

Lipid Transfer Proteins Enhance Cell Wall Extension in Tobacco ^W

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Plant cells are enclosed by a rigid cell wall that counteracts the internal osmotic pressure of the vacuole and limits the rate and direction of cell enlargement. When developmental or physiological cues induce cell extension, plant cells increase wall plasticity by a process called loosening. It was demonstrated previously that a class of proteins known as expansins are mediators of wall loosening. Here, we report a type of cell wall-loosening protein that does not share any homology with expansins but is a member of the lipid transfer proteins (LTPs). LTPs are known to bind a large range of lipid molecules to their hydrophobic cavity, and we show here that this cavity is essential for the cell wall-loosening activity of LTP. Furthermore, we show that LTP-enhanced wall extension can be described by a logarithmic time function. We hypothesize that LTP associates with hydrophobic wall compounds, causing nonhydrolytic disruption of the cell wall and subsequently facilitating wall extension.

INTRODUCTION

Plant cells are enclosed by a cell wall that counteracts the internal osmotic pressure of the vacuole. Although the wall is rigid, it is able to extend to allow for turgor-driven growth. This unique combination of strength and extensibility is determined by the composition and architecture of the wall. One of the most relevant mechanical properties during cell elongation is the wall's viscoelasticity. The wall shows both plastic and elastic deformation upon force application. Elastic wall behavior is exhibited mainly in reaction to rapidly changing external forces, whereas plastic or viscous behavior manifests itself in reaction to slowly varying forces (Ward and Hadly, 1993). The classic way to describe the viscoelastic behavior of materials under stress is by combining terms that linearly depend on the applied stress and that represent either elastic or viscous behavior. However, such linear models cannot describe the mechanical properties of many materials (e.g., of biological origin). To this end, empirical relations describing the experimental data can be a valuable alternative. In this way, Cleland (1971) observed that the extension of isolated *Avena sativa* walls behaves logarithmically in

time. Others have used logarithmic functions to describe wall creep, the long-term and continuous deformation of cell walls (Büntemeyer et al., 1998; Thompson, 2001).

One way for plant cells to change the extensibility of the wall is by regulating the activity of expansin proteins. These proteins have been identified as mediators of acid growth (Cosgrove, 1989; McQueen-Mason et al., 1992). They are secreted into the wall and activated at low pH by a mechanism that remains unclear. Nevertheless, it has been proposed that expansins work by binding to cellulose via a carbohydrate binding domain followed by the breaking of hydrogen bonds between cellulose and xyloglucan by a separate, pH-activated catalytic domain (Cosgrove, 2000). As a result, the cellulose microfibrils in the wall may slide along each other, allowing turgor pressure to cause wall expansion. This reaction can be measured by an extensometer, which follows the extension of wall material over time (Cosgrove, 1989).

Several members of the β -expansin subfamily have been found in grass pollen, and it has been proposed that they may facilitate pollen tube growth by loosening the cell walls in the pistil (Cosgrove et al., 1997). Grasses belong to the angiosperms, which possess dry stigmas, and their pollen is characterized by a pollen coat composed of lipids and proteins that surrounds the exine (Dickinson, 1993). The pollen coat becomes liquid at pollination and is necessary for pollen germination (Edlund et al., 2004). In wet stigma-type plants (e.g., tobacco [*Nicotiana tabacum*]), the function of the pollen coat is carried by the stigmatic exudate (Sanchez et al., 2004). This exudate is secreted by the secretory zone of the stigma and is composed of polysaccharides, proteins, and lipids. We have shown previously that this lipid fraction is essential and sufficient for directional pollen tube growth (Wolters-Arts et al., 1998). As part of our work to investigate the function of proteins present in the exudate, we isolated the β -expansin homolog Pistil Pollen Allergen-Like (PPAL) in

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tobacco (Pezzotti et al., 2002). In this study, we tested the exudate and purified PPAL for cell wall-loosening activity, and we show that although the exudate has this activity, it is not mediated by PPAL but by a lipid transfer protein (LTP).

LTPs are small, basic proteins with eight Cys residues at conserved positions (Kader, 1996). Studies of the three-dimensional structure of several LTPs demonstrated that the protein has four α -helices cross-linked by four disulfide bridges (Shin et al., 1995; Heinemann et al., 1996; Charvolin et al., 1999). Positively charged amino acids are exposed on the surface of the protein, which is hydrophilic. Hydrophobic residues line the internal cavity, which is a characteristic conserved in all LTPs. However, beyond these similarities, LTPs are highly divergent. In *Arabidopsis thaliana*, for example, 71 putative LTPs with highly divergent sequences have been identified (Beisson et al., 2003).

LTPs were discovered as proteins capable of lipid transfer in vitro, and it was shown that lipids can enter and occupy an LTP's hydrophobic pocket (Kader, 1996). The transfer activity and the broad lipid specificity of the cavity suggested that LTPs may be involved in intracellular lipid transport. However, as LTPs were found to be located extracellularly (Thoma et al., 1993), this function in vivo became questionable. As an alternative, it was proposed that LTPs are involved in cutin deposition by carrying the cuticular components toward the extracellular matrix. Garcia-Olmedo et al. (1995) suggested that LTPs in plants could function as a defense against pathogen attack, but it is unknown whether this mechanism of antibiotic activity is related to the lipid transfer properties. Recently, Maldonado and coworkers (2002) reported that the LTP-like protein DIR1 of *Arabidopsis* participates in plant defense mechanisms by presumably moving a lipid-derived signaling molecule for the induction of systemic acquired resistance. Furthermore, an LTP-like protein named SCA from lily (*Lilium longiflorum*) exudate was shown to function in pollen tube adhesion (Park et al., 2000). Together, these examples, the great sequence diversity, and the expression pattern of LTPs suggest that their functions are very diverse.

In this study, we analyzed LTP-mediated cell wall loosening. Our results provide evidence that LTPs isolated from tobacco and wheat (*Triticum aestivum*) tissues are able to enhance the extension of different wall specimens. Furthermore, we show that the availability of the hydrophobic cavity in the LTP molecule is necessary for its cell wall-loosening activity. Wall extension in the absence of wall-loosening proteins can be described by a logarithmic function in time (Büntemeyer et al., 1998; Thompson, 2001). When LTP is added, only the rate of logarithmic extension is increased, implying that LTP does not initiate new pathways for cell wall extension but rather facilitates the ongoing extension process. Our results provide new insight into the mechanism of cell wall loosening as such but also suggest an important role for LTPs in plant growth and development.

RESULTS

Tobacco Stigma Exudate Has Cell Wall–Loosening Activity That Is Not Caused by the β -Expansin Homolog PPAL

As tobacco stigma exudate contains the β -expansin homolog PPAL (Pezzotti et al., 2002), which could have cell wall-loosening

activity, we assayed the exudate in a custom-built extensometer using cucumber (*Cucumis sativus*) hypocotyls and wheat coleoptiles as wall specimens. Figure 1A shows that the exudate has cell wall-loosening activity and that this is destroyed by papain protease treatment, demonstrating that the activity is determined by a protein. The exudate contains a moderate number of proteins that can be easily separated using SDS-PAGE (Figure 1B). Among these, PPAL is the 32-kD protein (Pezzotti et al., 2002). Alignment with other members of the β -expansin family shows that PPAL possesses all of the typical, conserved β -expansin regions (data not shown). It shares 43% sequence identity with Zm-EXPB1, the purified β -expansin that was shown to have in vitro cell wall-loosening activity (Cosgrove et al., 1997). We purified PPAL by HPLC and tested its purity by two-dimensional gel electrophoresis (Figure 1C). However, purified PPAL did not show any cell wall-loosening activity in the extensometer assay on any of the wall substrates tested (Figure 2A). To exclude the possibility that the purification procedure had somehow destroyed the expansin activity of PPAL, we made transgenic plants in which PPAL expression was silenced by RNA interference (RNAi). Although neither PPAL mRNA nor its protein could be detected in the transgenic plants (Figures 3A and 3B), the exudate without PPAL protein was still active in the extensometer (Figure 1A), indicating that another cell wall-loosening protein is active in the exudate.

A Tobacco LTP Is a Cell Wall–Loosening Protein and Accumulates in the Tobacco Pistil

To identify the cell wall-loosening protein in the tobacco exudate, we fractionated the exudate again and tested each fraction in the extensometer. Only one fraction had cell wall-loosening activity, and when subjected to two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis, it was shown to contain a single protein of 8.8 kD with a pI of \sim 9 (Figure 1D; data not shown). This purified protein showed clear cell wall-loosening activity on cucumber hypocotyls (Figure 2A). Analysis of its N-terminal sequence revealed a sequence 100% identical to the deduced amino acid sequence of a cDNA encoding the tobacco LTP TobLTP2, which was isolated previously from a flower cDNA library (Masuta et al., 1992). On the basis of the LTP protein sequence, we cloned the corresponding cDNA by RT-PCR on tobacco pistil mRNA and used it as a probe in RNA analyses. Figure 4A shows that TobLTP2 mRNA accumulates abundantly and increasingly in the pistil during flower development. Very low signals were detected in stem, leaf, and petal and also in the stylar tissues at later stages of flower development. No transcript was detected in anthers, sepals, and ovaries, suggesting that TobLTP2 expression is stigma-specific. In situ hybridization revealed the accumulation of TobLTP2 mRNA mainly in the cells of the secretory zone bordering the cortex and transmitting tissue (Figures 4B and 4C).

To test whether other LTPs also cause cell wall loosening, we purified LTP from wheat seeds and assayed it in the extensometer. Figure 2B shows that this protein, purified under nonhydrophobic and nondenaturing conditions, also has cell wall-loosening activity. To confirm that the cell wall loosening observed was attributable to LTP (and not to undetected

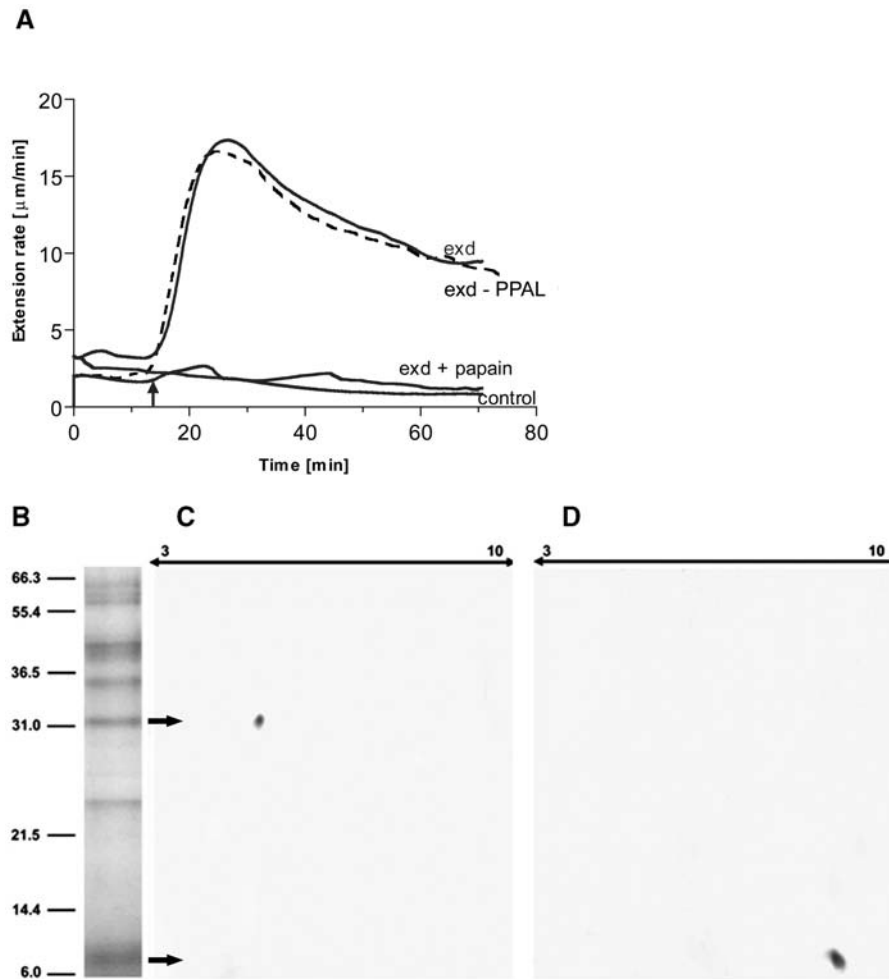


Figure 1. Cell Wall-Loosening Activity by the Tobacco Pistil Exudate and Purification of Two of Its Proteins.

(A) Samples of exudate (exd), exudate extract treated with papain protease (exd + papain), exudate extract from transgenic plants lacking PPAL expression (exd - PPAL), and 50 mM NaAc, pH 4.5, extension buffer (control) were applied separately to cucumber hypocotyl cell wall specimens at the time indicated by the arrow. The extension rate graph shows representative results from five independent measurements. The average relative increases in extension rate were 7.8 ± 1.3 (exd), 8.2 ± 1.4 (exd - PPAL), 1.0 ± 0.3 (exd + papain), and 1.0 ± 0.4 (control).

(B) SDS-PAGE of crude exudate extract from one stigma containing 20 µg of total protein. Approximately eight to nine proteins are detectable after staining with Coomassie blue. The protein of 32 kDa is PPAL (arrow), and the most abundant is the 8.8-kDa LTP (arrow).

(C) Two-dimensional SDS-PAGE of 5 µg of purified PPAL.

(D) Two-dimensional SDS-PAGE of 10 µg of purified LTP.

contaminants in the purified fraction), we silenced LTP expression in tobacco exudate using an RNAi approach. RNA gel blot analyses confirmed the absence of the LTP transcript in the transgenic plants (Figure 3C). Exudate extracted from these transgenic plants showed no cell wall-loosening activity in the extensometer (Figure 2B). We did not observe any obvious phenotype in stigma morphology or pollen tube growth in transgenic plants harboring the LTP RNAi construct.

It has been shown that the α -expansin CsExp1 is also active on artificial composite material consisting of cellulose and xyloglucan (Whitney et al., 2000). This material is formed when cellulose, synthesized by *Acetobacter xylinum*, associates with xyloglucan added to the medium during bacterial growth. Although its mole-

cular organization is similar to that of plant cell walls, it is molecularly more defined and far less complex. A strip of this cellulose/xyloglucan composite was used in the extensometer as substrate. Figure 2B shows that LTP is also active on this cell wall analog material. Together, these data establish that LTPs can act as cell wall-loosening proteins on wall specimens of different origins.

The Hydrophobic Cavity of LTP Is Necessary for Its Cell Wall-Loosening Activity

Alignment of the deduced amino acid sequence of TobLTP2 (excluding its signal peptide) with other LTPs demonstrated that

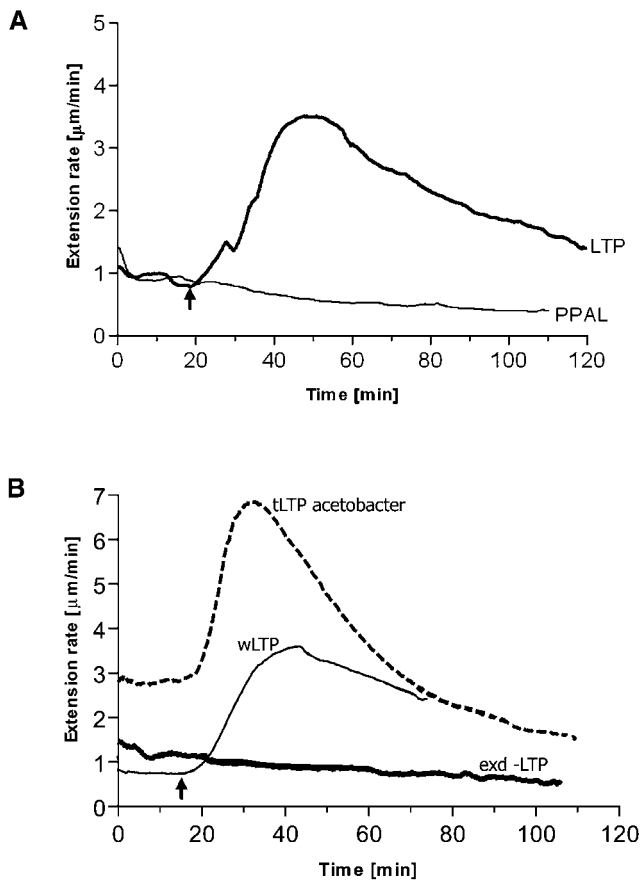


Figure 2. LTP Is a Cell Wall-Loosening Protein.

(A) Purified tobacco LTP or purified tobacco PPAL (each 10 $\mu\text{g}/\text{mL}$) was applied to hypocotyl cell wall specimens at the time indicated by the arrow to establish their cell wall-loosening activity. The same results were obtained using high concentrations of LTP and PPAL (40 $\mu\text{g}/\text{mL}$; data not shown).

(B) Purified tobacco LTP was tested on wall analogous material produced by *Acetobacter xylinum* (10 $\mu\text{g}/\text{mL}$; tLTP; broken line). Cucumber hypocotyl wall specimens were used to measure the loosening activity of wheat LTP (wLTP; 10 $\mu\text{g}/\text{mL}$) and of exudate extract from transgenic plants lacking LTP expression (exd - LTP). The arrow indicates the time at which the samples were applied. LTP has the same cell wall-loosening effect on wheat coleoptiles (data not shown). All extension rate graphs show representative results from four to five independent measurements. The average relative increases in extension rate were 3.7 ± 0.8 (LTP), 1.0 ± 0.2 (PPAL), 3.5 ± 1.0 (tLTP *A. xylinum*), 3.9 ± 0.6 (wLTP), and 1.0 ± 0.2 (exd - LTP).

the protein has features conserved among LTPs, such as the spacing of the eight Cys residues and the small (~ 9 kD) molecular mass (Figure 4D). TobLTP2 shares 50 to 53% amino acid sequence identity with maize (*Zea mays*), barley (*Hordeum vulgare*), and wheat LTPs, for which the structures have been resolved (Shin et al., 1995; Heinemann et al., 1996; Charvolin et al., 1999). This enabled us to model the TobLTP2 three-dimensional structure by homology (Figures 4E and 4F) (Vriend,

1990). As expected, this model indicates four α -helices, a hydrophobic cavity, four disulfide bridges, a high pI, and positively charged surface residues, demonstrating that TobLTP2 shares all of the signature motifs of the LTP family. Furthermore, Masuta et al. (1992) demonstrated that the recombinant TobLTP2 has lipid transfer activity in vitro. We were not able to find any sequence or structural similarities between LTPs and expansins. LTP consists of four α -helices, whereas the dominant secondary structure of expansins is predicted to be β -strand (Cosgrove, 2000).

Because the hydrophobic pocket is strictly conserved among all LTPs, we investigated whether this feature is involved in cell wall extension. Hydrophobic molecules associate with the pocket with different affinities, depending on their size and structure (Zachowski et al., 1998). This association does not cause any alteration of the protein three-dimensional structure (Liepinsh et al., 1999). To assess the possible role of the hydrophobic cavity in LTP's cell wall-loosening activity, we preincubated the protein with the hydrophobic molecules benzene and β -sitosterol and then assayed these mixtures in the extensometer. Figure 5A shows that these treatments completely inhibited the activity of LTP. Subsequent addition of fresh LTP restored wall extension, showing that benzene and β -sitosterol did not alter the intrinsic extensibility of the tissue and that the availability of the hydrophobic cavity is required for cell wall-loosening activity.

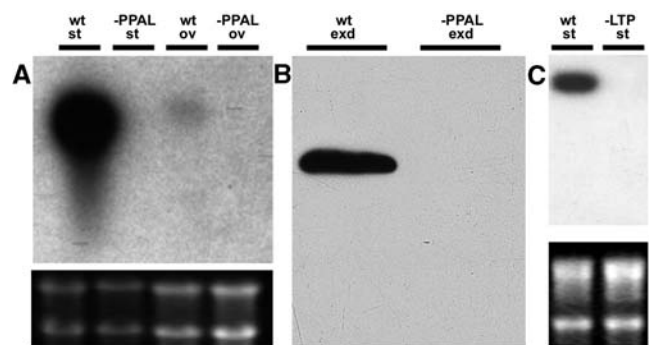


Figure 3. Silencing of PPAL and LTP Expression.

(A) RNA gel blot analysis of PPAL transcript levels in wild-type stigmas (wt st), transgenic stigmas (-PPAL st), wild-type ovaries (wt ov), and transgenic ovaries (-PPAL ov). Stigmas were collected from mature flowers, and ovaries were collected 48 h after pollination. Ten micrograms of total RNA was loaded in each lane, and RNA integrity was checked by ethidium bromide staining (bottom gel). Tobacco PPAL cDNA was used as probe.

(B) Detection of PPAL protein in the stigmatic exudate by protein gel blot analysis using anti-PPAL antibody. Fifty micrograms of total exudate protein collected from wild-type plants (wt exd) and from transgenic plants (-PPAL exd) was loaded.

(C) RNA gel blot analysis of LTP transcript isolated from stigmas of wild-type (wt st) and transgenic (-LTP st) plants harboring an LTP-RNAi construct. Ten micrograms of total RNA was loaded in both lanes, and RNA integrity is shown by ethidium bromide staining (bottom gel).

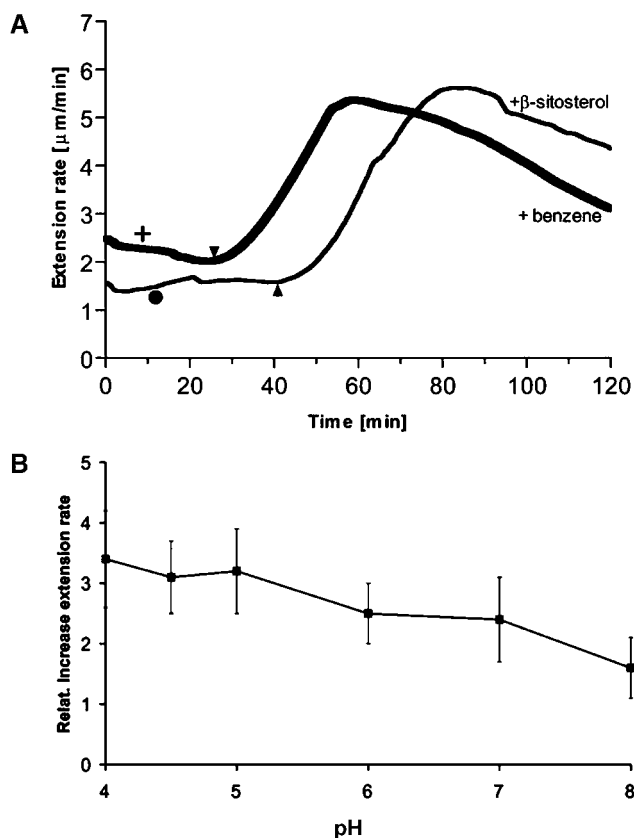


Figure 5. Characteristics of TobLTP2-Mediated Cell Wall Loosening.

(A) Extension rate of cucumber hypocotyl cell walls upon incubation with 5 $\mu\text{g}/\text{mL}$ LTP inactivated by benzene (plus sign) or β -sitosterol (closed circle) and applied at the time indicated by the symbols. After 20 to 25 min of incubation, the LTP inhibited by benzene or β -sitosterol was removed by washing the hypocotyls. Fresh LTP (5 $\mu\text{g}/\text{mL}$) was then applied (arrowheads) to confirm that the hypocotyl cell wall was still responsive to LTP activity. All extension rate graphs show representative results from five independent measurements. The average relative increases in extension rate were 0.9 ± 0.2 (LTP preincubated with β -sitosterol), 3.8 ± 1.0 (fresh LTP, β -sitosterol), 0.9 ± 0.2 (LTP preincubated with benzene), and 3.9 ± 0.8 (fresh LTP, benzene).

(B) Cell wall-loosening activity of LTP at different pH values. The relative increase in extension rate was calculated by dividing the maximum extension rate by the rate just before LTP addition.

DISCUSSION

LTPs and Plant Growth

Our data presented here focused on establishing LTP as a cell wall-loosening protein using a well-established *in vitro* assay for cell wall extension. Our results indicate a role for LTP *in vivo* in plant growth and development, which is sustained by many data published on LTP expression, particularly by the observations that LTPs are extracellular proteins and that LTP1 from *Arabidopsis* is localized in the cell wall (Thoma et al., 1993; Kader, 1997). Although the data available in the literature on RNA

expression are quite extensive, many studies focus on just one aspect. The most comprehensive studies have been performed in *Arabidopsis*, rice (*Oryza sativa*), and tobacco. Notwithstanding the scattered expression data, they are similar in the fact that expression in roots is rare and LTPs are frequently detected in young tissues. For example, some LTPs are expressed in the protoderm during embryogenesis (Sterk et al., 1991; Thoma et al., 1994; Yadegari et al., 1994; Dodeman et al., 1997; Sabala et al., 2000). The protoderm is the layer that restricts the expansion of the embryogenic cells (Jurgens et al., 1991; Dodeman et al., 1997). Furthermore, Sabala and coworkers (2000) studied the effect of LTP overexpression and underexpression in transgenic proembryogenic cell clusters and found that clusters underexpressing LTP did not contain elongated cells, whereas in clusters with overexpression, highly elongated cells could be detected. In the expression study of LTP1 of *Arabidopsis*, the authors concluded that LTP1 expression is increased in young and expanding tissue (Thoma et al., 1994). It should be noted, however, that there are also LTP expression data that do not support a function for LTP in cell wall loosening. In potato (*Solanum tuberosum*) plants, for example, LTP transcripts have been detected in phloem tissue (Horvath et al., 2002). Thus, we argue that the existing LTP expression data are compatible with a function in cell wall loosening, but they confirm the known diversity in LTP function, reflected by their divergent amino acid sequences and expression patterns (Kader, 1997; Beisson et al., 2003). Further experiments are needed to establish the role for LTP in plant growth. For example, the use of RNAi technology with LTP1-4 in *Arabidopsis* resulted in dwarfed plants and a disruption of flower development and/or sterility (E. Lord and J. Dong, unpublished data).

TobLTP2 and Pollen–Pistil Interaction

Cosgrove et al. (1997) isolated an active β -expansin from the coat of maize pollen and suggested that these expansins may facilitate pollen tube growth by softening the female tissue. Drawing on the similarity between the pollen coat and exudate, cell wall-loosening proteins in the exudate may have the same function. However, we could not detect any difference in pollen tube growth rate or direction in pistils of PPAL- and LTP-silenced plants compared with wild-type plants. This finding indicates that pollen tube growth does not require cell wall-loosening proteins in the exudate. This is in agreement with previous results obtained by Wolters-Arts et al. (1998), who demonstrated that the lipids of the exudate are sufficient for pollen tube growth in the pistil. We did not see an effect on pistil morphology in transgenic plants lacking PPAL or TobLTP2 expression. However, it is possible that the cell wall-loosening activity of LTP has an effect on the cells of the secretory zone of the stigma. Such an effect may be obscured by redundant cell wall-loosening activity, for example, α -expansins, whose transcripts were detected in the tobacco pistil (de Graaf, 1999). In addition, because >70 members of the LTP family have been annotated in *Arabidopsis* (Beisson et al., 2003), it is likely that other tobacco LTPs cause functional redundancy. At any rate, this conclusion cannot be extrapolated to other plant species, because pistil morphologies differ considerably between angiosperms. Stigmatic and stylar

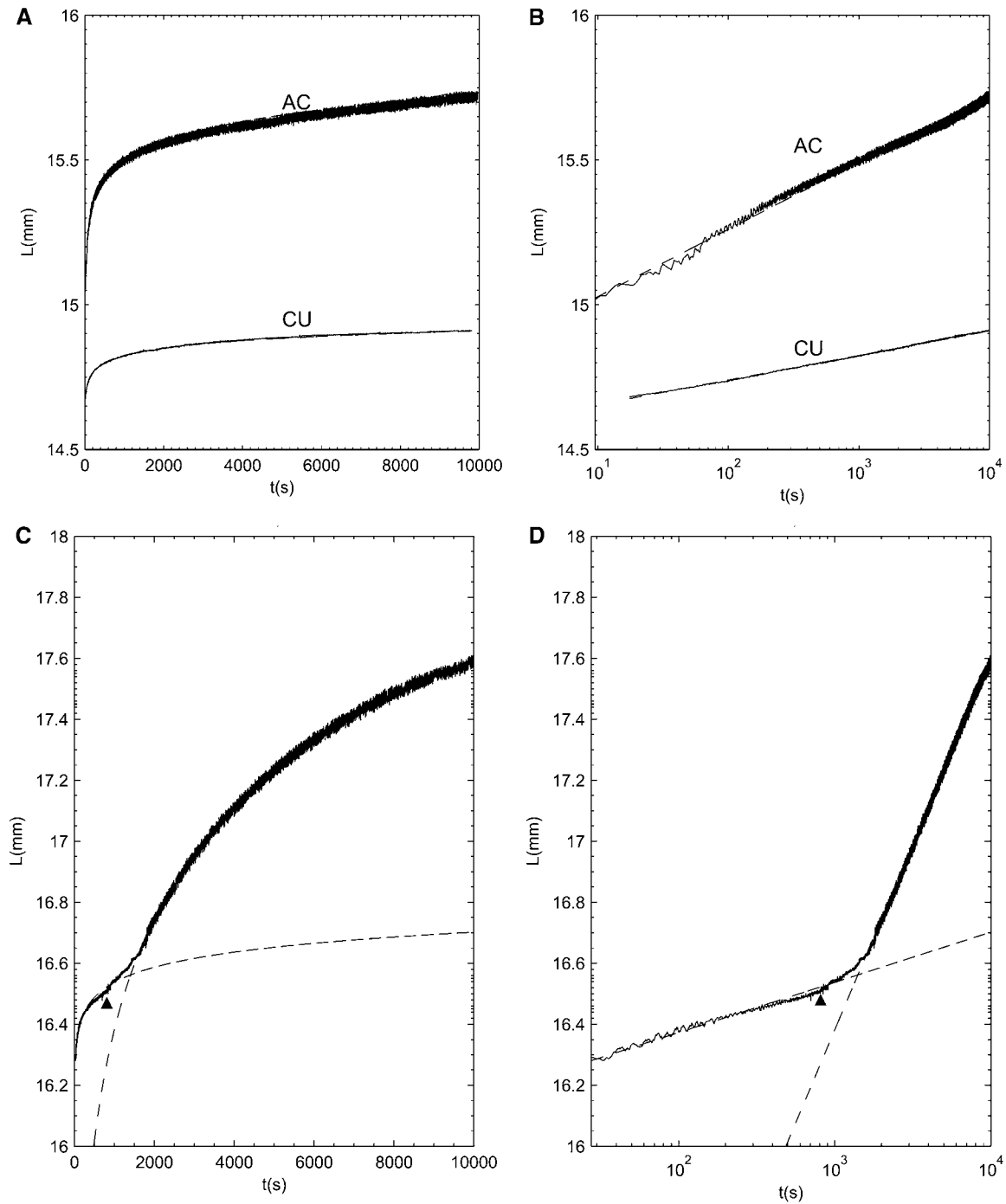


Figure 6. Wall Extension Is Logarithmic in Time.

(A) Length of cucumber hypocotyl (CU) or *A. xylinum* composite material (AC) upon a unidirectionally applied force of 0.1 N versus time. Material was incubated in 50 mM NaAc, pH 4.5, and the force was applied at time 0.

(B) The same data from **(A)** semilogarithmically plotted to show log-like behavior. The logarithmic fit using Equation 1 is shown by the dashed line though the data points.

(C) Length of *A. xylinum* composite material upon a unidirectionally applied force of 0.1 N versus time. At the time indicated by the arrowhead, 20 μ g/mL purified LTP was added.

(D) The same data from **(C)** semilogarithmically plotted to show log-like behavior. The logarithmic fit using Equation 1 with its appropriate N_{creep} is shown by the dashed line.

tissues from solanaceous species have a loose structure with large intercellular spaces through which pollen tubes grow (Cresti et al., 1986; Kandasamy and Kristen, 1990; Hudak et al., 1993; Atkinson et al., 1995). By contrast, the cellular organization of maize styles is compact with small intercellular spaces (Kroh et al., 1979); therefore, it is more likely that a cell wall-loosening protein facilitates pollen tube growth in these types of pistils.

Another function in pollen-pistil interaction attributed to LTP is that of pollen tube adhesion and guidance. Lily flowers have a hollow style along which pollen tubes adhere and grow toward the ovary. The LTP-like protein SCA from lily exudate was shown to hold and guide lily pollen tubes but had no effects on tobacco pollen tubes (Park et al., 2000; Park and Lord, 2003). We failed to detect tobacco pollen tube adhesion mediated by tobacco stigmatic exudate. In addition, we could not detect cell wall-loosening activity in the lily exudate, which indicates that SCA and TobLTP2 differ in function.

LTP-Mediated Cell Wall Loosening

Based on our findings, we suggest a model for LTP-mediated cell wall loosening similar to the model proposed for expansins by McQueen-Mason and Cosgrove (1994). It has been demonstrated previously that benzene only occupies the hydrophobic cavity and does not alter protein structure (Liepinsh et al., 1999); hence, our results show that the availability of LTP to engage in hydrophobic interactions is essential for its action in cell wall loosening. After secretion, the hydrophobic cavity of LTP may interact with hydrophobic molecules in the cellulose/xyloglucan network of the wall. Subsequently, the complex, with its charged surface, could disrupt the surrounding hydrogen bonds between cellulose and hemicellulose. Because the wall extension becomes faster but remains logarithmic, we hypothesize that LTP slightly reduces the energy barriers for rearrangements in the cellulose/xyloglucan network toward lower energy configurations, thereby facilitating wall extension under the applied force.

We do not know with which site in the cell wall LTP associates, but the protein can be extracted from plant material easily, indicating that the LTP-wall interaction is not strong. The hydrophobic cavity can bind hydrophobic molecules with wide ranges of size, hydrophobicity, and structure. A possible ligand in the cell wall might be β -sitosterol, which was shown to be a primer for cellulose synthesis (Peng et al., 2002). In addition, undefined lipid-linked 1,4- β -glucans that have been found in cell walls of *kor* mutants of *Arabidopsis* (Sato et al., 2001) may also be putative ligands. However, it is not clear whether and how many lipids linked to cellulose are present normally in the cell wall. Therefore, the question of which hydrophobic cell wall components associate with LTP remains open.

Although both LTP and expansins are cell wall-loosening proteins, there are some differences in their activity. Expansins have been shown to be activated by low pH and therefore are good candidate mediators of acid growth (Cosgrove, 2000). By contrast, LTP is still active at neutral pH and does not show a clear pH optimum (Figure 5B). The models proposed for the mechanism of cell wall loosening by LTPs and expansins share some similarities. Both models argue that after secretion, LTPs and expansins associate with the cellulose/xyloglucan network: ex-

pansins by their putative carbohydrate binding domain and LTPs by their hydrophobic cavity. Expansins have an endoglucanase-like domain (Cosgrove, 2000), but this has never been shown to possess hydrolytic activity; LTPs lack an enzymatic domain. It is quite possible that these cell wall-loosening proteins do not require hydrolytic activity but interfere sterically with the cellulose-hemicellulose wall matrix. However, the observations that the induction of extension by LTP appeared to be slower than the expansin-mediated induction and that LTP, unlike expansins, does not show a pH optimum suggest differences between LTPs and expansins in their kinetics and modes of action.

If the underlying cell wall-loosening mechanisms were identical for LTPs and expansins, one would expect expansin-mediated extension to be logarithmic in time, as we found for LTP. The data published to date, however, are not conclusive on this point and are difficult to interpret. There are many examples of linear extension after expansin addition (McQueen-Mason et al., 1992; Li and Cosgrove, 2001), but the effect of expansin on *A. xylinum* material extension is not linear and seems logarithmic (Whitney et al., 2000). Interestingly, when only the effect of endogenous, untreated (e.g., not boiled) cell wall-loosening proteins was measured in the extensometer, by not treating (e.g., boiling) plant material, the extension application appeared logarithmic (Cleland, 1971; McQueen-Mason et al., 1992). It would be interesting to compare the activity of LTP and expansin directly.

Our observation that cell wall extension remains logarithmic after LTP addition implies that LTP does not initiate new pathways for cell wall extension but merely facilitates the endogenous process of cell wall extension under an applied force. The fact that the creep is logarithmic in time (i.e., the time it takes to increase the length by a certain factor is the square of the time it takes to increase it by half of this factor) indicates that the cell wall becomes more and more rigid and resistant to the force applied to it. Consequently, the effect of LTP depends heavily on the amount of time the wall has been subjected to a certain force. Cell elongation, therefore, would be more efficient when a cell increases N_{creep} before rather than during turgor pressure increase. It is unlikely that cell wall loosening by LTP alone is sufficient for total plant elongation. Expansins are mediators required for acid growth, whereas LTPs, because they also work at neutral pH, may not be regulated by wall acidification. An interesting question is where and at what stages in plant development LTPs would specifically work and how do they affect plant growth in vivo. Cell wall loosening by LTP may be instrumental for the initiation of cell expansion or for local and directional growth leading to cell specialization.

METHODS

Collection of Exudate

Tobacco (*Nicotiana tabacum* cv Petit Havana SR1) plants were grown under greenhouse conditions. To collect the secreted exudate, flowers were emasculated 1 d before anthesis and stigmas were collected after 2 d. The stigmas were incubated for 15 min in 50 mM NaAc buffer, pH 4.5, called extension buffer (10 stigmas/0.1 mL buffer), and then removed. The remaining buffer with exudate was either used directly in cell wall-loosening assays or further processed for protein purification.

Exudate Protein Purification

Proteins in the exudate extracts were analyzed by standard 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue R 250 (Amersham-Pharmacia Biotech, Uppsala, Sweden). For protein purification, the exudate extract was clarified by centrifugation (10,000g for 15 min) and filtration and then concentrated with a 10-kD Centricon concentrator (Millipore-Amicon, Bedford, MA). To isolate LTP, the concentrate was loaded on a MonoS cation-exchange column (Amersham-Pharmacia Biotech) preequilibrated with extension buffer and eluted with a linear gradient from 0 to 1 M NaCl in 50 mM NaAc, pH 4.5. Fractions putatively containing LTP were loaded on a Superdex-200 gel filtration column (Amersham-Pharmacia Biotech), and protein was eluted with extension buffer. Fractions containing a single protein of ~9 kD were pooled.

PPAL was purified by loading the concentrated exudate on a MonoQ anion-exchange column (Amersham-Pharmacia Biotech) preequilibrated with 20 mM Pipes, pH 5.7, and eluted with a linear gradient from 0 to 1 M NaCl in 20 mM Pipes, pH 5.7. Fractions that cross-reacted with anti-PPAL antibody were pooled and loaded on a Superdex-200 gel filtration column (Amersham-Pharmacia Biotech). Proteins were eluted with extension buffer, and fractions containing PPAL were pooled. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA).

Wheat LTP Purification

To isolate wheat (*Triticum aestivum*) LTP, we used a conventional procedure (Guerbette et al., 1999) but avoided the generally used reverse-phase chromatography, because this method involves hydrophobic solvents that may occupy the hydrophobic cavity of LTP. In short, the procedure was as follows. Seeds were soaked for 36 h at 4°C in 100 mM KCl, 5 mM EDTA, 8 mM 2-mercaptoethanol, and 100 mM Tris-HCl buffer, pH 7.8. After grinding in the same buffer and filtering through a nylon mesh, the homogenate was centrifuged for 30 min at 5000g. The collected supernatant was adjusted to pH 5.0 and centrifuged (for 30 min at 10,000g), and the pH of the supernatant was readjusted to 7.8 with 2 M Tris base. Purification started by selective ammonium sulfate precipitation at 40 and 80% saturation. After dialysis at 5°C overnight to allow proper refolding of the protein, it was further purified through a Superdex-200 gel filtration column (Amersham-Pharmacia Biotech) with extension buffer as eluent. Fractions containing proteins with a molecular mass in the range of 8 to 10 kD, as tested by SDS-PAGE, were pooled and loaded onto a MonoS cation-exchange column (Amersham-Pharmacia Biotech) preequilibrated with extension buffer. Proteins were eluted with a linear gradient from 0 to 1 M NaCl in extension buffer. Fractions containing a protein of ~9 kD were tested for purity by SDS-PAGE and MALDI-TOF, which confirmed the protein to be the size of LTP (i.e., 8.8 kD). This fraction was desalted by elution with extension buffer from a FastDesalt column (Amersham-Pharmacia Biotech).

MALDI-TOF Mass Spectrometry Analysis

LTP was characterized by MALDI-TOF mass spectrometry (Biflex III; Bruker, Bremen, Germany). One microgram of purified LTP was mixed with 5 μ L of trifluoroacetic acid (0.1%, v/v) and 5 μ L of matrix solution (a saturated solution of synaptic acid in a 50:50 mixture of acetonitrile and 0.1% [v/v] trifluoroacetic acid). One microliter of this mixture was spotted on the target plate and was analyzed in the linear mode.

Cell Wall-Loosening Assays

Assays were performed essentially as described (McQueen-Mason et al., 1992) using a custom-built extensometer equipped with two cuvettes for

controls or duplicate measurements. The relative increase in extension rate was calculated by dividing the maximum extension rate by the rate just before LTP addition. Three types of cell wall specimens were used for the cell wall-loosening assays: cucumber (*Cucumis sativus*) hypocotyls, wheat coleoptiles, and pellicles of cellulose/hemicellulose composite from *Acetobacter xylinum*. We chose these wall materials because they have been used previously to assay expansin activity and are therefore more suitable for comparison with the expansin data. Hypocotyls of cucumber and coleoptiles of wheat were obtained from 4-d-old etiolated seedlings that were germinated in moist vermiculite. Apical segments of 20 mm were cut, frozen at -20°C, thawed, abraded with carborundum slurry, and boiled for 15 s. *A. xylinum* was grown at 30°C in Hestrin Schramm medium in the presence of 0.5% tamarind xyloglucan (Megazyme, Bray, Ireland) (Whitney et al., 2000). The pellicles were removed from the plates and cut into 2 \times 20-mm strips, dried on filter paper for 15 min, and then rehydrated in extension buffer for use in the extensometer. Hypocotyls, coleoptiles, and bacterial strips were incubated individually in a cuvette containing 800 μ L of extension buffer (50 mM NaAc buffer, pH 4.5). The extension was measured every 20 s, under a constant load of 30 g, for a period of 1 h until a stable base extension was established. This extension represents the intrinsic extension of the wall. Approximately 15 min after the extension appeared linear, the buffer was replaced by the test sample. Samples were tested in comparison with a control or treated sample. Protease sensitivity was tested by pre-digestion of the sample with 2 mg/mL papain protease (Sigma-Aldrich, St. Louis, MO) for 2 h at 65°C. Protein degradation after papain treatment was confirmed by SDS-PAGE (10 μ L of treated extract per lane). Protein was denatured by incubating for 15 min at 100°C with 5% 2-mercaptoethanol. Unless stated otherwise, purified TobLTP2, wheat LTP, and PPAL were used in the extensometer assay at a concentration of 10 μ g/mL in extension buffer for all three types of wall specimens. Phosphate buffers were used to measure LTP-mediated wall extension at different pH levels. Alternatively, up to 2 mg/mL of 4- to 15-kD poly-D-Lys (Sigma-Aldrich) was dissolved in extension buffer and assayed on cucumber hypocotyls. LTP was incubated for 1 h at room temperature in extension buffer saturated with benzene or β -sitosterol (Sigma-Aldrich) to test the possible role of the hydrophobic cavity of LTP. Before these assays, we confirmed that benzene or β -sitosterol did not inhibit the endogenous extension of the wall. The extensometer assays were performed with 5 μ g/mL purified LTP. After measuring the extension for 25 min, the wall specimens were washed three times and replaced in fresh extension buffer containing 5 μ g/mL new LTP protein, and further extension was measured. For the logarithmic time dependence measurements, extension was recorded over a minimum period of 4 h with a minimum interval of 1 s. Temperature was maintained constant within 0.1°C.

Protein Analysis and Sequencing

Amino acid sequencing of purified LTP was performed by automated Edman degradation at the Protein Analysis Laboratory of the University of Alabama (Birmingham). To test protein purity, two-dimensional electrophoresis was performed by first separating proteins on the basis of their pI values using a 7-cm Immobiline DryStrip pH 3-10 on an IPGphor as recommended by Amersham-Pharmacia Biotech. After isoelectric focusing, the strip was incubated in 50 mM Tris-HCl, pH 8.8, 6 M urea, and 30% glycerol for 15 min, placed on a 12.5% SDS-PAGE gel, and run using standard procedures. Coomassie Brilliant Blue R 250 staining was performed using the standard protocol. Protein gel blot analysis was performed essentially as described (Pezzotti et al., 2002), using polyclonal antibodies against PPAL and horseradish peroxidase-conjugated goat anti-rabbit IgG at a 1:100,000 dilution as secondary antibodies. Detection was performed using Supersignal West Pico (Pierce, Rockford, IL) for 30 min, and blots were exposed to film for 5 min and developed.

Construction of PPAL and LTP RNAi Plasmids

To silence PPAL by RNAi, the PPAL (accession number AF333386) full-length cDNA in the sense orientation was cloned under the control of the 35S *Cauliflower mosaic virus* promoter. A cDNA clone of 389 bp was amplified from the TobLTP2 transcript sequence by RT-PCR on tobacco stigma RNA with primers 5'-AATCCTGACACTACTCTATTG-3' and 5'-GGACCTTAGAGCAGTCTGTGGA-3' and cloned into the pGEM-T Easy vector (Promega, Madison, WI). The RNAi plasmid for LTP, based on pGSA1165 (ABRC CD3-450), was made of 2 units with either PPAL or STIG1 promoters, replacing the 35S promoter of pGSA1165. These two pistil-specific promoters regulate the transcription of an inverted repeat of the LTP cDNA (nucleotides 44 to 433 of EMBL D13952).

Plant Transformation

The PPAL-RNAi and LTP-RNAi binary vectors were introduced into *Agrobacterium tumefaciens* LBA4404 by freeze-thaw transformation (Chen et al., 1994). Tobacco plants were transformed using a standard leaf disc transformation and regeneration method. Transgenic plants were selected in vitro on the basis of kanamycin resistance and subsequently in the greenhouse.

RNA Extraction and RNA Gel Blot Analysis

RNA extraction was performed as described previously (Pezzotti et al., 2002). RNA gel blot analysis was performed under stringent conditions with LTP cDNA as probe.

In Situ Hybridization

Stigmas/styles were dissected from tobacco flowers of developmental stage 6 (Goldberg, 1988). Fixing, embedding, sectioning, and hybridization were performed as described (Pezzotti et al., 2002). The antisense and sense LTP RNA probes were synthesized using digoxigenin-UTP by in vitro transcription with the T7 and T3 RNA polymerases of the digoxigenin RNA labeling kit using LTP cDNA as template (Boehringer Mannheim, Mannheim, Germany).

Data Fitting

We fitted the data as a function of the logarithm of time by a straight line and discarded the first 10 s of data after the application of force. To avoid overfitting during long time measurements, only measurements close to regular intervals in the logarithm of time were used. The noise was estimated by piecewise removing the trend for every 25 measurements and determining the SD. Then, a linear least-squares fit was used, in logarithmic time space, to determine N_{creep} and its error.

Sequence data from this article have been deposited with the Swiss-prot data library under accession number Q03461 (TobLTP2).

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