..N.G....L..T.G.............K..      114
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Figure 2. Deduced amino acid sequence alignment of nsLTPs from wild tomato (*L. pennellii*), cultivated tomato (*L. esculentum*), and tobacco (*Nicotiana tabacum*). Tomato sequences are from genes *le16* (accession no. U81996) and TSW12 (accession no. X56040); tobacco sequences are from genes *TobLTP1* (accession no. D13952) and *NTLTP1* (accession no. X62395). The alignment was performed using ClustalW (1.60). Positions of identity with respect to *LpLTP1* are indicated by dots. The asterisks mark identical residues; the arrowheads indicate conservative substitutions in all six genes. Eight Cys and four Pro residues at highly conserved positions in all plant nsLTPs are underlined. The number of amino acid residues is indicated to the right of each sequence.

Table I. *Percent amino acid sequence identity of nsLTPs from L. pennellii, L. esculentum,* and tobacco

nsLTP	LpLTP2	TSW12	LE16	TobLTP	NTLTP1
LpLTP1	82	99	84	85	74
LpLTP2		81	78	78	76
TSW12			83	84	74
LE16				87	72
TobLTP					74

tive mature polypeptides are 8.94 and 8,970 D for LpLTP1, and 8.48 and 9,116 D for LpLTP2, respectively. Further, LpLTP1 and LpLTP2 have eight Cys and four Pro residues at highly conserved positions found in all other nsLTPs (Yamada, 1992; Shin et al., 1995) (Fig. 2).

A ClustalW alignment of LpLTP1 and LpLTP2 with nsLTPs from *L. esculentum* and tobacco is presented in Figure 2. The *nsLTP* sequences from *L. esculentum* were isolated as cDNA clones of a NaCl-induced gene, *TSW12* (Torres-Schumann et al., 1992), and an ABA- and drought-induced gene, *le16* (Plant et al., 1991). In the case of tobacco, *TobLTP* was isolated as a cDNA clone (Masuta et al., 1992) and *NTLTP1* was isolated as a genomic clone (Fleming et al., 1992). All gene products have the same length and are highly homologous: 62% of the residues are identical in all six LTPs, whereas an additional 15% are conservative substitutions. Percent amino acid residue identities among the six LTPs are shown in Table I. LpLTP1 exhibits 99% amino acid sequence identity to TSW12, strongly suggesting that they are alleles. LpLTP2 and LE16 have 82 and 84% sequence identity with LpLTP1, respectively, but only 78% with each other. These data, together with additional evidence presented in the following section, indicate that these two genes are not alleles. In the case of tobacco, TobLTP and NTLTP1 exhibit 85 and 74% amino acid sequence identity, respectively, to LpLTP1. When TobLTP and NTLTP1 are compared with LpLTP2, percent identities are 78 and 76, respectively.

Comparison of *nsLtp* Gene Families in *L. pennellii* and *L. esculentum*: Generation of Three *nsLTP* Gene-Specific Probes

Genomic DNA fragments partially encompassing the 3′ UTR of *LpLtp1* and *LpLtp2* were used as gene-specific probes to describe their patterns of expression. In addition, a 250-bp DNA fragment within the coding sequence of *LpLtp1*, downstream from the putative signal peptide, was used as the conserved-region probe (Cod250; nucleotides 189–439) to detect all of the gene family members. We used the probes for Southern analysis to confirm their specificity and to study the *nsLtp* gene family organization (Fig. 3). An equal number of restriction fragments hybridize to the probe Cod250 in *L. pennellii* and *L. esculentum*, exhibiting several polymorphisms. When the gene-specific probes for *LpLtp1* and *LpLtp2* were used, a single major band hybridized to each probe in *L. pennellii* and *L. esculentum*: 10.2 kb versus 4.3 kb for *LpLtp1* and 7.0 kb versus 8.1 kb for *LpLtp2*. In all cases, the bands for the gene-specific probes comigrate with a band detected by the probe Cod250.

The hybridization of the gene-specific probes to tomato DNA demonstrates that there is sequence conservation within the 3′ UTRs of the *nsLtp* alleles in these two species. Based on this observation, a third gene-specific probe for *LpLtp3* was generated through PCR amplification of a fragment in the 3′ UTR of the *L. esculentum* cDNA pLE16. This probe hybridizes to two Cod250-related fragments (approximately 3 and 4 kb) in the *L. esculentum* genome and to a single 2.5-kb fragment in *L. pennellii* (Fig. 3). Based on the *le16* genomic sequence in *L. esculentum*, there are no *Hin*dIII restriction sites within the amplified fragment of the 3′ UTR. In fact, the 3-kb *Hin*dIII fragment corresponds to the genomic fragment encompassing *le16* (Plant et al., 1991). The 4-kb *Hin*dIII fragment hybridizing to the *LpLtp3*-specific probe corresponds to an additional *nsLtp* gene or pseudogene in *L. esculentum*, which is closely related to *le16*. Aside from these three *nsLtp* genes in *L. pennellii*, there are four additional *Hin*dIII fragments (3.8, 5.5, 6.2, and 22 kb) hybridizing to the probe Cod250.

Spatial and Drought-Induced Expression of Three *nsLTP* Genes in *L. pennellii*

Gene-specific probes were used to separately describe the expression of three individual members of the *nsLtp* family in different organs of normal and wilted *L. pennellii* plants. *nsLtp* transcripts accumulated differentially throughout leaf-blade expansion in normal and wilted plants (Fig. 4). Whereas transcription of *LpLtp1* and *LpLtp2* was constitutive in well-watered plants, decreasing as the leaves matured, *LpLtp3* transcription was detectable at only very low levels in younger leaves (L1–L3) and was not detected in leaf stages L4 and L5. Transcript accumulation of all three genes was affected differently during drought-stress conditions. *LpLtp1* transcript levels increased and were approximately the same for all leaf-growth stages in

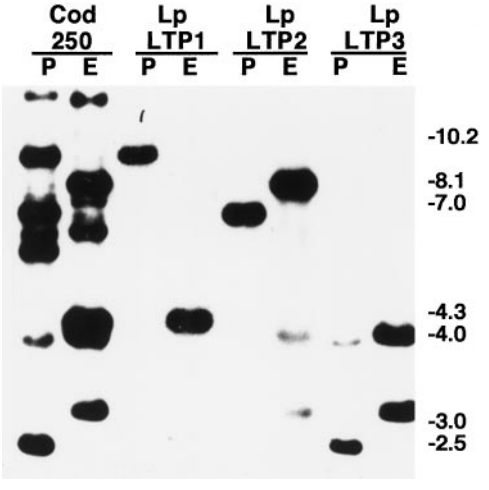

Figure 3. Genomic Southern blot of *L. pennellii* (P) and *L. esculentum* (E). Genomic DNA (8 μg) was digested with *Hin*dIII. Blots were probed with either an *Ltp* conserved-region probe (Cod250), or gene-specific probes for *LpLtp1*, *LpLtp2*, and *LpLtp3*. The sizes of *LpLtp*-hybridizing fragments are indicated in kb.

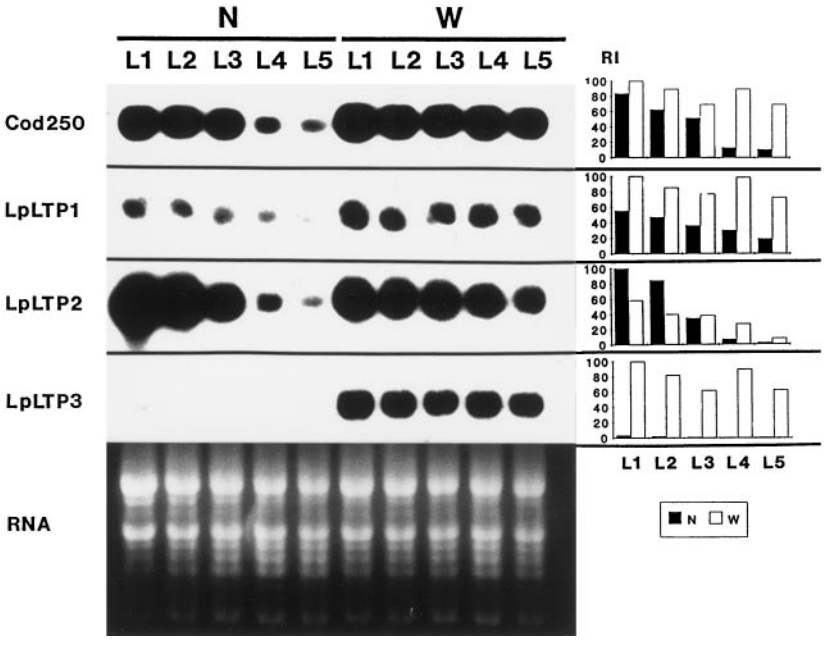

Figure 4. *nsLtp* transcript accumulation during late development of leaves from normal (N) or wilted (W) *L. pennellii* plants. RNA (7.5 μg) isolated from the smallest leaf (L1) to a fully expanded leaf (L5) was probed with either the *nsLtp* conserved-region probe (Cod250), or gene-specific probes for *LpLtp1*, *LpLtp2*, or *LpLtp3*. The relative intensity (RI) of hybridization to each probe is graphed to the right of each autoradiogram. A photograph of one of the ethidium-bromide-stained gels is shown at the bottom for load comparison.

wilted plants, equaling about twice the level found in the youngest leaves from well-watered plants. In wilted plants, *LpLtp2* transcription was repressed in younger leaves and induced in older leaves relative to the levels in well-watered plants, resulting in a similar but less pronounced developmental profile. In the case of *LpLtp3*, water deficit induced transcription at comparable levels in all leaf sizes. The probe Cod250 monitored the accumulation of transcripts from all *nsLtp* genes.

Northern analyses of individual *nsLtp* gene expression was also performed in other aerial tissues of normal and drought-stressed plants (Fig. 5). As in the case of leaf tissue, transcript distribution was unique for each gene. Transcripts for all three genes accumulated to higher levels

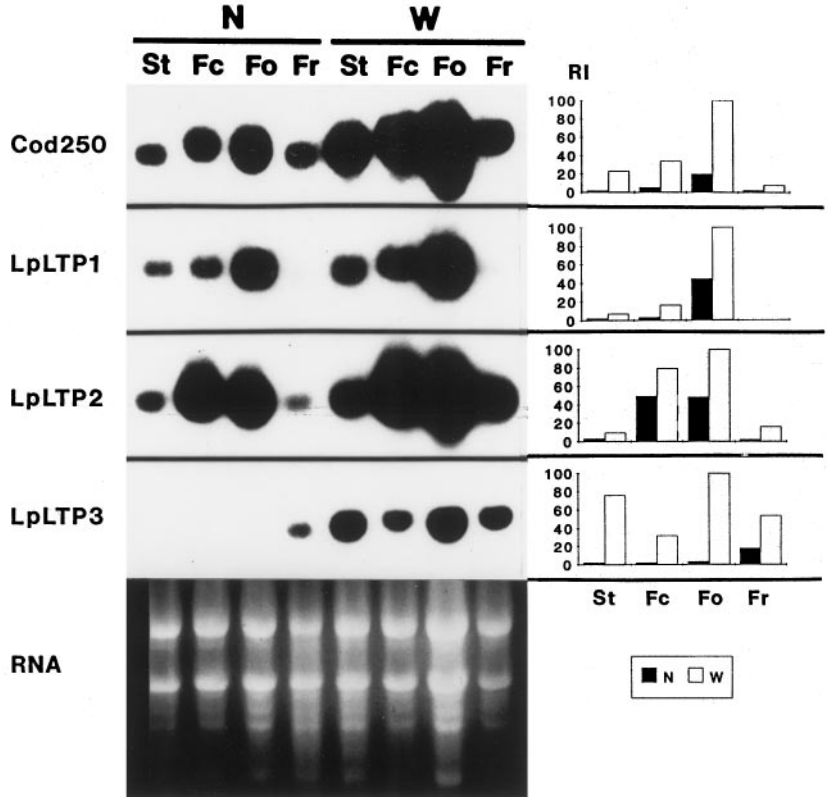

Figure 5. *nsLtp* transcript accumulation in stem, flower, and fruit from normal (N) or wilted (W) *L. pennellii* plants. RNA (7.5 μg) isolated from stems (St), closed (Fc), and open (Fo) flowers and immature fruits (Fr) was probed and analyzed as in Figure 4.

in stem and in both open and closed flowers in wilted plants. *LpLtp1* and *LpLtp2* were distinguished by their patterns of expression in fruit; *LpLtp1* had no detectable expression in fruit, whereas *LpLtp2* was expressed in all organs tested including fruit. *LpLtp3* was expressed in all drought-stressed organs, and in well-watered plants, *LpLtp3* appreciably accumulated only in fruit. The absence of *nsLtp* transcription in roots has been previously demonstrated (Kahn et al., 1993), and thus roots were not included in this study.

ABA-Induced Expression of Three *nsLtp* Genes in *L. pennellii*

nsLtp transcript accumulation was assessed using gene-specific probes in detached, fully expanded leaves that had been exposed to a range of ABA concentrations (Fig. 6). These treatments resulted in intracellular ABA levels comparable to those that occur in the plant as a result of drought stress (Kahn et al., 1993). Transcript levels for all three genes were increased to 10- to 20-fold over the buffer control by exogenous ABA, in a dose-dependent manner. In the case of *LpLtp1* and *LpLtp2*, transcript levels induced by exogenous ABA were higher than those resulting from drought stress in whole plants. In contrast, *LpLtp3* transcript levels were higher in droughted than in ABA-treated leaves.

DISCUSSION

The gene-specific patterns of expression for three members of the *nsLtp* gene family were characterized in the drought-resistant tomato species *L. pennellii*. The gene-specific probes used for this analysis were developed following isolation and complete characterization of the genomic clone form of *LpLtp1* and *LpLtp2*. The third gene-specific probe was developed from a cDNA form of a gene member from cultivated tomato. The results of this investigation demonstrated that although members of this gene family are inducible by drought stress, development plays the primary role in the regulation of expression of at least two members of this gene family.

LpLtp1 and *LpLtp2* are oriented in tandem in the *L. pennellii* genome, with *LpLtp1* located upstream of *LpLtp2*, and the transcribed regions separated by approximately 3.4 kb. Both genes consist of two exons of conserved lengths, interrupted by a single intron located at identical positions but differing in length. The intron location is the same as in most plant *nsLtp* genes for which a genomic sequence is available (Kader, 1996). Genomic Southern data indicated the existence of a *nsLTP* family in *L. pennellii* and *L. esculentum*, composed of at least five to seven members. In most plant species thus far analyzed, nsLTPs are encoded by a small gene family and linkage of some members of the gene family has been observed, e.g. tobacco, sorghum, and barley, among others (Fleming et al., 1992; Pelèse-Siebenbourg et al., 1994; White et al., 1994; Kader, 1996). In the case of maize, additional nsLTP isoforms have been reported to occur via alternative splicing of a *nsLtp* gene (Arondel et al., 1991).

The deduced amino acid sequences for *LpLtp1* and *LpLtp2* share a number of characteristics common to all

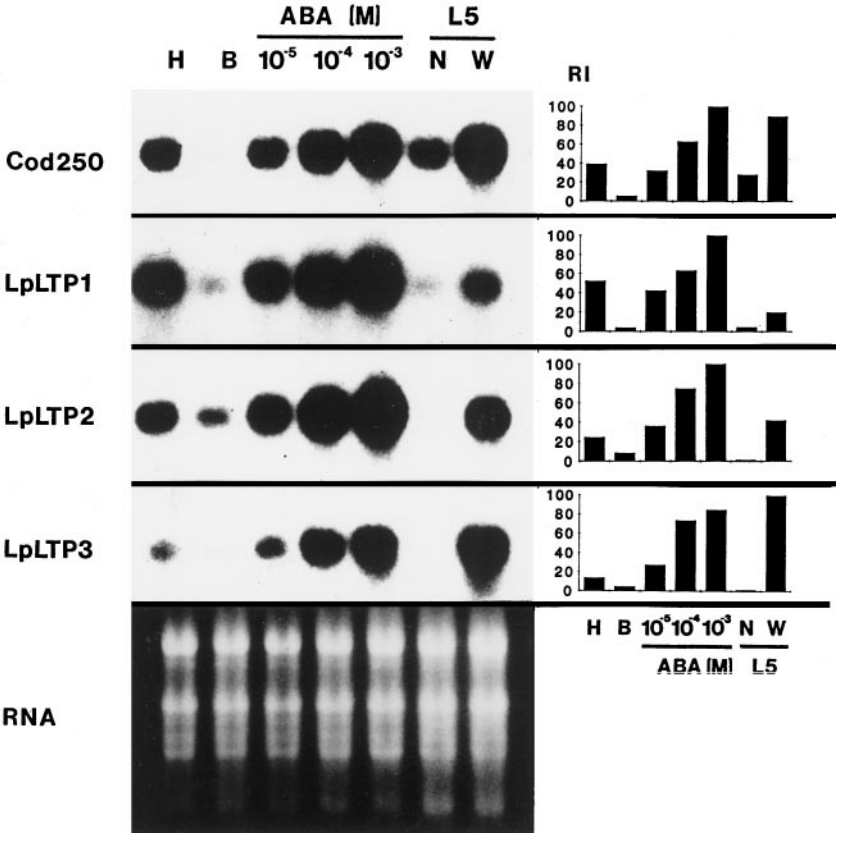

Figure 6. *nsLtp* transcript accumulation in ABA-treated leaves from *L. pennellii*. RNA (7.5 μg) isolated from detached, fully expanded leaf petioles that had been immersed for 6 h in either water (H), 10 mM Mes buffer (B), or increasing concentrations of ABA in 10 mM Mes buffer; or from fully expanded leaves (L5) of normal (N) or wilted (W) plants, was probed and analyzed as in Figure 4.

plant nsLTPs: they have a low M_r, a basic pI, eight Cys and four Pro residues at conserved positions, and an amino-terminal signal peptide for translocation into the ER.

LpLTP1 and LpLTP2 showed the highest degree of amino acid sequence identity (72–99%) with nsLTPs from tomato and tobacco. *TSW12* is a cDNA clone isolated from cultivated tomato leaves in a screen for salt-inducible transcripts (Torres-Schumann et al., 1992). At the DNA level, *LpLtp1* differs from the tomato gene *TSW12* at five nucleotide positions within the coding region, and only one of them represents a missense substitution in the amino acid sequence. Moreover, homology in the nucleotide sequence of these two genes extends through the 3′ UTR (data not shown), suggesting that they are alleles. *le16* is a cDNA clone isolated in a screen of cultivated tomato leaves for ABA and drought-inducible transcripts (Plant et al., 1991). A lower percentage in amino acid sequence identity between LpLTP2 and LE16, as well as the lack of cross-hybridization between their corresponding 3′ UTRs, suggests that they are not alleles. In fact, their deduced polypeptides show a higher degree of sequence identity to LpLTP1 than to each other. Amino acid sequence comparisons with the tobacco nsLTPs suggest that TobLTP (Masuta et al., 1992) is more closely related than NTLTP1 (Fleming et al., 1992) to all of the nsLTPs thus far described in *Lycopersicon* spp.

The 3′ UTRs of the *nsLtp* alleles in *L. pennellii* and *L. esculentum* have a high degree of nucleotide sequence conservation, indicating that the gene family members were generated in a common ancestor prior to the differentiation of the two species. This high degree of sequence homology in the 3′ UTRs allowed their use as gene-specific probes in both species. In addition, the 3′ UTR from the *L. esculentum* gene *le16* was used as a probe to detect a third member (designated *LpLtp3*) of the *nsLtp* gene family in *L. pennellii*. These three *nsLtp* family members are probably physically linked in the genome. *LpLtp1* and *LpLtp2* were mapped within 7 kb on a phage clone (Fig. 1). The gene-specific probes for *LpLtp2* and *LpLtp3*, as well as the Cod250 probe, all hybridized to a common 12-kb fragment in the *L. esculentum* genome (data not shown). Altogether, these results suggest that at least three members of the *nsLtp* family are physically contiguous in *Lycopersicon* spp.

A variety of possible roles for nsLTPs has been proposed based on their in vitro properties and their spatial expression patterns (Pelèse-Siebenbourg et al., 1994; Shin et al., 1995; Kader, 1996). Secretion of a nsLtp (EP2) from carrot somatic embryos has been reported by Sterk et al. (1991), who have proposed a role for nsLTPs in cutin biosynthesis by effecting the transport of cutin monomers through the extracellular matrix. In accordance with this notion, we found that transcription of *nsLtp*s in *L. pennellii* is restricted to the aerial tissues of the plant. Moreover, accumulation of *LpLtp1* and *LpLtp2* transcripts in leaves is also developmentally regulated, with levels being the highest in young leaves, when biosynthesis of the cuticular membrane is required for leaf expansion, and decreasing as the leaves mature, when the demand for epidermal components declines. A number of studies have reported epidermal cell-specific expression of nsLTPs in various plant tissues (Sterk et al., 1991; Fleming et al., 1992; Thoma et al., 1994). It is interesting that Pyee et al. (1994) have reported a nsLTP to be a major surface wax protein in broccoli leaves.

Organ- and tissue-specific expression of *nsLtp* genes has been reported in several plant species (Tsuboi et al., 1991; Kotilainen et al., 1994; Thoma et al., 1994; Soufleri et al., 1996). Although none of the three *nsLtp* genes studied in *L. pennellii* were found to be organ specific, their transcript accumulation patterns in leaves, stems, flowers, and fruit were different. Accumulation of *LpLtp1* and *LpLtp2* transcripts in leaves was primarily developmentally regulated and *LpLtp1* and *LpLtp2* were distinguished by their patterns of expression in fruit. In contrast, accumulation of transcripts from *LpLtp3* was rarely observed in unstressed tissues. Transcripts for *nsLtp*s were not detected in roots of *L. esculentum*, *L. pennellii*, or the interspecific F_1, using the cDNA clone pLE16 as a probe (Kahn et al., 1993).

Drought stress affected transcript accumulation of the three *nsLtp* genes in a different manner, but an overall increase in total *nsLtp* transcript levels was observed as a result of the stress. This observation also seems to be in agreement with the proposed involvement of nsLTPs in cuticle biosynthesis (Sterk et al., 1991), since the cuticle plays an important role in the water balance of plants (Lemieux, 1996). Presumably, the induction of *nsLtp* expression represents an adaptive response to drought stress, in which the plant may be able to reduce water loss by increasing the cuticle thickness. Induction of *nsLtp*s by several forms of dehydrative stress has been previously reported (Dunn et al., 1991; Torres-Schuman et al., 1992; White et al., 1994; Soufleri et al., 1996). In all of these cases, ABA responsiveness was implicated in the induction of *nsLtp* expression.

Although leaf transcript levels for all three *nsLtp*s in *L. pennellii* were increased in response to exogenous ABA, each gene had a unique dose response. ABA responsiveness appears to be the result of two distinct *cis* elements, a G-box class element, ABRE, and a coupling element, CE, and the diversity of ABA-mediated responses in planta appears to be the result of combinations of ABREs and different relative locations of unique coupling elements (Shen et al., 1996). Putative ABREs and CE-1-like *cis*-elements were found in the upstream regulatory regions of *LpLtp1* and *LpLtp2*, yet *LpLtp1* appears to be much less responsive to ABA. It is conceivable that in the case of *LpLtp1* an additional physiological signal is required for ABA to affect induction of transcription. Elevated leaf transcript levels in the water control relative to the buffer treatment control were seen for all three genes. We are unable to explain this anomalous response.

In summary, it appears that, although members in the *nsLtp* gene family can be developmentally expressed in different plant organs, their expression may also be mediated by ABA during dehydrative stress. The characterization of all of the members in the *nsLtp* gene family, as well as their expression patterns and subcellular localization, should help in the elucidation of their functions in the plant.

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