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Journal

Journal of Experimental Botany, 61(15)

ISSN

0022-0957

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Publication Date

2010-10-01

DOI

10.1093/jxb/erq228

Peer reviewed

Journal of Experimental Botany, Vol. 61, No. 15, pp. 4277–4290, 2010
doi:10.1093/jxb/erq228 Advance Access publication 28 July, 2010
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

A multifaceted study of stigma/style cysteine-rich adhesin (SCA)-like *Arabidopsis* lipid transfer proteins (LTPs) suggests diversified roles for these LTPs in plant growth and reproduction

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Received 12 May 2010; Revised 2 July 2010; Accepted 6 July 2010

Abstract

Lily stigma/style cysteine-rich adhesin (SCA), a plant lipid transfer protein (LTP) which is secreted into the extracellular matrix, functions in pollen tube guidance in fertilization. A gain-of-function mutant (*ltp5-1*) for *Arabidopsis* LTP5, an SCA-like molecule, was recently shown to display defects in sexual reproduction. In the current study, it is reported that *ltp5-1* plants have dwarfed primary shoots, delayed hypocotyl elongation, various abnormal tissue fusions, and display multibranching. These mutant phenotypes in vegetative growth are recessive. No abnormality was found in *ltp5-1*/+ plants. In a phylogenetic analysis of plant LTPs, SCA-like *Arabidopsis* LTPs were classified with conventional plant LTPs. Homology modelling-based electrostatic similarity index (ESI) clustering was used to show diversity in spatial distributions of electrostatic potentials of SCA-like LTPs, suggestive of their various roles in interaction in the extracellular matrix space. β-Glucuronidase (GUS) analysis showed that *SCA*-like *Arabidopsis LTP* genes are diversely present in various tissues. *LTP4* was found specifically in the guard cells and *LTP6* in trichomes as well as in other tissues. *LTP1* levels were specifically abundant in the stigma, and both *LTP3* and *LTP6* in the ovules. *LTP2* and *LTP4* gene levels were up-regulated in whole seedlings with 20% polyethylene glycol (PEG) and 300 mM NaCl treatments, respectively. *LTP5* was up-regulated in the hypocotyl with 3 d dark growth conditions. *LTP6* was specifically expressed in the tip of the cotyledon under drought stress conditions. The results suggest that SCA-like *Arabidopsis* LTPs are multifunctional, with diversified roles in plant growth and reproduction.

Key words: *Arabidopsis thaliana*, electrostatics, extracellular matrix (ECM), lipid transfer protein (LTP), small secreted peptide, stigma/style cysteine-rich adhesin (SCA).

Introduction

Plant non-specific lipid transfer proteins (nsLTPs) are small (9–10 kDa), basic (pI 8.8–10) (Kader, 1996), secreted proteins (Bernhard *et al.*, 1991; Thoma *et al.*, 1993). The conserved features of plant LTPs are four disulphide bonds with eight cysteine residues and two consensus pentapeptide motifs (Thr/Ser-X1-X2-Asp-Arg/Lys and Pro-Tyr-X-Ile-Ser) (Douliez *et al.*, 2000). The three-dimensional structures of plant LTPs have a typical globular shape of an

orthogonal four-helix bundle architecture and a hydrophobic core (Shin *et al.*, 1995; Gomar *et al.*, 1996, 1998; Heinemann *et al.*, 1996). This hydrophobic cavity runs through the whole molecule and is capable of interacting with a phospholipid molecule and fatty acids *in vitro* (Zachowski *et al.*, 1998; Hamilton, 2004).

Stigma/style cysteine-rich adhesin (SCA), a lily LTP, is secreted from both pollen and the pistil transmitting tract epidermis (TTE), where pollen tubes grow and are guided to the ovules (Lord, 2000). SCA and pectins are involved in pollen tube adhesion-mediated guidance (Park *et al.*, 2000). By studying the mechanism of action of this adhesion event, it was learned that positive SCA binds negative pectin moieties to form an adhesive matrix between pollen tube walls and surfaces of the TTE (Mollet *et al.*, 2000). The fact that this charge interaction is critical to make the SCA–pectin matrix functionally adhesive suggests that SCA acts as a lectin-like molecule (Mollet *et al.*, 2007). In addition, it was found that SCA produced in the pistil enters the pollen tube tip through an endocytotic pathway (Kim *et al.*, 2006) and may act as a signal for pollen tube tip growth (Chae *et al.*, 2007). SCA is also involved in pollen tube guidance by facilitating chemotrophic activity of chemocyanin, a small (~9.8 kDa) secreted molecule from the lily stigma (Kim *et al.*, 2003). SCA may have diverse binding partners for cell–cell communications between growing pollen tubes and the pistil TTE.

In addition to lily SCA, several LTPs from crop plants have been studied to determine their biological functions using biochemistry or gene overexpression. These functions include antifungal activity for Ace-AMP1 from onion seeds (Phillippe *et al.*, 1995), antibacterial activity for barley LTP4 (Molina and GarciaOlmedo, 1997), and *in vitro* cell wall-loosening activity for tobacco LTP2 (Nieuwland *et al.*, 2005). In recent years, genetic approaches revealed several new functions of LTP-like molecules in *Arabidopsis*. Defective in induced resistance 1 (DIR1) and Azelaic acid induced 1 were shown to play roles in plant defence (Maldonado *et al.*, 2002; Jung *et al.*, 2009). Glycosylphosphatidylinositol-anchored LTP 1 (LTPG1) was shown to function in cuticular wax deposition (DeBono *et al.*, 2009). More recently, a study of a gain-of-function mutant for *Arabidopsis* LTP5, an SCA-like molecule, revealed a role for LTPs in pollen tube tip growth and seed formation (Chae *et al.*, 2009).

There is a large literature on plant LTPs, but only a few publications on expression patterns of *Arabidopsis* LTPs (Thoma *et al.*, 1994; Clark and Bohnert, 1999; Arondel *et al.*, 2000). *Arabidopsis* has conventional plant LTPs as a small multigene family (Arondel *et al.*, 2000). Their extracellular presence negated their initially assumed function in intracellular lipid transfer (Thoma *et al.*, 1993). A broad look at gene expression patterns will help to lay a foundation for further study of these small extracellular matrix (ECM) proteins. In this paper, it is suggested that *Arabidopsis* LTP5, an SCA-like LTP, is multifunctional in plant growth and organ development, based upon examination of *ltp5-1* plants. Phylogenetic analysis and homology modelling-based electrostatic similarity index (ESI) clustering showed that SCA-like *Arabidopsis* LTPs co-evolved with conventional plant LTPs. β-Glucuronidase (GUS) analysis of the *SCA*-like *Arabidopsis LTP* genes sheds light on their multifunctional roles.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (ecotype Columbia) plants were grown in a growth chamber in the Department of Botany and Plant Sciences at the University of California, Riverside. The *ltp5-1* T-DNA insertion line (SALK_104674) was obtained from Ohio State University ABRC, USA (Alonso *et al.*, 2003) and examined for mutant phenotypes in plant growth. Seeds were grown to 6-week-old plants in soil (Sunshine Mix #1, Sun Gro Horticulture) at 22 °C under a 16 h light/8 h dark photoperiod (200 $\mu Em^{-2}\ s^{-1}$). For examination during seedling development, *ltp5-1* seeds were grown in solid MS medium under normal growth conditions up to 10 d after germination.

Phylogenetic analysis of plant LTPs

The amino acid sequences of 104 *Arabidopsis* LTPs were aligned with 29 plant LTPs from other species using default parameters (an open gap penalty of 10 and an extended gap penalty of 0.1 in pairwise alignments, an extended gap penalty of 0.2 in the multiple alignment, and a delay divergent setting of 30%; Gonnet 250 protein weight matrix) of ClustalX 2.0.10 (Thompson *et al.*, 1997), and the aligned sequences were analysed using PAUP* 4.0 (Swofford, 2003). Pairwise amino acid divergence was calculated and a Neighbor–Joining (NJ; Saitou and Nei, 1987) tree was constructed using PAUP*. Support for groups was examined by 1000 bootstrap replicates (Felsenstein, 1985). Parsimony analysis was also performed using the heuristic search option with tree–bisection–reconnection (TBR) branch swapping and the multiple parsimony (MULPARS) option on. A total of 591 aligned characters were used for maximum parsimony (MP) analysis. There are 84 constant characters, 222 variable but parsimony uninformative characters, and 285 parsimony informative characters in the data matrix. The heuristic search found 232 equally most parsimonious trees (tree length=7584, consistency index=0.4248, retention index=0.5050), and tree topologies were very similar to the NJ tree. Therefore, the discussion was based on the strict consensus tree of MP analysis.

Gene names of SCA-like Arabidopsis *LTPs shown in this study*

LTP1, At2g38540; *LTP2*, At2g38530; *LTP3*, At5g59320; *LTP4*, At5g59310; *LTP5*, At3g51600; *LTP6*, At3g08770; *LTP7*, At2g15050; *LTP8*, At2g18370; *LTP9*, At2g15325; *LTP10*, At5g01870; *LTP11*, At4g33355; *LTP12*, At3g51590.

Homology modelling-based ESI clustering of Arabidopsis *LTPs*

Structural homology modelling for SCAs, tobacco LTP2, and *Arabidopsis* LTPs was performed using the SWISS-MODEL web server (http://swissmodel.expasy.org) (Schwede *et al.*, 2003). The crystal structure of maize LTP with Protein Data Bank (PDB) (Berman *et al.*, 2000) code 1MZL (Shin *et al.*, 1995) was used as a structural homology template (~56% identity to SCA, a lily LTP) (Chae *et al.*, 2007). The ESI clustering was performed using the computational protocol AESOP (Analysis of Electrostatic Similarities Of Proteins) (Kieslich *et al.*, 2010). The spatial distributions of electrostatic potentials were calculated for all generated SCA-like *Arabidopsis* LTP homology models and the crystal structure of maize LTP (PDB Code 1MZL) using the

Adaptive Poisson–Boltzmann Solver (APBS) (Baker *et al.*, 2001). Prior to the calculations, the three-dimensional coordinates for the 19 homology models and 1MZL were converted from the PDB format to the PQR format using PDB2PQR (Dolinsky *et al.*, 2004). The generated PQR files incorporated van der Waals radii and partial charges obtained from the PARSE forcefield (Sitkoff *et al.*, 1994). The linearized Poisson–Boltzmann equation (Lu *et al.*, 2008) was solved in a 129^3 grid with the same dimensions for all protein structures. After superimposition of their three-dimensional coordinates, the structures were centred in an identical way in the grid. A probe sphere with radius of 1.4 Å, representing a water molecule, was used to determine the protein molecular surface. An internal protein dielectric coefficient of 2 and solvent dielectric coefficient of 78.54 were used. A probe sphere with radius of 2.0 Å, representing a monovalent counterion, was used to determine the ion accessibility surface. An ionic strength corresponding to 50 mM salt concentration was used, which is the physiological ionic strength for SCAs with respect to pectin binding. SCAs can bind the pectin matrix until 50 mM NaCl is added (Fig. 8 in Chae *et al.*, 2007). The temperature was set to 298K and pH to 7.0. The validity of the calculations extends to lower pH values in the range of 4–7. The normal pH of the cell wall is neutral. However, according to the acid-growth hypothesis, this can be lowered to 4.5 by an activated proton pump in the plasma membrane. The low pH can facilitate activity of an ECM protein such as expansin (Cosgrove, 2005). In a previous study of SCA proteins (Chae *et al.*, 2007), the calculated titration curves of SCAs showed plateau regions of about the same net charge in the pH range of 4 to ~7. Pair-wise comparisons between all of the generated electrostatic potentials were calculated, based on electrostatic potential values outside the 2 Å ion-accessibility surface. The normalized scalar product ESI (Wade *et al.*, 2001) was used, defined by $ESI_{a,b} = \frac{\sum_{i,j,k} \phi_a(i,j,k)\phi_b(i,j,k)}{\sum_{i,j,k} (\phi_a(i,j,k))^2 + \sum_{i,j,k} \phi_b(i,j,k))^2}$, where $\phi(i,j,k)$ is the electrostatic potential value at grid point (i,j,k) and $\sum_{i,j,k} \phi_a(i,j,k)\phi_b(i,j,k)$, $\sum_{i,j,k} \phi_a(i,j,k))^2$, and $\sum_{i,j,k} \phi_b(i,j,k))^2$ are scalar products for proteins a and b. A 20×20 distance matrix was generated, where 20 refers to the number of proteins used for clustering. The distance $D_{a,b}$ between proteins *a* and *b* was defined by $D_{a,b} = \sqrt{\frac{1}{2} - ESI_{a,b}}$. The generated distance matrix was imported into Matlab (The Mathworks, Inc., Natick, MA, USA), where hierarchical clustering was performed using average linkage. Isopotential contours were generated to visualize the spatial distributions of electrostatic potential using Chimera (UCSF) (Pettersen *et al.*, 2004).

Transgenic plants and GUS analysis

The promoter sequences of LTPs were cloned from *A. thaliana* (ecotype Columbia) genomic DNAs using PCR amplification (Supplementary Table S2 available at *JXB* online). The LTP_{pro}:GUS plasmid constructs were introduced into *Arabidopsis* (ecotype Columbia) plants using *Agrobacterium tumefaciens* strain GV3101 with a floral dip method (Clough and Bent, 1998). Transgenic plants were selected on soil by spraying with a 1000-fold dilution of Finale (AgrEvo Environmental Health, Montvale, NJ, USA; BASTA). Spraying was initiated at 10 days after germination (DAG) and was performed three times every 2 d. For $LTP5_{pro}$:GUS, plants were selected on solid MS medium containing 30 mg ml^{-1} kanamycin. Homozygous LTP_{pro}:GUS transgenic lines were obtained at the T_3 generation and further analysed for gene expression in developing seedlings, flowers, and in vegetative tissues in response to growth stresses such as dark, high salt (300 mM NaCl), and drought [20% polyethylene glycol 8000 (PEG8000)].

LTP_{pro}:GUS seedlings and flowers were incubated for 16 h at 37 °C in a GUS reaction buffer: 10 mM EDTA, 100 mM sodium phosphate, pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 0.1% Triton X-100 with 1 mM 5-bromo-4-chloro-β-D-glucuronide. GUS-stained tissues were decolourized with 70% EtOH three times in a 12 h incubation at 37 °C. Developed GUS signals were examined under a Leica dissecting microscope. For examination of gene expression in response to abiotic stresses, all seedlings were germinated on sterilized filter paper soaked with liquid MS under normal growth conditions for 2 d. Young seedlings were then moved to new MS plates with each stress condition and grown further for 3 d. GUS reaction was performed for only 5 h to minimize saturation of GUS signals.

Results

ltp5-1*, a gain-of-function mutant for Arabidopsis* LTP5*, showed abnormal vegetative growth*

Lily SCA, a small secreted plant LTP, is known to be involved in plant compatible pollination (Lord, 2003). *Arabidopsis* LTP5, an SCA homologue, was recently shown to be present at a low level in pollen and to have a biological role in pollen tube tip growth and seed formation (Chae *et al.*, 2009). Here, it is reported that *Arabidopsis LTP5* gene transcripts were also found ubiquitously in almost all vegetative and floral tissues (Supplementary Fig. S1 at *JXB* online) and its gain-of-function mutation, *ltp5-1* (Chae *et al.*, 2009), resulted in significantly disturbed plant growth (Figs 1–3). The heterozygous mutant plant (*ltp5-1*/+) did not show any abnormality, suggesting that the *ltp5-1* mutation acts recessively in plant growth, unlike its dominant effect on reproduction.

Six-week-old *ltp5-1* plants had a significantly shorter stature, compared with the wild type (Fig. 1A). The heights of *ltp5-1* plants were about half that of the wild type (Supplementary Table S1 at *JXB* online). In addition, *ltp5-1* plants had a larger number of nodes and axillary branches in the primary stem, and their internode lengths were significantly shorter than those of wild-type plants (Supplementary Table S1). To evaluate plant growth precisely, both *ltp5-1* (n=19) and wild-type (n=9) plants were measured for their heights for a period of 12 d after the first flower opened (day 1, stage 6.0) (Boyes *et al.*, 2001) (Fig. 1B). Both *ltp5-1* (white bars) and wild-type (grey bars) plants showed quite similar statures at day 1. However, *ltp5-1* plants started to show a delayed growth of the primary axis from day 2, compared with wild-type plants, and the difference in stature between them was ~2-fold at day 5 (Fig. 1B, C). Up to day 5, *ltp5-1* and wild-type plants grew ~2 cm tall and 3–4 cm tall per day, respectively. The difference in stature at day 5 (~10 cm) was maintained until day 12 at plant maturity (stage 6.5, 50% flowers opened) (Boyes *et al.*, 2001) (Fig. 1B).

Delayed growth defects of *ltp5-1* were also found in seedling development. In normal growth conditions, *ltp5-1* showed small cotyledons and a short primary root at 3 DAG, compared with the wild type and *ltp5-1*/+ (Fig. 2A). The defect in primary root growth of *ltp5-1* was shown to be consistent for ~10 DAG (Supplementary Fig. S2 at *JXB*

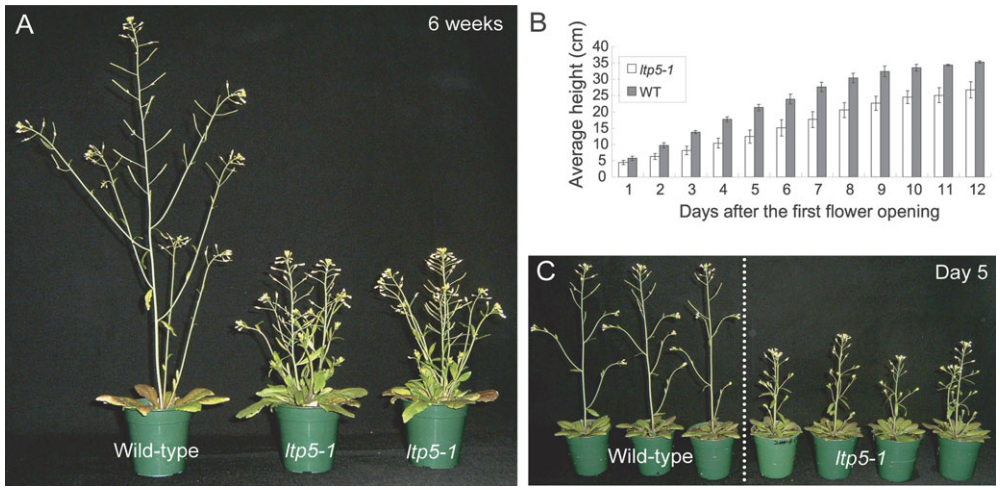

Fig. 1. Delayed plant growth and reduced stature of *ltp5-1*. (A) Six-week-old wild-type and *ltp5-1* plants. (B) Growth rates of wild-type and *ltp5-1* plants from stage 6.0 (day 1, first flower opening) to 6.5 (day 12, 50% of flowers opened) (Boyes *et al.*, 2001). Bar graphs represent average heights obtained from nine wild-type (grey) and 19 *ltp5-1* (white) plants. Data are shown as the mean ±SD. (C) Wild-type and *ltp5-1* plants at day 5 in B. Plants were grown *in vivo* in soil.

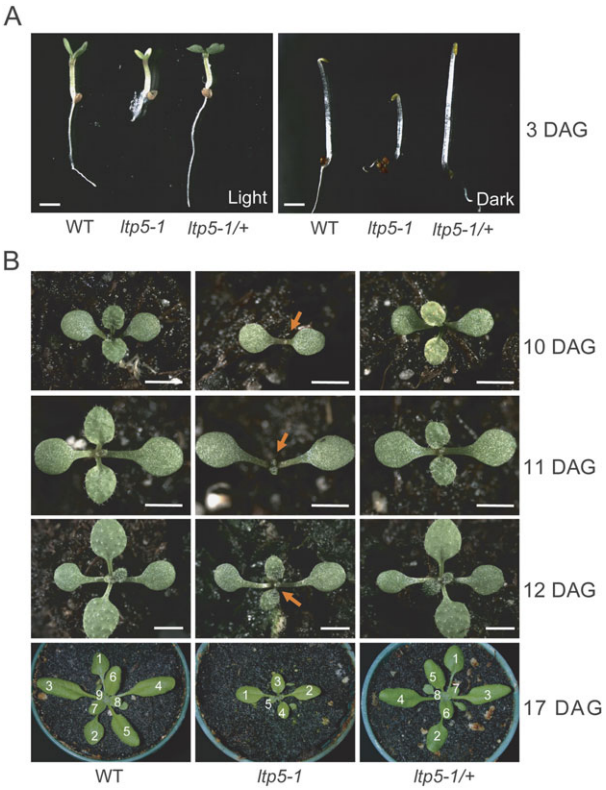

Fig. 2. Delayed seedling growth and abnormality in true leaf formation of *ltp5-1*. (A) Wild-type, *ltp5-1*, and *ltp5-1*/+ seedlings were grown *in vitro* on solid MS medium for 3 d in normal light conditions and in dark conditions, respectively. DAG, days after seed germination. Scale bars=2 mm. (B) Wild-type, *ltp5-1*, and *ltp5-1*/+ seedlings (n=20 for each) were grown *in vivo* in soil under normal growth conditions. Leaf generation was examined at 10–12 DAG. The numbers of leaves were counted at 17 DAG. Scale bars=2 mm.

online). In complete dark conditions, wild-type and *ltp5-1*/+ seedlings had typically elongated hypocotyls, but the hypocotyls of *ltp5-1* seedlings remained significantly shorter (Fig. 2A). Leaf development was also examined in *ltp5-1* (Fig. 2B). Both wild-type and *ltp5-1*/+ seedlings were shown to have mature leaves at 10 DAG. However, this was not the case for *ltp5-1* until 12 DAG. This delayed leaf growth in *ltp5-1* resulted in significantly low numbers of leaves, compared with those of the wild type and *ltp5-1*/+ at 17 DAG.

In addition, a certain level of abnormality was found in the organ development of mature *ltp5-1* plants (Fig. 3). About 40% of the examined *ltp5-1* plants (n=50) showed abnormal organ fusions such as two leaves congenitally fused onto a single petiole (Fig. 3B, arrowheads, C) or a single plant having two inflorescences (Fig. 3D, arrows) fused into a single primary axis (Fig. 3E, arrow). Some showed two primary stems separately emerging from the shoot apical meristem at the bolting stage (Fig. 3F). Fused petioles (Fig. 3G) and supernumerary branches at a single node (Fig. 3H) were often found. Each type of tissue fusion occurred randomly in *ltp5-1* plants: some displayed all forms of mutant phenotype and others showed only one or two.

Phylogenetic analysis shows that SCA-like Arabidopsis *LTPs are classified with conventional plant LTPs*

In *Arabidopsis*, there are >100 molecules annotated as an LTP or putative LTP (Chae *et al.*, 2009). This high number may reflect redundancy in the *Arabidopsis* LTP family, which has been an obstacle in studies of biological function using a genetic approach. Here, phylogenetic analysis was performed to learn what LTPs in *A. thaliana* (ecotype Columbia; Brassicaceae) are most closely related to conventional LTPs, previously known from diverse plant species of monocots and eudicots: *Hordeum vulgare* (barley; Poaceae),

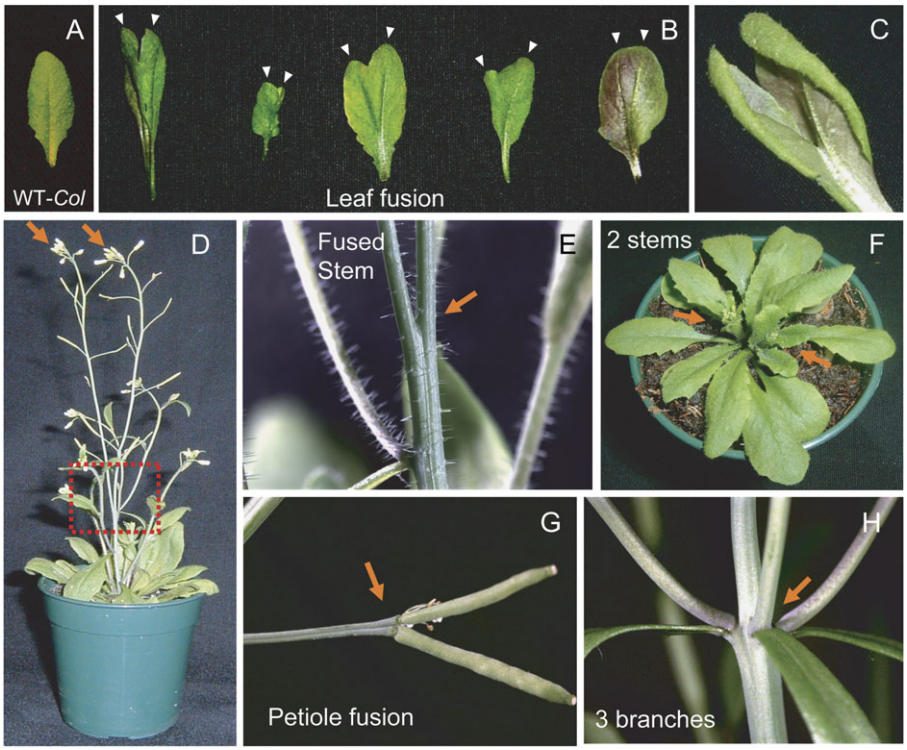

Fig. 3. Abnormal tissue fusions and supernumerary branching of *ltp5-1*. (A) A 5-week-old wild-type rosette leaf. (B) Two leaves (arrowheads) congenitally fused onto a single petiole. (C) Close-up of a fused leaf. (D) Two inflorescences (arrows) fused into a single primary axis. (E) Magnified view of the area in D. (F) Two primary stems (arrows) emerging at the bolting stage. (G) Two congenitally fused pedicels (arrow). (H) Three axillary branches (arrow) at a node. Plants were grown *in vivo* in soil.

Triticum aestivum (bread wheat; Poaceae), *Ricinus communis* (castor bean; Euphorbiaceae), *Vigna unguiculata* (cow pea; Fabaceae), *Oryza sativa* (rice; Poaceae), *Zea mays* (maize; Poaceae), *Nicotiana tabacum* (tobacco; Solanaceae), *Spinacia oleracea* (spinach; Amaranthaceae), *Prunus armenianca* (apricot; Rosaceae), *Daucus carota* (carrot; Apiaceae), *Brassica napus* (rapeseed; Brassicaceae), and *Lilium longiflorum* (lily; Liliaceae) (Fig. 4).

In the phylogenetic analysis, four important major groups can be identified based on the strict consensus tree of MP analysis. First, group I showed that lily SCA1 (Park *et al.*, 2002) and 13 *Arabidopsis* LTPs (some previously classified as SCA-like molecules) (Chae *et al.*, 2009) were related to most of the conventional plant LTPs. Lily SCA1 was sister to rice LTP5, and this group is, in turn, sister to other core eudicot LTPs such as tobacco, spinach, apricot, and carrot. This subgroup was shown to be related to other core eudicot *Arabidopsis* and rapeseed LTPs in group I. Groups II and III were separated mostly as *Arabidopsis* LTP-like proteins by themselves. Interestingly, these two groups contain DIR1 (Maldonado *et al.*, 2002), LTPG1 (DeBono *et al.*, 2009), and AZI1 (Jung *et al.*, 2009), which were previously studied for their biological functions using genetic mutants. They are all very different from conventional LTPs in their protein sizes or pIs, and therefore in phylogenetic relationships (Fig. 4). In addition to these three, *Arabidopsis* LTP5, an SCA-like LTP in group I, has been studied for a biological role using a gain-of-function mutant (Chae *et al.*, 2009), the only conventional LTP mutant with a phenotype. In group IV, four *Arabidopsis* LTP-like proteins were classified as closely related to several conventional LTPs from bread wheat, barley, and cowpea. Phylogenetic analysis suggests that plant LTP duplications and subsequent functional diversification pre-dates the divergence between monocots and eudicots.

Homology modelling-based ESI clustering of Arabidopsis *LTPs*

The electrostatic potential is important in charge interactions between two molecules, especially when they are positioned in close proximity, as are SCA and pectins in the ECM of the lily pistil TTE. The fact that an ionic interaction between a plant LTP and pectic polysaccharides is critical for formation of a biologically active ECM (Lord, 2001) prompted the performance of homology modelling-based hierarchical clustering analysis of spatial distributions of electrostatic potentials according to the ESI. Two lily SCAs, 12 *Arabidopsis* LTPs from group I, four *Arabidopsis* LTPs from group IV, maize LTP (Shin *et al.*, 1995), and tobacco LTP2 were used for the analysis (Fig. 5). The ESI clustering results were visualized in a dendrogram, with the corresponding isopotential contours shown at four axial rotations for each LTP.

The resulting dendrogram consists of two primary clusters: (i) containing the three LTPs with an overall negative charge and (ii) containing the remaining 17 LTPs

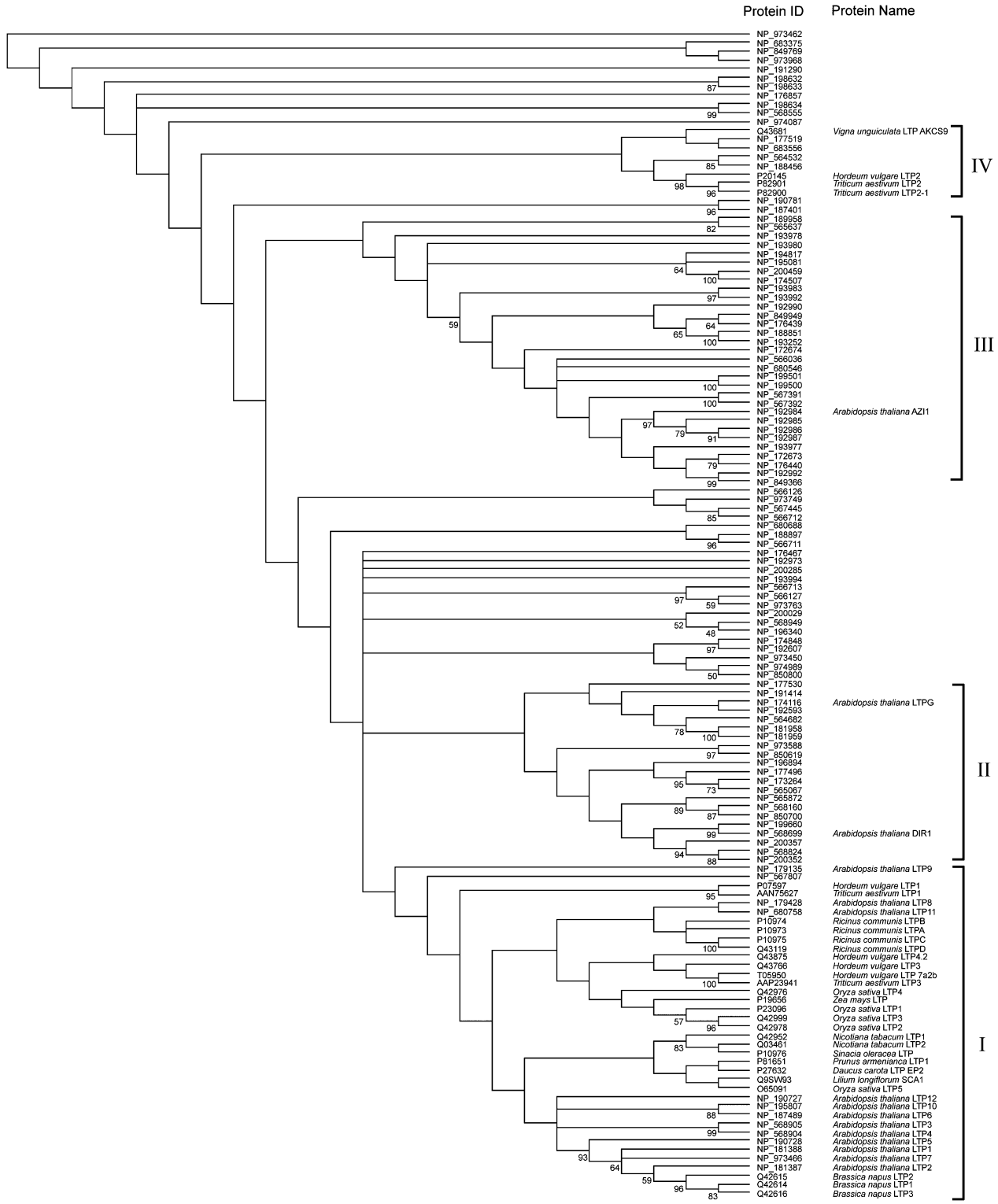

Fig. 4. Phylogenetic relationships of plant LTP and LTP-like proteins. This tree represents a strict consensus tree of 232 equally most parsimonious trees based on maximum parsimony analysis. The values on the branches indicate the number of bootstrap replicates supporting the branch. Only bootstrap replication values >50 are shown.

with an overall positive charge. Of the proteins of cluster (ii), seven *Arabidopsis* LTPs from phylogenetic group I (Fig. 4) were predicted to have SCA-like properties (Fig. 5, asterisk). The spatial distributions of the electrostatic potential of *Arabidopsis* LTP3 and LTP4 were most similar to those of SCA and maize LTP. *Arabidopsis* LTP1 and LTP2 belong to the SCA-like subset and were clustered with tobacco LTP2. Compared with the other LTPs, Arabidopsis LTP5, 7, and 10 showed very high positive charges (over +10), with that of LTP5 being the highest (+14). Some

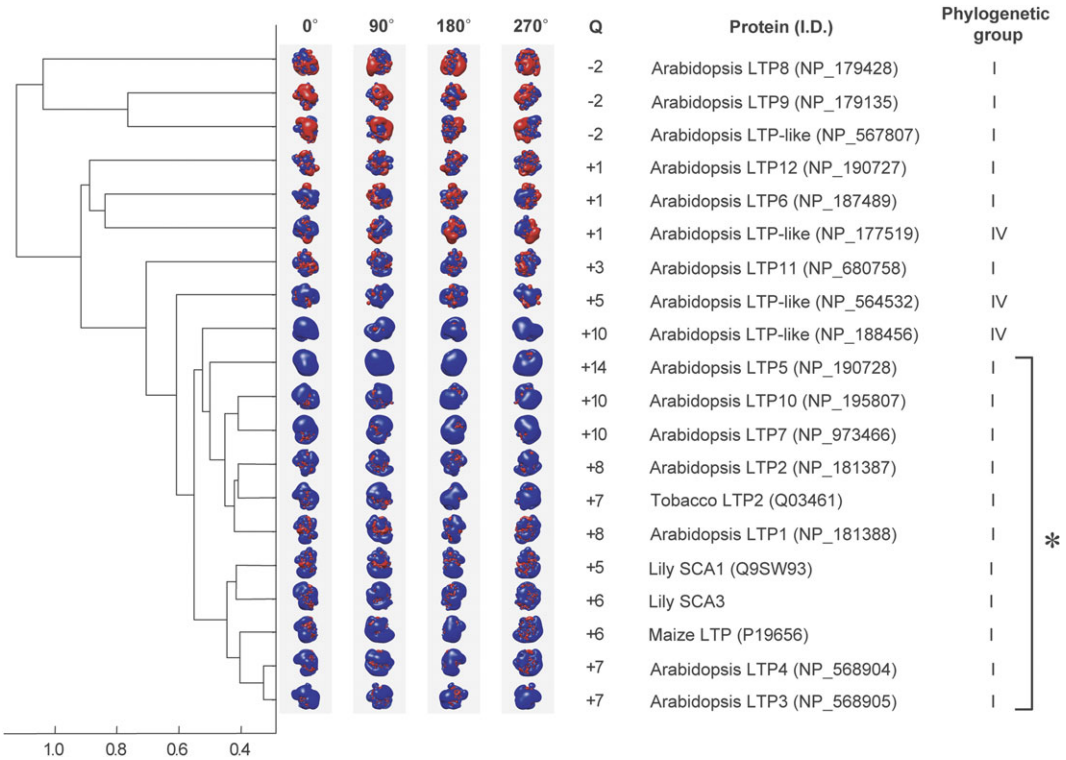

Fig. 5. ESI clustering of *Arabidopsis* LTPs. A dendrogram for the hierarchical clustering of the spatial distributions of electrostatic potentials for the generated LTP homology models is presented. Spatial distributions of electrostatic potentials are shown in the form of isopotential contours, plotted at ±1 k_BT/e. The colour code is blue for positive and red for negative electrostatic potential. Four different orientations are shown at rotations of 0, 90, 180, and 270 ° around the vertical axis. The net charge (Q) of each protein is marked. The SCA-like *Arabidopsis* LTPs were classified by the clustering of the spatial distributions of their electrostatic potentials in a supercluster marked by an asterisk.

Arabidopsis LTPs from phylogenetic group I were shown to have relatively low positive charges (LTP6, 11, and 12) or were even negative (LTP8 and 9).

Expression patterns of the SCA-*like* Arabidopsis LTP *genes in seedling growth*

To understand expression patterns of *SCA*-like *LTP* genes in *Arabidopsis* plants, promoter–GUS fusion lines were generated for seven *Arabidopsis LTP* genes (*LTP1–7*) (Materials and methods, and Supplementary Table S2 at *JXB* online). The homozygous LTP_{pro}:GUS seedlings at the T_3 generation were grown in MS–agar plates under normal plant growth conditions and examined for GUS expression levels and patterns for 10 d from seed germination. The GUS analysis showed that the overall expression of *SCA*-like *LTP* genes was dynamic and redundantly overlapping in various tissues, with a certain level of diversity (Fig. 6, Table 1, and Supplementary Figs S3, S4 at *JXB* online).

LTP1, *LTP5*, and *LTP7* have consistently strong gene expression levels and patterns in the shoot apex (Fig. 6A, E, and Supplementary Fig. S4A at *JXB* online). However, gene expression of *LTP2* in the shoot apex at 3 DAG became concentrated in leaf petioles at 8 DAG (Fig. 6B). In contrast, *LTP3* expression became specific to stipules (Fig. 6C). *LTP4* expression was found in the shoot apex only after 8 DAG (Fig. 6D).

Various gene expression patterns were also found in the cotyledons at 5 DAG and in the young leaves at 10 DAG for *LTP1*, *LTP4*, *LTP5*, *LTP6*, and *LTP7* (Supplementary Fig. S4B at *JXB* online). *LTP1* was present in the cotyledons (Fig. 6F), except for guard cells, and in young leaves and the trichomes. In contrast, *LTP4* gene expression was highly specific to guard cells on the cotyledons (Fig. 6G), but was not found in trichomes of leaves. For *LTP5*, *LTP6*, and *LTP7*, a high level of gene expression was found broadly in all tissues of the cotyledon and leaves (Fig. 6H). Also, only *LTP6* was shown to be highly specific to trichomes on the young leaves at 10 DAG (Fig. 6I).

In the developing root system, all examined *SCA*-like *LTP* genes, except for *LTP2* and *LTP7*, were diversified in their gene expression (Supplementary Fig. S3 at *JXB* online). Only *LTP1*, *LTP3*, and *LTP5* were highly expressed at the tip of the primary root at 1 DAG (Fig. 6J, K). *LTP1*, *LTP3*, and *LTP4* genes were specifically expressed at the lateral root initiation sites from 5 DAG (Fig. 6L), but their expression occurred in all parts of the lateral root during its growth (Fig. 6M). *LTP5* expression occurred in a broad area where lateral roots initiate (Supplementary Fig. S3), but then became highly specific to the tip of growing lateral roots (Fig. 6N). The *LTP6* expression pattern was similar to that of *LTP5* in lateral root tips (data not shown), but it was not found at the lateral root initiation sites.

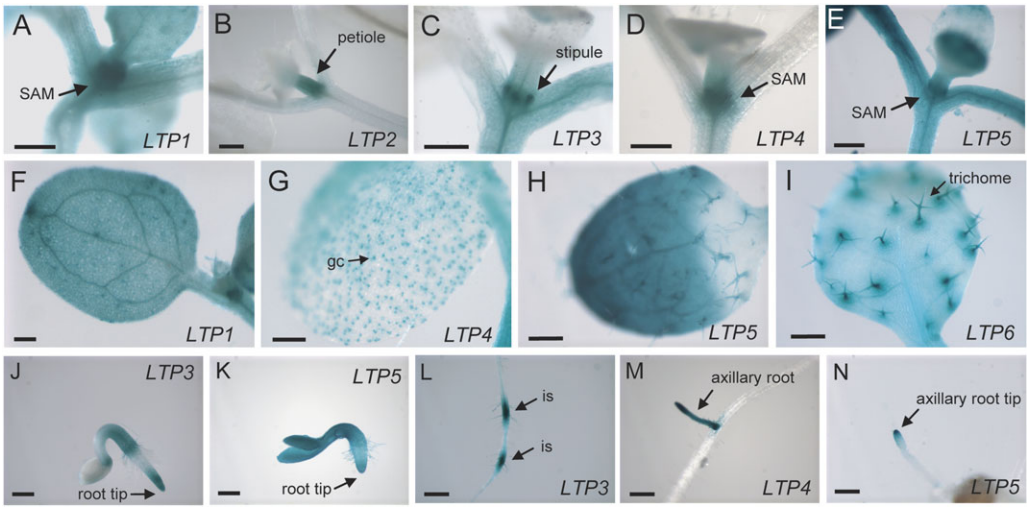

Fig. 6. Expression patterns of *SCA*-like *Arabidopsis LTP* genes in seedling tissues. (A–E) Diversified gene expression patterns of *LTP* genes in the various tissues (arrows) of the shoot apex at 8 DAG. Scale bars (A–E)=500 μm. (F) Ubiquitous gene expression of *LTP1* in the cotyledon at 5 DAG. (G) Specific gene expression of *LTP4* in the guard cells (arrow) of the cotyledon at 5 DAG. (H) A high level of *LTP5* gene expression in the true leaf at 10 DAG. (I) Specific gene expression of *LTP6* in the trichomes (arrow) in the true leaf at 10 DAG. (J) A high level of *LTP3* gene expression in the root tip (arrow) at 1 DAG. (K) Overall presence of the *LTP5* gene including the root tip (arrow) at 1 DAG. (L) *LTP3* gene expression in the axillary root initiation sites (arrows) at 5 DAG. (M) A high level of *LTP4* gene expression in the growing axillary root (arrow) at 8 DAG. (N) Specific gene expression of *LTP5* in the axillary root tip (arrow) at 8 DAG. Scale bars (F–N)=200 μm. Plants were grown *in vitro* on solid MS medium. GUS signals were developed for 16 h. SAM, shoot apical meristem; gc, guard cell; is, initiation site for the axillary root.

Table 1. An overview of SCA-like *Arabidopsis LTP* gene expression patterns examined in *Arabidopsis* LTP_{pro}:GUS plants

	Vegetative tissues (seedling)						Floral tissues				
	Root tip	Lateral roots is/tip	hyp	Shoot apex	Cotyledons bl/gc/v	First leaves bl/v/tr	ant/fl	Pollen[a]	Pistil st/sy/ov	se	pe
LTP1	+	+/+	+	+	+/–/+	+/+/+	+/+	ND	+/+/–	+	+
LTP2	–	–/–	+	+	–/–/–	–/–/–	–/–	–	–/–/–	–	–
LTP3	+	+/+	+	+	–/–/–	–/–/–	–/+	–	–/–/+	+	–
LTP4	–	+/+	+	+	–/+/–	+/–/–	–/–	–	–/+/–	+	–
LTP5	+	+/+	+	+	+/+/+	+/+/+	–/–	+	–/+/–	+	+
LTP6	–	–/+	+	+	+/+/+	–/–/+	–/+	–	–/+/+	+	–
LTP7	–	–/–	+	+	+/+/+	+/+/+	+/+	ND	–/–/–	–	–

LTP_{pro}:GUS seedlings were grown *in vitro* in MS–agar plates in normal growth conditions, and gene expression patterns in vegetative tissues were examined for 10 d after germination. Gene expression patterns in floral tissues were examined in mature open flowers at stage 13 (Smyth *et al.*, 1990). Plus (+) represents gene expression in each line and minus (–) indicates no expression.
Is, initiation sites for lateral roots; hyp, hypocotyl; bl, blade; gc, guard cells; v, veins; tr, trichomes; ant, anthers; fl, filaments; st, stigma; sy, style; ov, ovules; se, sepal; pe, petal;
[a] Gene expression in pollen was based upon previous work (Chae *et al.*, 2009); ND, not determined.

Expression patterns of SCA-*like* Arabidopsis LTP *genes were highly diversified in floral reproductive tissues*

In addition to vegetative tissues, *SCA*-like *Arabidopsis LTP* gene expression patterns were examined in floral tissues (Fig. 7). *LTP1* was expressed in almost all floral tissues, with the highest level in the stigma and the style (Fig. 7A). However, *LTP2* expression was found only in the receptacle (Fig. 7B). *LTP3* was present mainly in the top and the base of the ovary (Fig. 7C). Interestingly, the dissected pistil showed specific *LTP3* expression in the ovules (Fig. 7H). *LTP4* was abundantly expressed in the style and sepals (Fig. 7D). *LTP5* expression levels were very low in the style, petals, and sepals (Fig. 5E). This weak level confirms a previous result showing that only with a 5 d GUS reaction was it possible to show *LTP5* gene expression in pollen and the transmitting tract (TT) in the pistil (Chae *et al.*, 2009). For *LTP6*, gene expression was found in the style, anther filaments, and sepals (Fig. 7F). *LTP6* also showed significant gene expression in the ovules (Fig. 7I), very similar to *LTP3*. *LTP7* was found only in anther filaments and at the top of the ovary (Fig. 7G).

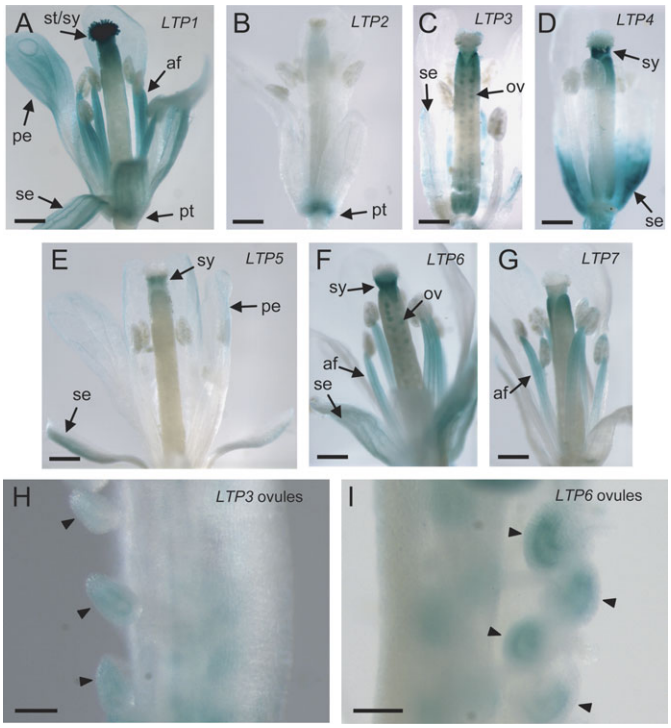

Fig. 7. Highly diversified expression of *SCA*-like *Arabidopsis LTP* genes in mature floral tissues. (A) Broad gene expression of *LTP1* in overall tissues (arrows). (B) Specific gene expression of *LTP2* in the petiole (arrow). (C) *LTP3* gene expression in the ovules and the sepals. (D) *LTP4* gene expression in the style and the sepals. (E) Low levels of *LTP5* gene expression in the style, the petals, and the sepals (arrows). (F) *LTP6* gene expression in the style, the ovules, anther filaments, and the sepals (arrows). (G) *LTP7* was found mainly in anther filaments. Scale bars (A–G)=400 μm. (H and I) Specific gene expression of *LTP3* (H) and *LTP6* (I) in the ovules (arrowheads). Scale bars (H and I)=50 μm. Plants were grown *in vivo* in soil. GUS signals were developed for 16 h. st, stigma; sy, style; ov, ovule; af, anther filament; pe, petal; se, sepal; pt, petiole.

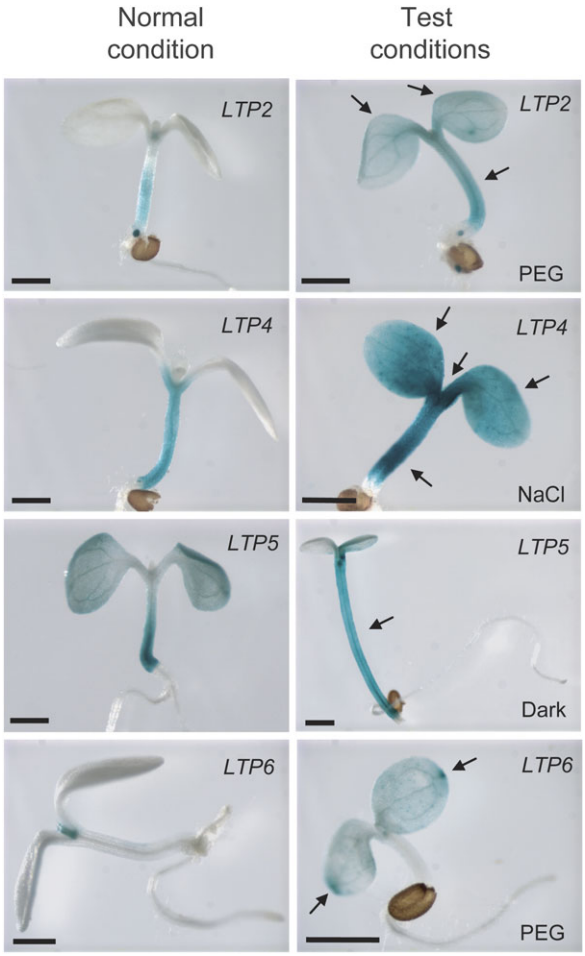

Fig. 8. Various responses in expression of *SCA*-like *Arabidopsis LTP* genes to stress conditions. Two-day-old seedlings were moved to each stress condition (dark, 20% PEG, or 300 mM NaCl) and grown further for 3 d. Plants were grown *in vitro* on the filter paper soaked with liquid MS medium. GUS signals were developed for 5 h. Arrows indicate specific tissues where the expression of each gene was significantly increased in the test conditions compared with normal conditions. Scale bars=500 μm.

Some SCA-*like* Arabidopsis LTP *genes are responsive to abiotic stresses*

Plant LTPs are also known as pathogenesis-related (PR) peptides (Sels *et al.*, 2008). *LTP* gene expression is often increased by various external stresses, hormones, and pathogen infection (Garcia-Olmedo *et al.*, 1995; Arondel *et al.*, 2000; Jung *et al.*, 2003; Trevor and Jocelyn, 2008), suggesting a role in plant defence and abiotic stress responses. Here, gene expression patterns were evaluated in LTP_{pro}:GUS seedlings in response to dark, drought (20% PEG8000), and high salt (300 mM NaCl) conditions (Fig. 8, and Supplementary Fig. S5 at *JXB* online). Overall, *LTP1*, *LTP3*, and *LTP7* did not show significant changes in gene expression patterns or levels in the test conditions, compared with controls in normal growth conditions. However, *LTP2* levels, expressed only in the hypocotyl at a low level in normal growth conditions, became strongly expressed in all above-ground tissues in response to PEG treatment. For *LTP4*, broad gene expression patterns in the hypocotyl became very concentrated to the base under drought stress and the level was highly increased in all above-ground tissues in response to salt stress. *LTP5* gene expression levels became stronger in the hypocotyl in response to dark treatment. For *LTP6*, gene expression levels were increased in the cotyledons, especially at the margins including the hydathode, in response to drought stress.

Discussion

SCA-like Arabidopsis *LTPs are multifunctional*

Small, secreted peptides play crucial roles in diverse signalling events in plant development, together with their membrane receptors: CLAVATA3 and CLAVATA1 in *Arabidopsis* meristem identity, *S*-locus cysteine-rich (SCR) peptide and *S*-locus receptor kinase (SRK) in the self-incompatibility (SI) reaction of *Brassica* plants (Matsubayashi *et al.*, 2001;

Matsubayashi, 2003), and EPIDERMAL PATTERNING FACTOR1 (EPF1) in stomata cell differentiation (Hara *et al.*, 2007). In addition, several small, secreted cysteine-rich peptides (CRPs) were also proposed to act as signalling cues in cell–cell communication in fertilization (Higashiyama, 2010).

Plant LTPs are small, secreted CRPs (Kader, 1996). SCA, a lily LTP, is abundantly found in the pistil TTE and functions in pollen tube adhesion-mediated guidance (Lord, 2003). The biological role of plant LTP in compatible pollination was further studied by utilizing a gain-of-function T-DNA mutant (*ltp5-1*) for an SCA homologue in *Arabidopsis* (Chae *et al.*, 2009). *LTP5* was shown to be present in both pollen and the pistil TT, participating in polar tip growth of pollen tubes and fertilized seed formation. The ballooned pollen tube tip of *ltp5-1* is highly similar to those of Rop signalling mutants (Li *et al.*, 1999; Fu *et al.*, 2001; Gu *et al.*, 2006) and its putative upstream receptor kinase (Zhang and McCormick, 2007), suggesting that LTP5 may act as a cue for pollen tube tip growth signalling. Small, secreted peptides are also crucial in polar tip growth of the mating yeast cell (Madden and Snyder, 1998) and in neuronal axon outgrowth (Baker *et al.*, 2006; Stumm and Hollt, 2007).

In addition to a role for *Arabidopsis* LTP5 in sexual reproduction (Chae *et al.*, 2009), the current study reveals that LTP5 is involved in diverse plant growth events. The most obvious features of the *ltp5-1* phenotypes in plant growth are the dwarfed inflorescence stature and the multi-branching at maturity. These suggest that the gain-of-function mutation of LTP5 could be related to a disturbance in apical dominance, by which the actively growing dominant shoot generates a signal to prevent the subordinate shoots from outgrowth (Leyser, 2005). In addition, some *ltp5-1* plants were shown to have two primary shoots that simultaneously appeared at the bolting stage. Leaf generation of *ltp5-1* seedlings was abnormal and severely delayed as well, possibly due to abnormal activity of the shoot apical meristem, where almost all *SCA*-like *LTP* genes examined in the present GUS assay were highly expressed in the shoot apex. The multieffect of *ltp5-1* on plant growth and reproduction suggests that *Arabidopsis* LTP5 may be involved in diverse cell–cell communication signalling events as a secreted CRP, possibly through interaction with various receptors, as was suggested for a mammalian β-defensin, a secreted peptide (Candille *et al.*, 2007; Dorin and Jackson, 2007). Finding a putative binding partner is now necessary for further understanding of LTP-mediated signalling.

SCA-like Arabidopsis *LTPs are conventional plant LTPs*

In this study, two combinatorial approaches were utilized to identify SCA-like *Arabidopsis* LTPs among >100 LTPs and LTP-like proteins (Chae *et al.*, 2009). First, the current phylogenetic study showed that only 13 *Arabidopsis* LTPs (LTP1–12 and a putative LTP, NP_567807) were highly related to conventionally known plant LTPs including lily SCA. These 13 LTPs were in group I. The second approach was to reclassify the phylogenetic group I, based upon isopotential contours using homology modelling-based ESI clustering. Since SCA functions in forming a biologically active ECM in the style through charge interactions with extracellular pectins (Mollet *et al.*, 2000), this approach allowed modelling of how each LTP might interact with a putative binding partner in the extracellular space.

The electrostatic analysis is based on an underlying two-step model for association of excessively and oppositely charged biomolecules, the first step being recognition and the second step being binding (Wu and Morikis, 2006; Zhang and Morikis, 2006; Zhang *et al.*, 2007; Cheung *et al.*, 2010). Recognition is driven by long-range electrostatic interactions which are responsible for the formation of a non-specific weak encounter complex. Binding involves short-range interactions (hydrogen bonds, salt bridges, hydrophobic interactions) which are responsible for the formation of a specific bound complex. Binding also includes entropic effects, involving solvent exclusion from the binding interface and side chain re-arrangement for induced fit complex formation. The recognition step contributes to the association rate constant and the binding step dominates the dissociation rate constant.

The role of positive overall electrostatic potential is to drive the recognition between those LTPs that are excessively positively charged and the negatively charged moieties of various ECM molecules (i.e. pectins, lipids, small secreted proteins, or extracellular motifs of plasma membrane receptors), which leads to complex formation. It is expected that charge variations would contribute to variable binding affinities, with a more positive charge producing a more favourable interaction with pectins. In contrast, it is predicted that a negative net charge or slightly positive net charge with even spatial distributions of positive/negative electrostatic potentials would result in significantly reduced or no binding affinities. It is also noted that the hydrophobic groove running through the protein core (Shin *et al.*, 1995; Gomar *et al.*, 1998; Charvolin *et al.*, 1999; Han *et al.*, 2001; Hamilton, 2004) is where another binding partner may occur.

The current ESI clustering analysis suggested that *Arabidopsis* LTP3 and 4 may have the most SCA-like activity in interacting with other ECM factors. LTP1 and 2 might share a similar mechanism to tobacco LTP2, which was reported to have cell wall-loosening activity (Nieuwland *et al.*, 2005). LTP5 has the highest net positive charge and the strongest electrostatic potential, suggesting that it has a unique mechanism for its biological role. With respect to both phylogenetic and ESI analyses, seven *Arabidopsis* LTPs can be classified as the most SCA-like molecules: LTP1–5, LTP7, and LTP10.

Diverse and redundant expression patterns of SCA-like Arabidopsis LTP *genes suggest their multifunctionality*

The GUS analysis of six *SCA*-like *Arabidopsis LTP* genes showed highly dynamic and redundant gene expression patterns during seedling growth in a spatial–temporal

manner. The dynamicity of gene regulation implies their possible involvement in various transient events that occur during the fast growth of seedlings. Some *LTP* genes overlap in expression, which may reflect functional redundancy in the SCA-like LTP group in *Arabidopsis*. This suggests that abnormalities in *ltp5-1* plant growth may be due to a disturbance in a redundant function of SCA-like LTPs. Nonetheless, some also showed specific patterns in gene expression in a certain growth stage.

In the *Arabidopsis* flower, the expression patterns of *SCA*-like *LTP* genes were more diversified than those in the vegetative tissues. Only *LTP1* was shown to be present abundantly in the stigma, where pollen adheres, hydrates, and germinates. *LTP2* was found only in the receptacle. In the style and ovary where pollen tubes grow, *LTP1*, *LTP4*, *LTP5*, and *LTP6* were redundantly expressed, suggestive of their roles in pollen tube growth and guidance in the TT. Further analysis should be done to detect any involvement of these LTP genes in a developmental process of the pistil tissues. Indeed, the *ltp5-1* pistil did not form a proper seed set, even when wild-type pollen was applied (Chae *et al.*, 2009). This may be due to dominant interference with redundant function of these other *LTP* genes in the pistil. In addition, *LTP5* is solely present at a low level in pollen, contributing to pollen tube cell polarity (Chae *et al.*, 2009). Interestingly, *LTP3* and *LTP6* showed specific gene expression in the ovules, implying a role in pollen tube attraction from the female gametophyte. SCA was shown to facilitate chemotropic activity of chemocyanin in the lily TT (Kim *et al.*, 2003). Plantacyanin, the *Arabidopsis* homologue of lily chemocyanin, was found in the TT and in the ovules (Dong *et al.*, 2005), where its role has not been addressed as yet. It will be interesting to see whether LTP3 or LTP6 can act with plantacyanin in pollen tube attraction to the ovule for fertilization.

The gene expression study suggested that some SCA-like LTPs may also be involved in plant growth during abiotic stresses. *LTP2* expression was significantly induced in the whole seedling by 20% PEG treatment, implying its specific involvement in resistance to drought. *LTP4* and *LTP6* were also shown to be significantly responsive to salinity and drought, respectively. Only *LTP5* gene expression was highly up-regulated in the fast elongating hypocotyl under dark conditions, which may explain a defect in hypocotyl elongation of *ltp5-1* seedlings grown in dark conditions.

Expression patterns of the seven *Arabidopsis LTP* genes were also analysed using the eFP browser (Winter *et al.*, 2007) (Supplementary Fig. S6 at *JXB* online). *LTP1*, *LTP5*, and *LTP7* are shown to be highly abundant in seedlings, similar to the GUS analysis. *LTP1* and *LTP6* are shown to be present most abundantly in the shoot apex, and *LTP1* and *LTP5* in leaves. *LTP2*, *LTP3*, and *LTP4* are not found in most vegetative tissues. *LTP5* is the most abundant gene in the internodes, suggestive of its role in inflorescence stem elongation. The *ltp5-1* plants were shown to have a dwarfed stature with delayed plant growth. In reproductive tissues, all *LTP* genes are found in seeds at early stages of embryo development, with *LTP1*, *LTP3*, *LTP4*, and *LTP6* at the highest levels. In contrast, *LTP2* is shown to be highly abundant in seeds at later stages. This suggests possible roles for these *LTP* genes in the female gametophyte function for embryo development. In the pistil, *LTP1*, *LTP3*, and *LTP6* were shown at high levels and *LTP4* at a significant level. This may be relevant to the abundant GUS levels identified in the stigma/style for *LTP1*, in the ovules for *LTP3* and *LTP6*, and in the style for *LTP4*.

In the abiotic stress map from the eFP browser (Supplementary Fig. S7 at *JXB* online), gene expression of *LTP2*, *LTP3*, *LTP4*, and *LTP6* is induced under osmotic stress at 300 mM mannitol or salinity at 150 mM NaCl. *LTP6* gene expression is very transient, compared with the other genes. These *LTP* genes may be involved in abiotic stress responses. However, the *LTP3* level was not increased in GUS assays in the present test conditions. Interestingly, the eFP browser showed that only *LTP5* gene expression increases in the dark during diurnal growth of *Arabidopsis* plants (Supplementary Fig. S8 at *JXB* online), correlating with the GUS analysis and the effect of *ltp5-1* on hypocotyl elongation.

Concluding remarks

The present study suggests that SCA-like LTPs share some common roles in *Arabidopsis* plant growth. No loss-of-function mutant has been identified for any of the conventional LTPs. Only the *Arabidopsis* gain-of-function mutant, *ltp5-1*, showed a mutant phenotype in both plant growth and sexual reproduction. Although SCA-like *Arabidopsis* LTPs appear to share redundant functions, diversified gene expression patterns in the *SCA*-like *LTP* group in various tissues indicate that each LTP participates in a biological event with its specific presence in a spatial and temporal way, or in different growth conditions. Two duplicated gene pairs, *LTP1*/*LTP2* and *LTP3*/*LTP4*, show very similar phylogenetic relationships and electrostatic potentials of their proteins (net charge +7 or +8). However, their gene expression patterns were very differentiated in floral tissues and in abiotic stress responses. *LTP1* was most abundant in the stigma and the style, *LTP2* in the pedicel, *LTP3* in the ovule, and *LTP4* in the style. *LTP2* gene expression was shown to be related to drought stress responses and that of *LTP4* to salinity. *LTP5* gene expression was specifically up-regulated in fast elongating hypocotyl of the young seedling in dark growth conditions. *LTP5* is the only plant *LTP* gene found in pollen so far. In addition, LTP5 protein was shown to be highly positive in electrostatic potential (net charge +14), indicative of a unique mode of action in interacting with a putative binding partner in the extracellular space. Detailed histological, biochemical, and genetic approaches are needed to elucidate further the biological roles for each *Arabidopsis* LTP.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. RT-PCR analysis of *LTP5* gene expression levels in various tissues in *Arabidopsis*.

Figure S2. Root growth of *ltp5-1* and wild-type *Arabidopsis* seedlings.

Figure S3. Gene expression patterns of *SCA*-like *Arabidopsis LTP* genes during seedling development.

Figure S4. Various gene expression patterns of *SCA*-like *Arabidopsis LTP* genes in developing seedling tissues.

Figure S5. Gene expression patterns of *SCA*-like *Arabidopsis LTP* genes under abiotic stress conditions.

Figure S6. An eFP overview of gene expression levels of *SCA*-like *Arabidopsis LTP* genes in various *Arabidopsis* plant tissues.

Figure S7. An eFP overview of gene expression levels of *SCA*-like *Arabidopsis LTP* genes in response to osmotic, salt, and drought stresses.

Figure S8. An eFP overview of gene expression levels of *SCA*-like *Arabidopsis LTP* genes in response to diurnal light changes.

Table S1. The *ltp5-1* plants have more abundant and shorter internodes, compared with the wild type, but their statures are reduced.

Table S2. PCR primer sets for *Arabidopsis* LTP promoters.

Acknowledgements

This research was funded by the National Science Foundation (grant no. IBM0420445 to E.M.L.).

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