

Developmental and pathogen-induced expression of three barley genes encoding lipid transfer proteins

Antonio Molina and Francisco García-Olmedo*

Laboratorio de Bioquímica y Biología Molecular, ETS Ingenieros Agrónomos-UPM, E-28040 Madrid, Spain

Summary

Clones for three barley non-specific lipid transfer proteins (LTP2, LTP3, and LTP4; formerly Cw18, Cw20 and Cw21, respectively) which had been previously shown to inhibit growth of plant pathogens, were selected and characterized from a cDNA library derived from young etiolated leaves. Genes *Ltp2* and *Ltp4* were located in chromosome 3H and gene *Ltp3* was assigned to chromosome 7H by Southern blot analysis of wheat-barley disomic addition lines, using gene-specific probes (3'-ends of cDNAs). These assignments were confirmed by the polymerase chain reaction, using specific primers. The three genes were expressed in stem, shoot apex, leaves and roots (at low levels) throughout development. Genes *Ltp3* and *Ltp4* were expressed at high levels, and *Ltp2* at low levels, in the spike (rachis, lemma plus palea and grain coats). Neither of the mRNAs was detected in endosperm. The proteins were localized by tissue-printing with polyclonal antibodies in the outer cell layer of the exposed surfaces of the plant, throughout the embryo, and in vascular tissues. Expression levels in leaves were moderately increased by 0.34 M NaCl and by 0.1 mM abscisic acid and were not affected by cold, drought, salicylate, 2,6-dichloro-isonicotinic acid, ethylene or ethephon. Methyl Jasmonate (10 μ M) switched off all three genes. Inoculation with *Av6* or *vir6* isolates of the fungal pathogen *Erysiphe graminis* increased the three mRNAs, especially that of LTP4, which reached a maximum nine-fold increase 12–16 h after infection.

Introduction

Non-specific lipid transfer proteins (LTPs) in plants are a family of homologous polypeptides of about 9 kDa which have been so designated because of their ability to shuttle different kinds of lipids between liposomes and mitochondria *in vitro* (Arondel and Kader, 1990; Breu *et al.*, 1989; Watanabe and Yamada, 1986). However, a possible cytoplasmic role *in vivo* for the LTPs has been

questioned because they are synthesized as precursors with typical signal peptides (Bernhard and Somerville, 1989; Sterk *et al.*, 1991; Tchang *et al.*, 1988), a cell wall localization has been found for some of them (Sterk *et al.*, 1991; Thoma *et al.*, 1993), and cell cultures have been shown to secrete them into the medium (Sterk *et al.*, 1991). They have been reported in a variety of plant tissues, including barley aleurone (Mundy and Rogers, 1986; Linnestad *et al.*, 1991), wheat seeds (Simorre *et al.*, 1991), maize endosperm and embryo (Tchang *et al.*, 1988), castor beans (Takishima *et al.*, 1988), spinach leaves (Bouillon *et al.*, 1987), tomato stems (Torres-Schumann *et al.*, 1992), tobacco anthers and shoot apex (Fleming *et al.*, 1992), ragi seeds (Campos and Richardson, 1984), and carrot embryos (Sterk *et al.*, 1991). It is becoming evident that multiple LTP genes are present in a given genome and that these are expressed in different specific temporal and spatial patterns (Sossountzov *et al.*, 1991). Involvement of LTPs in cutin deposition has been suggested based on the cell wall location of the carrot EP2 LTP (Sterk *et al.*, 1991), although a cytoplasmic location has been proposed for the maize protein (Sossountzov *et al.*, 1991).

Four LTPs present in crude cell wall preparations from barley leaves are potent growth inhibitors of bacterial and fungal plant pathogens (Molina and García-Olmedo, 1991; Molina *et al.*, 1993). We cloned cDNAs encoding three of these proteins and studied the developmental and pathogen-induced expression of their genes. Deposition of LTPs in the outer cell layer, and in vascular tissues of different parts of the plant, and higher steady-state levels of their mRNAs in response to pathogens were in line with the proposed protective role.

Results

Cloning of leaf LTP cDNAs

Nucleotide sequences presented in Figure 1 correspond to cDNA clones encoding LTPs isolated from a library which was derived from 7-day-old etiolated barley leaves. A total of 45 clones were selected out of about 4000 screened, using two degenerate oligonucleotides deduced from appropriate regions (shaded in Figure 1) of the known amino acid sequences of proteins LTP2 and LTP4 (formerly Cw18 and Cw21, respectively). The sequence encoding protein LTP2 was identified in nine of the 24 clones sequenced, while one of the clones

absence of cross-hybridization by the Southern blot technique (not shown).

Genomic organization and mapping of LTP genes

Southern blot analysis of barley DNA digested with the *EcoRI* endonuclease, using a mixture of the complete cDNA inserts (LTP2, LTP3 and LTP4) as probe, yielded a pattern with four main bands (15, 9.4, 6.8 and 1.8 kb in Figure 2a). Sequence-specific probes for LTP2 and LTP3 hybridized with the 15 and 6.8 kb bands, respectively, and gave practically no signals with wheat DNA (Figure 2a). The LTP2 band was associated with chromosome 3H of barley, as it was detected only in the wheat–barley disomic addition line for that chromosome, and similarly, the LTP3 fragment was located in chromosome 7H (Figure 2a). The LTP4-specific probe hybridized with

three fragments, including the 15 kb fragment detected with the LTP2 probe (Figure 2a). The sizes of the fragments (15, 9.4 and 1.8 kb) hybridized by the short (300 b) LTP4 probe indicated that there was at least a second gene or pseudogene of this type in the barley genome. The same probe detected up to 17 fragments in wheat, which did not overlap with those in barley, except for the 9.4 kb band (Figure 2a). The two non-overlapping bands (15 and 1.8 kb) were detected only in the DNA of the 3H addition line, whereas an enhancement of the overlapping band (9.4 kb), with respect to the preceding one, was also observed in the same addition line (Figure 2a). To confirm the chromosomal localizations, DNAs from the wheat–barley addition lines were amplified by the polymerase chain reaction (PCR), using the sequence-specific primers indicated in Figure 1. Single bands associated with chromosomes 3H and 7H were obtained with the primers for LTP2 and LTP3, respectively (Figure 2b). The LTP2 band was of the same size as that obtained with the cDNA (not shown), while the LTP3 band was about 170 b longer than expected, which suggested the presence of an intron. The primers for LTP4 gave two closely migrating bands with barley DNA as template; one of them had the predicted size, while the other was slightly smaller and overlapped with the single band obtained with wheat DNA as template. The barley amplification pattern was reproduced when the DNA from the 3H addition line was used as template (Figure 2b). The amplification results indicated again the presence of at least one more gene or pseudogene of the LTP4 type in barley and allowed confirmation that the LTP4 gene was located in chromosome 3H.

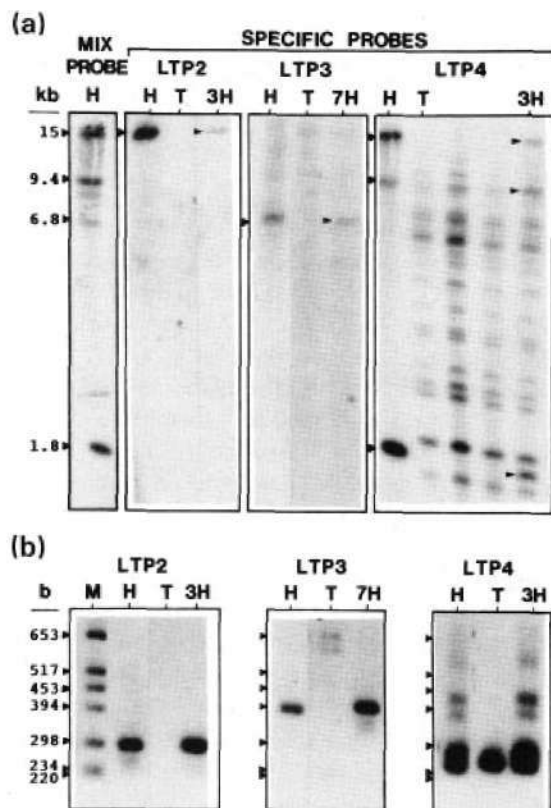


Figure 2. Chromosomal locations of *Ltp* genes.

(a) Southern blot analysis of wheat–barley addition lines. DNAs (15 µg) were digested with the *EcoRI* nuclease. The sequence-specific probes (LTP2, LTP3 and LTP4; see Figure 1) and a mixture of equal amounts of the three complete cDNA inserts (MIX) were hybridized. Markers used to deduce fragment sizes were fragments of λ phage digested with nucleases *EcoRI* and *HindIII*.

(b) PCR amplification of the indicated DNAs. Size markers (M) used were fragments of plasmid pBR328 digested with nucleases *BglI* and *HinfI*.

(a and b) Only the patterns of Betzes barley (H), Chinese Spring wheat (T), and of the pertinent addition line (3H or 7H) are shown in each case.

Developmental expression of genes encoding barley LTPs

Total RNA was extracted from different barley tissues and organs at the times indicated in Figure 3. The three sequence-specific probes were used to hybridize these RNAs by the Northern blot technique and positive signals were obtained after overnight exposure for all samples shown in Figure 3, except for grain coats with the LTP2 probe, which gave a weak signal even after a week-long exposure. This was also the case for RNAs from roots collected at different stages and hybridized with the three probes (not shown); whereas developing endosperm did not give any signal with these probes even after 7 day exposures (not shown). The main differences among the expression patterns of the three genes were the low steady-state levels of the LTP2 mRNA in different parts of the spike and of the LTP4 mRNA in older leaves. Otherwise, steady-state mRNA levels were similar in all cases, although somewhat higher in stems and, to a lesser extent, in young leaves.

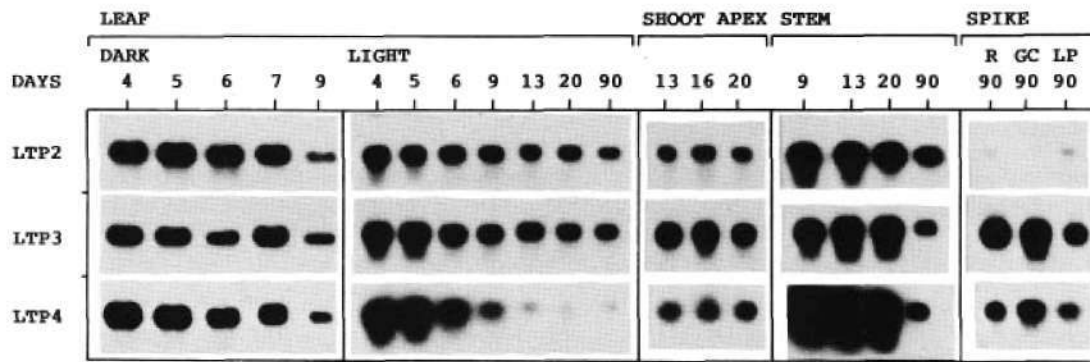


Figure 3. Northern blot analysis of total RNAs (7.5 µg per lane) collected at the indicated ages (days) from different plant organs and tissues. Spikes were separated into rachis (R), lemma plus palea (LP), endosperm plus embryo (not shown) and grain coats (GC). Sequence-specific probes (1×10^7 c.p.m.) were used in each case for the whole set of RNAs and the films exposed overnight. Longer exposures are not shown.

Polyclonal antibodies were raised against a mixture of the previously purified proteins LTP2 and LTP4 and found to recognize all four purified LTPs from barley leaves (not shown). Concentrations of LTPs in different tissues were estimated from the Western blots in the range of $1-4 \times 10^{-5}$ mol kg fresh weight⁻¹.

The distribution of LTPs in different parts of the barley plant was investigated by the tissue-printing technique (Figure 4a-g). These proteins were mainly accumulated in the outer cell layer of exposed surfaces of the plant, throughout the mature embryo, and in vascular tissues. A substantial fraction of the LTPs could be readily extracted just by dipping an intact leaf (without homogenization or infiltration by vacuum treatment) in extraction buffer, indicating an external location of these proteins in the cell walls of the epidermal layer (Figure 5).

Effects of physical and chemical treatments on steady-state levels of LTP mRNAs

Treatment with abscisic acid increased steady-state levels of all three LTP mRNAs in parallel with that of thionin DG3 (Figure 6a), whereas methyl jasmonate had the opposite effect on the LTP mRNAs under conditions that greatly increased the thionin mRNA (Figure 6b). Exposure to ethylene, ethephon, salicylate or 2,6-dichloro-isonicotinic acid had no significant effects on LTP-mRNA levels (not shown).

As it has been reported that some *Ltp* genes respond to cold and to drought in barley (Dunn *et al.*, 1991) and to salinity in tomato (Torres-Schumann *et al.*, 1992), response of the *Ltp* genes, under study, to these environmental factors was investigated. Neither of the genes responded to cold treatment, under conditions that caused a 20-fold induction of the sucrose synthase *Ss1* gene, a gene which has previously been shown to respond to cold (Maraña *et al.*, 1990), or to drought,

under conditions that stimulated four-fold the thionin *Th DG3* gene. A small, but significant increase (about twofold) in the steady-state levels of the mRNAs of LTP2 and LTP4 in response to salinity was observed, and no response was seen to wounding in either of the genes.

Response of LTP genes to fungal infection

Barley line PO3 (cv. Pallas background with gene *Mla-6*) was inoculated with either isolate CC142 (*Av6*) or isolate CC143 (*vir6*) of powdery mildew (*Erysiphe graminis*) by L. Boyd, P.H. Smith and J.K.M. Brown, John Innes Centre, Norwich, UK, and total RNAs were isolated from leaves at different time intervals over 72 h and subjected to Northern blot analysis, using the three sequence-specific LTP-probes and that for thionin DG3 (Figure 7). All three LTP-mRNA levels were significantly increased, especially that for LTP4, and reached a maximum at 12-16 h after inoculation. No significant differences were observed between the effects of the avirulent and the virulent isolates. The mRNA increases observed for the *Ltp* genes were equal or greater than those of the thionin mRNA used for comparison and all of them took place before differences in growth between the virulent and the avirulent isolates could be detected under the microscope, as reported by Boyd (1993).

Discussion

The three cDNA sequences reported here clearly correspond to three of the four LTPs previously purified by us from crude cell wall preparations isolated from young etiolated barley leaves and selected because of their strong inhibitory capacity against plant pathogens *in vitro* (Molina and García-Olmedo, 1991; Molina *et al.*, 1993). The amino acid sequences of these LTPs were similar to other LTPs isolated from ragi seeds or maize

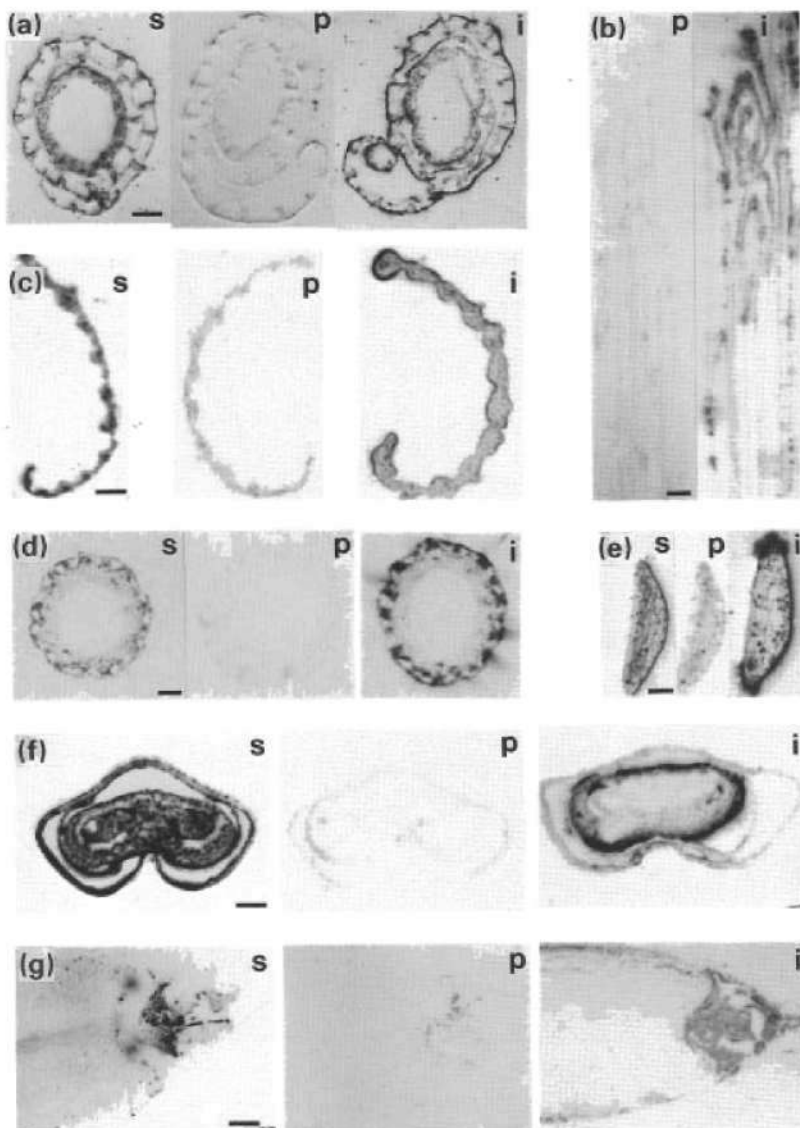


Figure 4. Tissue-printing detection of LTP proteins in different sections of the barley plant using an anti-LTP2/LTP4 polyclonal antibody (i); sections stained with amido black (s) and treated with pre-immune serum (p) were used as controls.

(a) Transverse section of leaves plus stem in the upper part of a 90-day-old plant. Epidermal layer and vascular tissues are differentially immuno-stained. Bar represents 0.4 mm.

(b) Longitudinal section of a 12-day-old plant, including the shoot apex. Bar represents 0.5 mm.

(c) Cross-section of leaf from a 12-day-old plant. The epidermal layer is preferentially immuno-stained. Bar represents 0.5 mm.

(d) Cross-section of stem from the same plant and region as in (a). Epidermal layer and vascular tissue are immuno-stained. Bar represents 0.25 mm.

(e) Cross-section of the rachis from the same plant. Bar represents 0.12 mm.

(f) Transversal section of developing kernel, including lemma and palea. Grain coats, lemma and palea are differentially immuno-stained. Bar represents 0.9 mm.

(g) Longitudinal section of mature kernel hydrated for 12 h. The embryo and the grain coats are immuno-stained. Bar represents 1.5 mm.

endosperm (61–62% identical positions; Campos and Richardson, 1984; Tchang *et al.*, 1988) but quite divergent from that described by Mundy and Rogers (1986) in barley aleurone (48% identical positions) and from that deduced from the cloned cDNA of a barley cold-induced gene (40% identical positions), designated *blt4* (Dunn *et al.*, 1991). However, the *blt4* cDNA would be almost identical (98%) to that encoding LTP2 if a number of insertions are introduced (bases numbers 149, 150, 222, 316, 438–449 and 548 in Figure 1). As the BLT4 protein has not been isolated and characterized, it is not possible to discern whether the divergence is real or is the result of sequencing errors.

The Southern hybridization patterns, as well as the PCR results, were consistent with the presence of four closely related *Ltp* genes in the barley haploid genome,

three of which would be located in chromosome 3H, probably within a few kilobases of each other, as suggested by a common hybridization band for two of the probes, and another in chromosome 7H. It is interesting to note that the gene for the aleurone LTP is in chromosome 5H (Cannell *et al.*, 1992) and that for BLT4 has been reported in chromosome 3H (Dunn *et al.*, 1991). The four members of this LTP subfamily (Cw18, 20, 21 and 22; now called LTP2, 3, 4 and 5) that we had previously purified (Molina *et al.*, 1993) would account for the minimum of four genes detected by Southern hybridization and PCR amplification in the present work, but the additional existence of the BLT4 protein and its gene (as separate entities with respect to those described above) cannot be totally excluded.

Both Northern and Western blot analyses indicate that

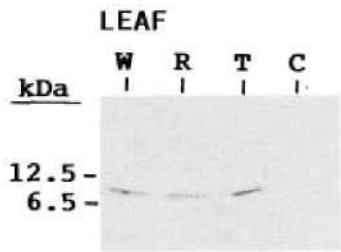


Figure 5. Extraction of LTPs from intact leaves. Western blot analysis of proteins washed from the leaf surface with extraction buffer (W), extracted from the washed leaf by homogenization with the same buffer (R), extracted similarly from the same weight of unwashed leaf (T), and washed from the intact leaf surface (same weight) with electrophoresis sample buffer, containing 6 M urea (C). Proteins were precipitated from wash and extract solutions with saturated ammonium sulfate and then dissolved in electrophoresis sample buffer, except in (C).

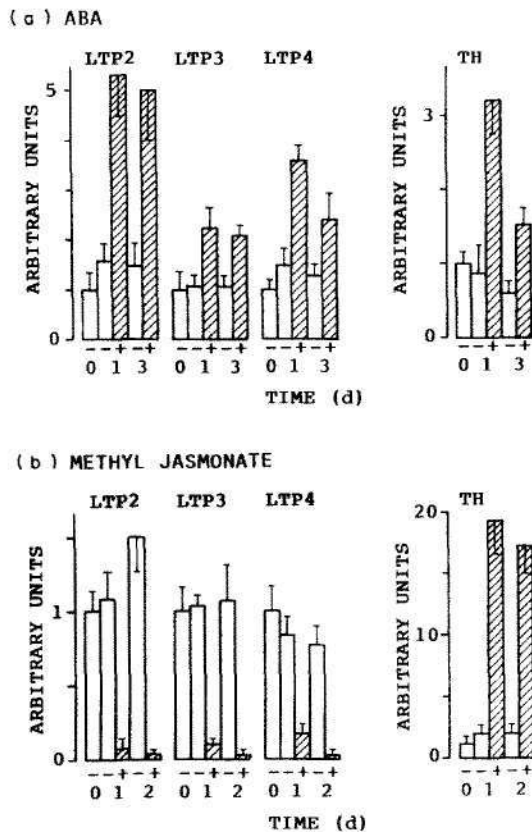


Figure 6. Responses of *Ltp* and *Th* genes to abscisic acid (a) and to methyl jasmonate (b). Three independent filters were processed and analyzed by densitometry. Densitometric reading at time 0 was taken as the arbitrary unit in each case. Values represented are the mean of the three experiments and the bars indicate the standard error of the mean.

the *Ltp* genes studied here are expressed at significant levels in all tissues investigated, except in roots, where they are expressed at very low levels, and in endosperm, where expression is not detected. As judged from immunological staining of tissue prints, in all parts of the

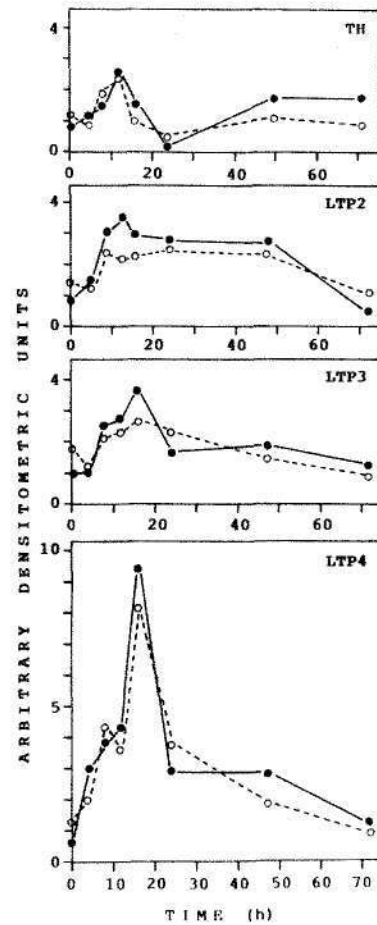


Figure 7. Responses of *Ltp* and *Th* genes to infection by virulent (●) and avirulent (○) isolates of the fungus *E. graminis*.

plant where they are present, the proteins are preferentially located in epidermal cells and in vascular tissues. Their preferential localization in cell walls of peripheral cell layers was corroborated by the fact that a substantial proportion of the proteins could be extracted just by a short dip of the intact organ in extraction buffer. The uneven distribution of LTPs within the different tissues implies that the actual concentrations of these proteins in those cells where they are deposited are indeed very high, well above the average concentrations deduced from the Western blots. The location of LTPs at the exposed surfaces of the plant is well in line with the defense role proposed for them based on their inhibitory properties (Molina *et al.*, 1993). This role would not be incompatible with other developmental role(s).

The negative effect of methyl jasmonate on the expression of *Ltp* genes was simultaneous with the positive one previously described for leaf thionins (Andresen *et al.*, 1992), which has been corroborated in this work, and indicated that the two sets of defense genes can be independently regulated. Jasmonate seems to act as a signaling molecule in stress responses,

including wounding and pathogen attack (Farmer and Ryan, 1990; Staswick, 1992). The lack of induction of *Ltp* genes by wounding and by elicitors of systemic acquired resistance, such as salicylate or 2,6-dichloroisonicotinic acid, further indicate an independent regulation of these genes with respect to other stress responses. Independent regulation does not imply that the two sets of genes cannot be simultaneously elicited, as observed when the plants were treated with ABA.

The response of *Ltp* genes to infection by the fungal pathogen *Erysiphe graminis* is also in agreement with the proposed defense role of the LTPs. This response was in contrast with the relative insensitivity of these genes to physical stresses. The lack of significant differences between the responses to the virulent and the avirulent isolates might mean that the elicitation is gratuitous in this particular plant/pathogen combination, or that a compatible interaction depends on resistance of the virulent isolate to the defense proteins. As in the case of the ABA treatment, simultaneous expression of both *Ltp* and thionin genes was observed.

Experimental procedures

Biological materials

Cultivated barley, *Hordeum vulgare* cv. Bomi, was used throughout this study, except for the inoculation with the virulent (CC143; vir6) and avirulent (CC142; Av6) isolates of *Erysiphe graminis*, which was carried out on barley cv. Pallas (line PO3 with gene Mla6) by Drs L. Boyd, P.H. Smith and K.M. Brown at the John Innes Centre (Norwich, UK). Disomic addition lines of barley cv. Betzes on a Chinese Spring wheat background were the gift of Drs A.K.M.R. Islam and K.W. Shepherd (Glen Osmond, Australia). Probes for the sucrose synthase gene *Ssf1* and the *DG3* thionin gene from barley were supplied by Dr P. Sanchez de la Hoz and by A. Segura (Madrid, Spain).

Cloning of cDNA

A cDNA library was constructed in vector pT7T3-18U (Pharmacia, Uppsala, Sweden) from poly(A)⁺ mRNA (Sambrook *et al.*, 1989) obtained from 7-day-old etiolated barley plants, using the Time Saver cDNA Synthesis Kit according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). The library was screened at 55°C, on Hybond N (Amersham, UK), with two degenerate oligonucleotides corresponding to regions (underlined in Figure 1) of the known amino acid sequences of proteins LTP2 and LTP4 (formerly Cw18 and Cw21; Molina *et al.*, 1993). Plasmid DNA from the selected clones was purified on Magic MiniPreps columns (Promega, Madison, WI) and sequenced according to Hattoni and Sakaki (1986).

DNA and RNA hybridizations

DNAs were isolated essentially as described (Taylor and Powell, 1982), digested with *EcoRI* endonuclease, subjected to

electrophoresis in 0.8% agarose, and transferred to Hybond N membranes (Amersham, UK) following standard procedures.

Hybridizations were carried out at 65°C in 0.5 M NaPO₄, 1% SDS, 1 mM EDTA, 100 µg ml⁻¹ salmon sperm DNA, and washed according to Sambrook *et al.* (1989). Polymerase chain reactions were carried out (94°C, 1 min; 56°C, 1 min; 72°C 1 min) with genomic DNA (100 ng per reaction) following standard procedures (Sambrook *et al.*, 1989), and the reaction products were subjected to electrophoresis in 1.8% agarose gel.

RNAs were purified from frozen tissues by phenol/chloroform extraction, followed by precipitation with 3 M lithium chloride (Lagrimini *et al.*, 1987). Electrophoresis was carried out on 5% formaldehyde/agarose gels, which were blotted to Hybond N membranes (Amersham, UK). Hybridization and washing were carried out at 65°C according to Church and Gilbert (1984). Ethidium bromide (40 µg ml⁻¹) was included in the sample loading buffer to allow photography. Equal sample loads were checked by densitometry (Joyce and Loeb microdensitometer, Gateshead, UK) of the negatives of the U.V. photographs. Quantitation of radioactive signals in Northern blot experiments was carried out by densitometry of three independent filters.

Hybridization probes were prepared either from the full inserts, or from their divergent 3'-ends (indicated in Figure 1), using the Random Primer Labeling Kit (Boehringer Mannheim, Germany) and [³²P]-ATP.

Western blots and tissue printing

Polyclonal antibodies against a mixture of proteins LTP2 and LTP4 were produced in rabbits (BAbCO, Richmond, CA). Proteins were extracted from different plant tissues and organs (5 mg fresh weight) with 2 volumes of electrophoresis sample buffer (Laemmli, 1970), subjected to electrophoresis on 12% SDS-polyacrylamide gels (Laemmli, 1970), and transferred to PVDF membranes (Immobilon, Millipore, Bedford, MA) according to Towbin *et al.* (1979). Intact leaves (15 mg) were washed with 3 volumes of extraction buffer (1.5 M LiCl, 0.01% SDS) for 15 min. The washing liquid was saturated with ammonium sulfate and the pellet resuspended in electrophoresis sample buffer. The washed leaf and an intact leaf (15 mg) were homogenized in extraction buffer and the extracts were processed as above.

Tissue-printing was carried out as described Hood *et al.* (1991), using pre-immune serum and amido black staining on control prints. Both for Western blots and for tissue prints, the first antibody was used at a 1/500 dilution and the anti-rabbit antibody coupled to alkaline phosphatase (Sigma, St Louis, MO) was used at a 1/5000 dilution. Visualization was with Nitroblue tetrazolium (Sigma, St Louis, MO) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (Sigma, St Louis, MO).

External treatments

Low-temperature treatment was carried out essentially as described (Dunn *et al.*, 1991): 15-day-old plants grown on vermiculite (22°C day/18°C night; 16 h light) were exposed to 4°C. Shoot apex and leaves were collected 2 and 7 days after. Water deprivation was achieved by leaving 7-day-old plants, grown as before, on the bench until the appearance of clear symptoms of wilting (12 h). Salinity treatment was carried out by irrigating 3-day-old plants with 0.34 M NaCl as described by Ramagopal (1987) and collecting leaves 18 h later. Abscisic acid (*cis-trans* isomer; Sigma, St Louis, MO) was sprayed (0.1 mM)

on 7-day-old plants at 6 h intervals during 24 h and leaf samples were collected at 1 day and 3 days. Leaves from 7-day-old plants were floated on a 10 μ M solution of methyl jasmonate (Bedoukian Research Inc., Danbury, CN) as described by Andresen *et al.* (1992) and collected at 1 day and 2 days. The experiment with sodium salicylate (1 mM; Sigma, St Louis, MO) was performed as that with abscisic acid. Ethephon treatment (20 mM in 0.05% v/v Tween 20) was also carried out by spraying 7-day-old plants and collecting leaves at 8 h, 1 day, 2 days, 3 days and 4 days. Ethylene (100 p.p.m) was applied on 7-day-old plants in a confined atmosphere during 17 h and 46 h. A solution of 2,6-dichloroisonicotinic acid (4 p.p.m.; Ciba-Geigy, Basel) was formulated with a wettable powder carrier and applied as a soil drench to 7-day-old plants; the wettable powder alone was used for the mock treatment. Leaves were collected at 2 days and 5 days after treatment.

Infection with *Erysiphe graminis*

Barley was planted two seeds per pot and grown according to the method of Martinelli *et al.* (1993). Plant material was inoculated in steel settling towers. Mildew spores were blown into the tower and allowed 2–5 min to settle on to the plants placed at the bottom of the tower. Spore inoculation densities were about 50 spores mm^2 . Leaf samples were taken up to 72 h after inoculation and immediately frozen in liquid nitrogen.

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