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A tomato xylem sap protein represents a new family of small cysteine-rich proteins with structural similarity to lipid transfer proteins

Martijn Rep[a,*], Henk L. Dekker[b], Jack H. Vossen[a], Albert D. de Boer[a], Petra M. Houterman[a], Chris G. de Koster[b], Ben J.C. Cornelissen[a]

[a] *Plant Pathology, Swammerdam Institute for Life Sciences, University of Amsterdam, P.O. Box 94062, 1090 GB Amsterdam, The Netherlands*
[b] *Mass Spectrometry, Swammerdam Institute for Life Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands*

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Abstract **The coding sequence of a major xylem sap protein of tomato was identified with the aid of mass spectrometry. The protein, XSP10, represents a novel family of extracellular plant proteins with structural similarity to plant lipid transfer proteins. The *XSP10* gene is constitutively expressed in roots and lower stems. The decline of XSP10 protein levels in tomato infected with a fungal vascular pathogen may reflect breakdown or modification by the pathogen.**
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Key words: Xylem sap; Lipid transfer protein; Cysteine-rich protein; *Fusarium oxysporum*

1. Introduction

The main function of the xylem is to provide water and minerals to the aerial tissues of land plants [1]. However, xylem sap does not just contain mineral salts. Its composition in sugar beet for instance comprises, besides mineral salts, amino acids, organic acids and sugars [2]. Proteins have been detected as well [3,4]. In cucumber xylem sap, several of these have been identified as glycine-rich proteins, a lectin-like protein and a chitinase [5–7]. In addition, new proteins appear in xylem sap during development of diseases affecting the vascular system [8–10].

We have recently shown that several pathogenesis-related (PR) proteins accumulate in tomato xylem sap upon infection with *Fusarium oxysporum* f. sp. *lycopersici*, a xylem-invading fungal pathogen [11]. In that study, we also found a strong decrease in abundance of a 10 kDa protein, the major low molecular weight protein in xylem sap of healthy plants, during the course of infection. Its relative abundance in healthy plants and its decline in infected plants prompted us to identify the protein with mass spectrometry.

*Corresponding author. Fax: (31)-20-5257934.
E-mail addresses: rep@science.uva.nl (M. Rep), hldekker@science.uva.nl (H.L. Dekker), vossen@science.uva.nl (J.H. Vossen), houterman@science.uva.nl (P.M. Houterman), dekoster@science.uva.nl (C.G. de Koster), cornelissen@science.uva.nl (B.J.C. Cornelissen).

2. Materials and methods

2.1. Plant material, fungal isolates, infection assay

Xylem sap was collected from 8-week old tomato (*Lycopersicon esculentum*) line GCR161 as described in [4,11]. Briefly, stems were cut off below the second true leaf, the first droplet appearing on the cut surface was removed with blotting paper and the plant was placed in a horizontal position. Sap dripping from the cut surface was collected in tubes placed on ice for a period of 3–6 h, generally yielding 2–10 ml of sap. *F. oxysporum* f. sp. *lycopersici* (Fol) isolate Fol007 (race 2) or Fol029 (race 3) was used for infection of tomato [12]. Fungal spores were collected from 5 day old cultures in potato dextrose broth and used for root-inoculation of 5 week old tomato plants at a spore density of 0.5×10^7/ml. Xylem sap was collected 3 weeks after (mock) inoculation.

2.2. SDS–PAGE of xylem sap proteins

Xylem sap was concentrated by freeze-drying, and the protein concentration was estimated with the bicinchoninic acid method (Sigma). Volumes were adjusted so that each sample contained 1 μg/μl of bovine serum albumin (BSA) equivalents. SDS–PAGE was done with Hoefer Mighty Small SE250 minigel equipment (Amersham Biosciences) using the Tris–Tricine buffer system [13]. Silver staining was used to visualize proteins as described in [14].

2.3. Mass spectrometry

For mass spectrometry gel slices containing XSP10 were S-alkylated with iodoacetamide and vacuum-dried using a speedvac. The in-gel digestion with trypsin (sequencing grade, Roche Molecular Biochemicals) and extraction of the peptides after the overnight incubation were done according to [14]. The collected eluates were concentrated and washed on a μC18 ZipTip (Millipore). The peptides were eluted or redissolved (speedvac) in 5–10 μl of 60% acetonitrile/1% formic acid. The peptide solutions were mixed 1:1 (v/v) with a solution containing 52 mM α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemie) in 49% ethanol/49% acetonitrile/2% TFA and 1 mM ammonium acetate. Prior to dissolving, the α-cyano-4-hydroxycinnamic acid was washed briefly with acetone. The mixture was spotted on a target plate and allowed to dry at room temperature. Reflectron matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectra were acquired on a TofSpec 2E time-of-flight mass spectrometer (Micromass Wythenshawe, UK). The resulting peptide mass spectra were used to search the ABCC Non-Redundant Protein Database release 20010401 (Advanced Biomedical Computing Center, Frederick, MD, USA)(http://www-fbsc.ncifcrf.gov/) with MassLynx ProteinProbe (Micromass Wythenshawe). Electrospray ionization tandem mass spectrometry (MS/MS) analysis was performed to obtain sequence tags, as follows. A gold-plated nanospray needle (Protana or New Objective) was filled with 2–5 μl of the peptide mixture and analyzed on a Micromass Q-TOF mass spectrometer using nano-electrospray ionization. Low-energy collision-induced dissociation experiments were performed by selecting peptide ions from the survey spectra and using argon as a collision gas. The resulting MS/MS spectra were analyzed with MassLynx Pepseq and Biolynx software. The Lycopersicon Gene Index (LGI) at The Institute for Genomic Research (TIGR) was searched with TblastN at http://tigrblast.tigr.org/tgi.

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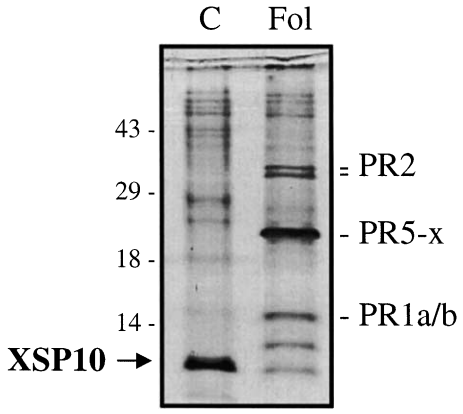


Fig. 1. A 10 kDa protein is the major low molecular weight protein in xylem sap of healthy tomato plants. Five week old GCR161 plants were either mock-inoculated (C) or inoculated with *F. oxysporum* (Fol). Xylem sap proteins were separated with SDS–PAGE on a Tris–Tricine gel and visualized by silver staining. Lanes represent sap from individual plants. Tomato xylem sap proteins identified in a previous study [11] are indicated on the right. XSP10 is a 10 kDa protein present in healthy plants (C) which declines in abundance in fungus-infected plants (Fol). Molecular weight markers are indicated on the left (in kDa).

2.4. Northern blot analysis

Five week old GCR161 tomato plants were mock-inoculated or inoculated with *F. oxysporum* race 3 (isolate Fol029). Two weeks after inoculation, roots, lower stems and apical growth tips were collected and frozen in liquid nitrogen. The selected organs were ground and nucleic acids were extracted in a mixture (1:1 volume) of Tris buffer saturated phenol (Life Technologies) and RNA extraction buffer (100 mM Tris–HCl pH = 8.5; 100 mM NaCl; 20 mM EDTA; 1% Sarkosyl). The extract was washed twice with phenol/chloroform (1:1 volume) and once with chloroform. Total RNA was precipitated with 2 M LiCl at 0°C for 4 h. 15 μg of total RNA was size-fractionated by electrophoresis on a 1.1% agarose gel supplemented with 6.6% formaldehyde in MEN buffer (20 mM MOPS; 5 mM NaAc; 10 mM EDTA, pH was adjusted to 7.0 with NaOH). The RNA was blotted onto Hybond-N nylon membranes (Amersham), and hybridized overnight at 65°C with selected probes in hybridization buffer (1% BSA; 7% SDS; 0.5 M $NaPO_4$ buffer pH = 7.2; 1 mM EDTA). After hybridization blots were washed three times with 0.2× saline sodium citrate; 0,1% SDS and hybridizing bands were visualized by phosphoimaging (Molecular Dynamics). Probe sequences used were a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Petunia hybrida*, and an extracellular PR-1 from tomato [15]. *XSP10* probe DNA was generated by PCR on a tomato cDNA library (Vossen et al., in preparation) using the primers FP274 (TACTTGTTGTGTGTTGTAGG) and FP275 (TACATGACATGATCGATCTG). Probe DNA fragments were labeled with [α^{32}P]dATP (Amersham) using the DecaLabel™ DNA labeling kit from MBI Fermentas.

2.5. Alignment software and tree construction

Sequence alignments and phylogenetic tree construction were done with MacVector (Oxford Molecular Group).

3. Results

As with cucumber and other plants [3–7], xylem sap of tomato contains various proteins. The most abundant one in the low molecular weight range was designated XSP10 for xylem sap protein of 10 kDa (Fig. 1, lane C). Upon infection with the vascular pathogen *F. oxysporum* additional proteins accumulate in xylem sap, including pathogenesis-related proteins PR-1, PR-2 and PR-5 [11]. Xylem colonization by *Fusarium* also leads to a strong decline in abundance of XSP10 (Fig. 1, lane Fol). This observation prompted us to identify this protein with the aid of mass spectrometry.

Peptide mass fingerprinting analysis of this protein with MALDI-TOF yielded a set of tryptic peptide masses that did not match any protein in the ABCC Non-Redundant Protein Database release 20010401 (Advanced Biomedical Computing Center, Frederick, Ml) (http://www-fbsc.ncifcrf.gov/). To obtain more information, two peptides of XSP10 were sequenced with MS/MS, yielding the following sequence tags: SS(K/GA)NPEVA(L/I)T(L/I)PK and (L/I)(L/I)PCAEAAS. These sequences were used for TblastN searches of translated cDNA sequences from the Lycopersicon Gene Index (release 8.0) at The Institute for Genomic Research (TIGR) [16] (this index is not accessible for peptide mass list searches). The first tag gave a match with the predicted translation product of the tentative consensus sequence TC102912 (*T7* in Fig. 2). The second tag and MS/MS data of a third peptide correspond to tryptic peptides of the same translation product (*T3* and *T9* in Fig. 2). Furthermore, five additional predicted tryptic peptides of the TC102912 translation product match peptides in the peptide mass fingerprint of the xylem sap protein. These data conclusively show that XSP10 is encoded by TC102912 (Fig. 2). In accordance with the presence of XSP10 in xylem sap, a predicted amino-terminal ER translocation signal sequence is found by SignalP (http://www.cbs.dtu.dk/services/SignalP) [17]. Disregarding this signal peptide, eight out of 10 predicted peptides were detected by MALDI-TOF MS, covering 88% of the predicted mature protein (Fig. 2). Only two predicted peptides were not observed. Since one of these is the N-terminal peptide, we could not confirm the predicted cleavage site of the signal sequence.

Although the protein itself has not been described before,

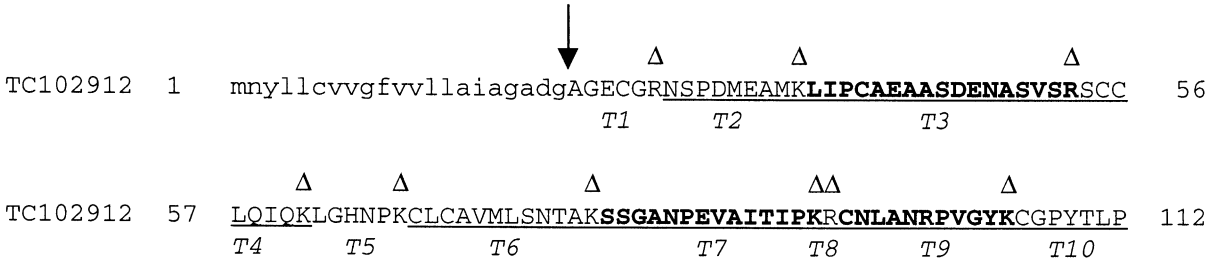
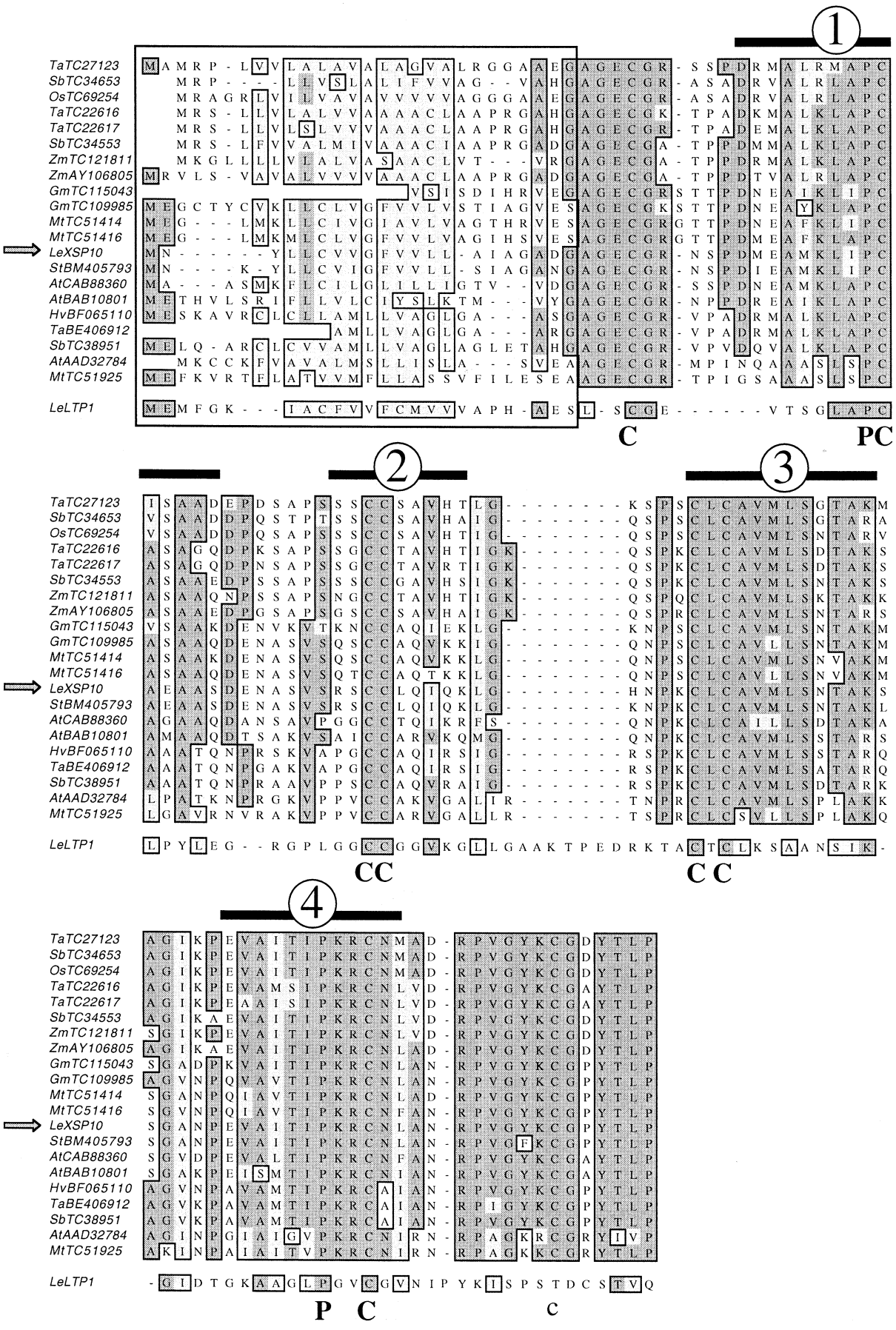


Fig. 2. Amino acid sequence of XSP10. MALDI-TOF and tandem MS analysis of XSP10 allowed identification of its coding sequence in the TIGR Tomato Gene Index [16] (see text). The predicted translation product of TC102912 is shown here. Predicted tryptic peptides are numbered (*T1–10*); triangles indicate their carboxyl-terminal amino acids. Three peptides that were sequenced with tandem MS are indicated in bold. All trypsin-generated peptides of XSP10 that match peptides in the MALDI-TOF spectrum are underlined (tryptic digestion at the lysine–arginine pair is a stochastic process, so that peptide masses corresponding to 'partial' digests, *T7–8* and *T8–9*, were also found in the fingerprint). The predicted signal sequence for translocation into the ER is in lower case; the arrow indicates the presumed cleavage site.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MtTC51414
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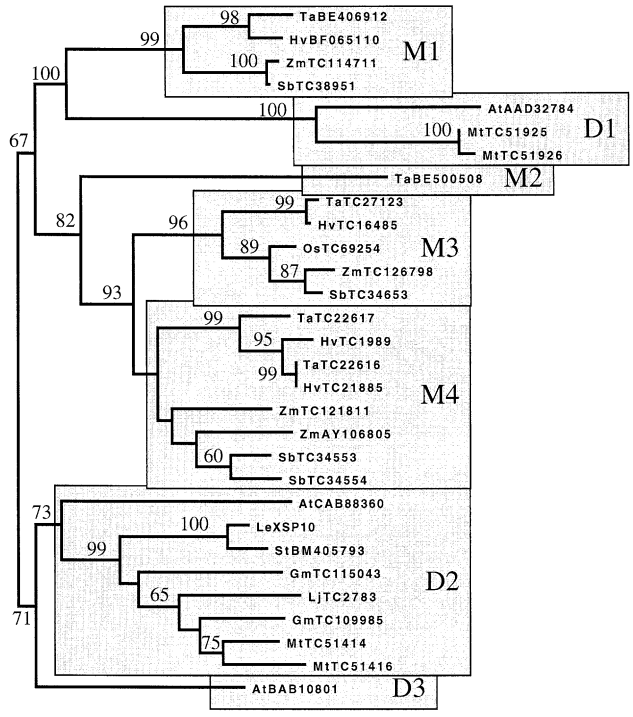

Fig. 4. The XSP10 family diversified early in angiosperm evolution. The aligned sequences of XSP10 family members found in TIGR Gene Indexes were stripped from N-terminal signal sequences and C-terminal extensions and used for phylogenetic analysis, using neighbor-joining and midpoint rooting. Dicot-specific (D1–3) and monocot-specific (M1–4) clades are indicated. Bootstrap percentages are provided for branches receiving 60% or more support. Branch length reflects the extent of sequence divergence.

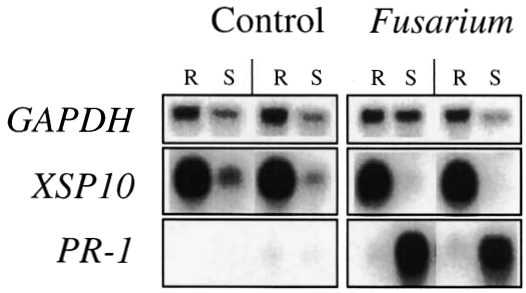

Fig. 5. *XSP10* is constitutively expressed in roots and *XSP10* RNA levels decrease in lower stems upon *Fusarium* infection. RNA was extracted from roots (R) or the lower part of stems (S) of 7 week old plants either mock-inoculated (Control) or infected with *Fusarium* at the age of 5 weeks. Northern blots were probed for *XSP10* and *PR-1*. A GAPDH probe served as loading reference. Each lane contains RNA from an individual plant.

blast searches of plant gene indexes at TIGR identified 30 XSP10 homologs in 11 higher plants, including tomato (Figs. 3 and 4). No homologs were found in other organisms. A phylogenetic tree of the XSP10 family harbors several dicot- and monocot-specific clades, which suggests that there were only a few (probably two) XSP10-like sequences before angiosperm diversification (Fig. 4).

All XSP10 homologs contain eight cysteines at corresponding positions. The spacing of these cysteines, as well as overall size and basic nature of the XSP10 family members, are similar to those of lipid transfer proteins (LTPs). The XSP10 sequence indeed conforms to the plant LTP consensus as defined in the SMART database [18] (http://smart.embl-heidelberg.de). Moreover, α-helices are predicted at similar positions as in LTPs, even though overall primary sequence similarity is quite low (Fig. 3). The fourth helix and part of the first helix were not consistently predicted by the software used (http://npsa-pbil.ibcp.fr), but their presence is suggested by comparison with LTP-like proteins. Crystal and nuclear magnetic resonance (NMR) structures of LTP-like proteins show helices in those positions despite the presence of central proline residues [19]. Proline residues are present at similar positions in XSP10-like proteins (Fig. 3). The overall fold of XSP10-like proteins may resemble that of the 'LTP2-class' of lipid transfer proteins, since the central residue in the 'CXC' motif in the third helix is hydrophobic (leucine), which was suggested to have an important influence on helix orientation and cysteine pairing [20]. Although the structural similarities between XSP10-like proteins and LTPs could suggest that the XSP10 family constitutes a novel class of LTPs, we do not designate it as such because of the low level of sequence similarity and the lack of experimental data concerning lipid transfer activity.

Previously, genes for several xylem sap proteins were found to be specifically expressed in roots [5,6,21]. As shown in Fig. 5, *XSP10* is strongly expressed in roots and moderately in the lower part of the stem. No expression was found in higher parts of the plant (data not shown). The strong expression in roots did not change upon *Fusarium* infection, but expression in stems was reduced to barely detectable levels. As a positive control for transcriptional responses to *Fusarium* infection, expression of the *PR-1* gene was found to be strongly induced in stems (Fig. 5). This corresponds to PR-1 protein accumulation in xylem sap during *Fusarium* colonization of xylem vessels (Fig. 1).

4. Discussion

During fungal colonization of tomato xylem vessels the abundance of a protein of 10 kDa, here called XSP10, declines

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Fig. 3. Tomato XSP10 and homologs in other plants show structural similarity to lipid-transfer proteins. Tomato (Le) XSP10 (arrow) was aligned with putative translation products of cDNA sequences found in TIGR Gene Indexes of several higher plants: the dicots *Arabidopsis thaliana* (At; release 8.0), *Medicago truncatula* (Mt; release 5.0), *Lotus japonicus* (Lj; release 1.0), potato (St; release 6.0) and soybean (Gm; release 8.0) and the monocots wheat (Ta; release 4.0), barley (Hv; release 3.0), sorghum (Sb; release 4.0), maize (Zm; release 9.1) and rice (Os; release 9.0) [16]. For clarity, only a representative subset of the sequences is shown here. The lipid-transfer protein LTP1 from tomato was included for comparison. TIGR tentative consensus sequences (TC) or Genbank accessions are preceded by species abbreviations. Signal sequences predicted by Von Heijne's method [29] or by homology with related sequences are boxed (note that not all N-termini are complete due to missing 5′ ends of cDNAs). Cysteine (C) and proline (P) residues conserved between XSP10 homologs and present at corresponding positions in LTP1 are indicated below the alignment. Only the last cysteine conserved between XSP10 homologs (lower-case c) does not easily align with the last cysteine in LTP1. Numbered bars (1–4) above the aligned sequences indicate putative α-helical regions (see text).

sharply in xylem sap [11]. This protein is by far the most abundant xylem sap protein of relatively low molecular weight (under 70 kDa) in healthy tomato plants. Using MS/MS sequence tags, we identified the coding sequence for this protein in the TIGR tomato gene index. XSP10 is very different from the few proteins thus far identified in xylem sap of various plants [5–7,9,10] and represents a new class of small, cysteine-rich plant-specific proteins bearing a structural relationship to plant LTPs (Figs. 3 and 4).

Apart from similarity in spacing of the eight cysteines, XSP10-like proteins are about the same size and have similarly high p*I* values as most LTPs. However, considering the low overall sequence similarity, it is unclear whether they share a common ancestry or are products of convergent evolution. Although LTPs have been shown to bind lipids, including phospholipids, and transfer them between membranes, their physiological function remains unknown [22]. Like many other small cysteine-rich proteins, LTPs can have antimicrobial activity [23,24]. This activity is not strictly coupled to lipid transfer properties, since at least one antimicrobial member of the LTP family, Ace-AMP1, does not bind or transfer lipids [19].

As in the case of several xylem sap proteins of cucumber [5,6,21], the *XSP10* gene is strongly expressed in roots. Presumably these proteins are secreted by root cells and travel upward with the sap stream. In contrast, none of the previously identified LTPs from tomato [25,26] or *Arabidopsis* [27] is expressed in roots. The moderate expression of *XSP10* in stems is suppressed during *Fusarium* colonization (Fig. 5). However, since root expression does not diminish, the decline of XSP10 protein level in xylem sap during disease development is most likely due to suppression of either translation or secretion, or to increased protein breakdown. Preliminary observations indicate that the XSP10 protein is modified by *Fusarium* grown in isolated xylem sap (M. Rep, unpublished results). An intriguing question is whether breakdown and/or modification of XSP10 is required for successful fungal colonization of xylem vessels. Future work to establish the function of XSP10 should include testing of purified protein for antimicrobial activity and the analysis of *XSP10*-silenced tomato lines. Recently, a role in transmission of a systemic disease resistance signal from pathogen-attacked *Arabidopsis* leaves was shown for a LTP-like protein [28]. In light of this finding, a possible role of XSP10 in systemic signaling from roots should be considered.

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