

# Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2

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## Summary

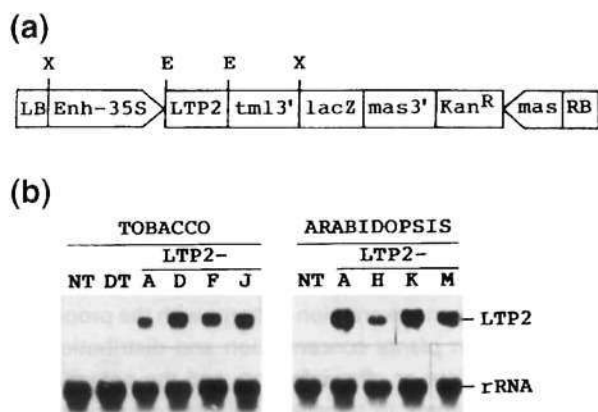
Purified lipid transfer protein LTP2 from barley applied on tobacco leaves eliminated symptoms caused by infiltration of *Pseudomonas syringae* pv. *tabaci* 153. Growth of the pathogen in leaves of transgenic tobacco plants was retarded when compared with non-transformed controls. The percentage of inoculation points that showed necrotic lesions was greatly reduced in transgenic tobacco (17–38% versus 78%) and the average size of these lesions was 61–81% that of control. The average total lesion area (necrosis and chlorosis) in the transgenic plants was also reduced (38% of control). *Arabidopsis thaliana* transgenic plants inoculated with *P. syringae* pv. *tomato* DC3000 also had lower percentages of necrotic lesions (22–38% versus 76%), a reduced average area for each lesion (53–67% of control), and a smaller total lesion area per inoculation (43% of control). These results further support the assignment of a defense role for LTPs and highlight their biotechnological potential.

## Introduction

It is generally assumed that a wide variety of proteins play different roles in the defense of plants against bacterial and fungal pathogens (Bowles, 1990). Thus, it has been proposed that some proteins enhance the strength of the extracellular matrix for passive resistance, others are enzymes involved in the biosynthesis of toxic compounds, such as phytoalexins, and a third group includes those that have direct anti-microbial activity. An increasing number of potential members of the latter group have been identified: thionins (Bohlman *et al.*, 1988; Fernández de Caleyra *et al.*, 1972; García-Olmedo *et al.*, 1992; Molina *et al.*, 1993a), glucanases and chitinases (Leah *et al.*, 1991; Mauch *et al.*, 1988), ribosome-inactivating proteins (Leah *et al.*, 1991), thaumatin-like proteins (Hejgaard *et al.*, 1991), zeamatin (Vigers *et al.*, 1991), osmotins (Woloshuck *et al.*, 1991), lipid transfer proteins (García-Olmedo *et al.*, 1995; Molina and García-Olmedo, 1991; Molina *et al.*, 1993b; Segura *et al.*, 1993; Terras *et al.*, 1992a), defensins (Moreno *et al.*,

1994; Terras *et al.*, 1992b), and others. However, the capacity to inhibit pathogens *in vitro* is not sufficient to postulate an *in planta* defense role for a particular protein, and additional criteria have been often used, such as the congruence of gene expression patterns with the proposed function, the *in planta* concentration and distribution of the protein before or after infection, and the potential to decrease symptoms in transgenic plants overexpressing the protein. The latter type of evidence, which is especially relevant, has been now provided for a number of proteins, including glucanases and chitinases (Broglie *et al.*, 1991; Jach *et al.*, 1995; Lin *et al.*, 1995; Zhu *et al.*, 1994), ribosome-inactivating proteins (Jach *et al.*, 1995; Logemann *et al.*, 1992), thionins (Carmona *et al.*, 1993), PR-1a (Alexander *et al.*, 1993), osmotin (Liu *et al.*, 1994), and defensins (Terras *et al.*, 1995).

Evidence supporting a defensive role for plant non-specific lipid transfer proteins (LTPs) has recently been reviewed (García-Olmedo *et al.*, 1995). These proteins were so named because of their ability to stimulate the transfer of a broad range of lipids between membranes *in vitro* (Arondel and Kader, 1990; Yamada, 1992). However, a cytoplasmic role is excluded for LTPs because they are generally secreted (Mundy and Rogers, 1986; Sterk *et al.*, 1991) and externally associated with the cell wall (Molina and García-Olmedo, 1991, 1993; Molina *et al.*, 1993b; Pye *et al.*, 1994; Segura *et al.*, 1993; Thoma *et al.*, 1993). It has been speculated that they might be involved in the secretion or deposition of extracellular lipophilic materials, such as cutin or wax (Pye *et al.*, 1994; Sterk *et al.*, 1991; Thoma *et al.*, 1993; Hendriks *et al.*, 1994). The *in vitro* antibiotic properties of LTPs against bacterial and fungal plant pathogens are now well established (Molina and García-Olmedo, 1991; Molina *et al.*, 1993b; Segura *et al.*, 1993; Terras *et al.*, 1992a) and their distribution at high concentrations over exposed surfaces and in the vascular tissue has been demonstrated (Fleming *et al.*, 1992; Molina and García-Olmedo, 1993; Pye *et al.*, 1994; Thoma *et al.*, 1993). Expression of *Ltp* genes has been shown to be induced well above basal levels in some plant-pathogen interactions, while in others the genes can be switched off by the pathogen (García-Olmedo *et al.*, 1995; Molina and García-Olmedo, 1993, 1994). We now present evidence that transgenic overexpression of the barley LTP2 protein under the control of a constitutive promoter in tobacco and *Arabidopsis* enhances tolerance to pathogens. This evidence further supports the assignment of a defensive role to LTPs and demonstrates their biotechnological potential in engineering plants that are resistant to pathogens.



**Figure 1.** Transgenic expression of *LTP2* from barley in tobacco and *Arabidopsis*.

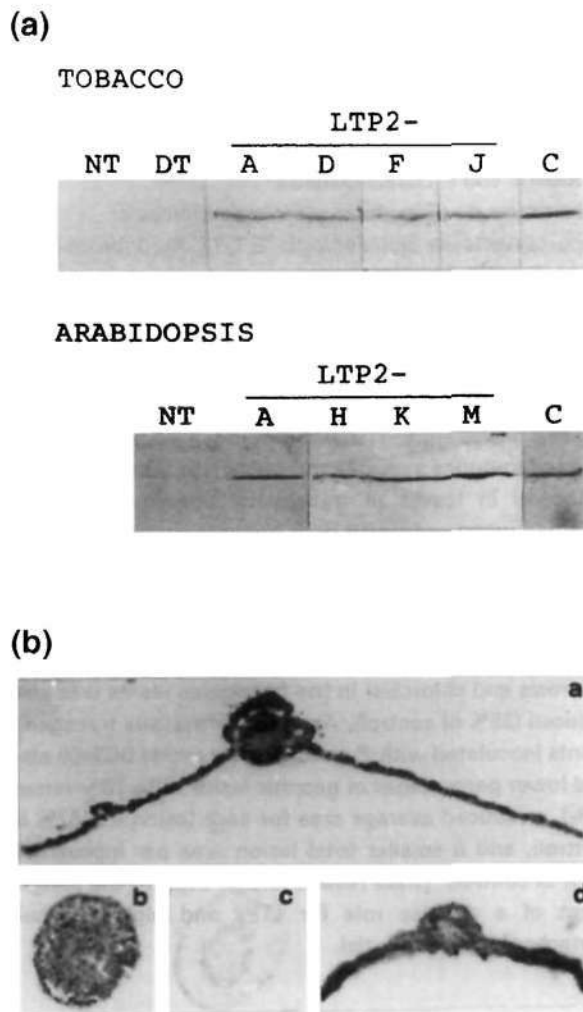
(a) Schematic representation of T-DNA in plasmid pCIB200LTP2. LB, left border; Enh-35S, enhanced 35S promoter; LTP2, barley LTP2 cDNA; tml3', *tml* gene 3' untranslated sequence; lacZ, sequence of the  $\beta$ -galactosidase  $\alpha$ -fragment; mas3', mannopine synthase 3' untranslated sequence; Kan<sup>R</sup>, neomycin phosphotransferase II gene conferring kanamycin resistance; mas, mannopine synthase promoter; RB, right border. The restriction sites for the endonucleases *Xba*I (X) and *Eco*RI (E) used for cloning are indicated. (b) Northern blot analysis (10  $\mu$ g of total RNA per lane) of non-transformed (NT) and transformed plants (LTP2-); plus a transformant with a partially deleted LTP2 cDNA (DT). Four independent homozygous lines of tobacco (A, D, F, J) and *Arabidopsis* (A, H, K, M) were tested. Filters were hybridized with a specific LTP2 probe. A 25S rRNA probe was used as sample loading control.

## Results

### Generation of LTP2 transgenic tobacco and *Arabidopsis*

To evaluate the defense potential of LTPs, transgenic tobacco and *Arabidopsis* plants were obtained that expressed the *LTP2* from barley under the control of a strong, constitutive promoter (Figure 1a). The nucleotide sequence encoding the signal peptide was included in the gene fusion to mediate export of the protein to the cell wall (Molina and García-Olmedo, 1993). Four homozygous lines with high mRNA levels from each species were chosen for further study (Figure 1b). These lines showed no differences in their morphology and growth characteristics with respect to non-transformed plants. Concentrations of LTP2 in the leaves of the selected homozygous lines range from 3–10  $\mu$ mol kg<sup>-1</sup>, as estimated from Western blots (Figure 2a). In tobacco, plants transformed with a partially deleted LTP2 cDNA sequence, which lacked the polyadenylation signal and did not produce detectable mRNA or protein, were used as negative controls.

Tissue-print analysis of the transgenic plants showed that, as expected, the protein was evenly distributed across leaf and stem sections (Figure 2b). This was in contrast with its location in barley, where it is preferentially distributed in the epidermis of exposed surfaces and in the vascular tissue (Molina and García-Olmedo, 1993), as well as with the epidermal location of endogenous LTPs in



**Figure 2.** Analysis of LTP2 expression in transgenic plants using a polyclonal antibody.

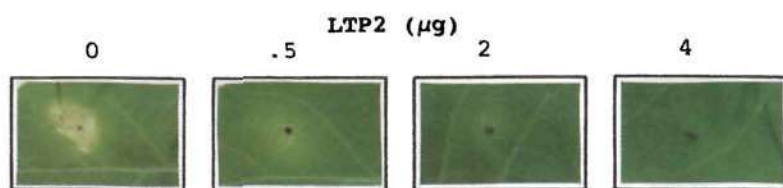
(a) Western blot of protein extracts from tobacco and *Arabidopsis*: NT, non-transformed; DT, partially deleted LTP2 transformant; LTP2-, LTP2 transformed homozygous lines; C, purified LTP2 protein (150 ng). (b) Tissue-printing detection of LTP2 protein in tobacco and *Arabidopsis*. Transverse sections of tobacco leaf (a) and stem (b) from transformant, stem from non-transformed tobacco (c), and leaf from *Arabidopsis* transformant (d).

tobacco (Fleming *et al.*, 1992) and *Arabidopsis* (Thoma *et al.*, 1993).

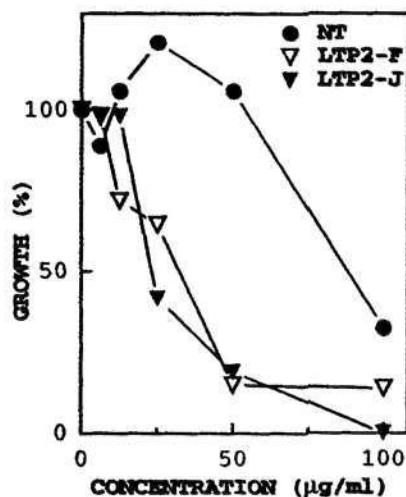
### Evaluation of LTP2 transgenic tobacco

The tobacco-pathogenic, LTP-sensitive bacterium *Pseudomonas syringae* pv. *tabaci* 153 was chosen to carry out detailed pathogenicity tests in tobacco as in initial tests it produced consistent symptoms in non-transformed tobacco cv. Xanthi, which could be accurately measured. These symptoms disappeared in a concentration-dependent manner when the purified barley LTP2 protein (Molina *et al.*, 1993b) was simultaneously applied with the inoculum to the leaf surface of non-transformed tobacco (Figure 3a).

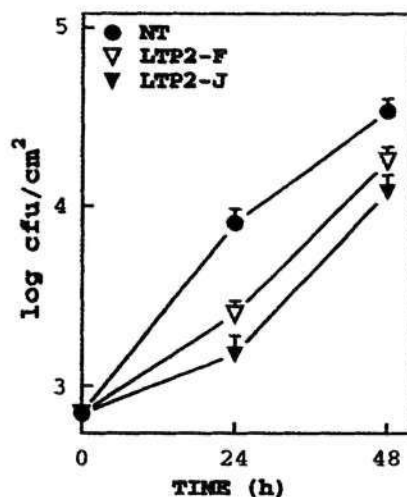
(a)



(b)



(c)



**Figure 3.** LTP2 effects on *Pseudomonas syringae* pv. *tabaci* 153.

(a) Effect of treatment of the tobacco surface with LTP2 on symptoms caused by the bacterium.

(b) *In vitro* inhibition of the bacteria by tobacco cell-wall protein extracts of non-transformed plants (NT) and LTP2 transformants (LTP2-). Concentrations of total cell-wall protein are given; LTP2 contents of extracts from transformed leaves were estimated by Western blot as 10–12% of total protein in the extract.

(c) Bacterial growth in leaves of non-transformed (NT) and transformed (LTP2-) tobacco. This experiment is one of three performed which gave similar results. Standard errors of the mean are represented by bars. Growth differences of the bacterium in the LTP2- versus the NT plants were significant ( $P < 0.05$ ).

Cell-wall extracts from LTP2 transgenic plants, which were checked for LTP2 content by Western blot (not shown), inhibited bacterial growth *in vitro* at concentrations at which extracts from non-transformed tobacco had no effect (Figure 3b). Furthermore, growth of the bacterium on the leaves of transgenic plants was retarded with respect to that in non-transformed leaves (Figure 3c).

Plants from the four selected tobacco transformed homozygous lines were infiltrated with *P. syringae* pv. *tabaci* 153 and the percentage of inoculation points in which necrosis could be detected was recorded over a period of 12 days (Figure 4). In preliminary experiments, the inoculum was adjusted to produce detectable necrosis in about 80% of the inoculations performed on non-transformed tobacco under the described experimental conditions. The four transformants presented significantly lower percentages of necrotic lesions (17–38%) compared with non-transformed tobacco control plants (78%) and the average area of these necrotic lesions was also reduced (61–81% of control). At the end of the experiment, the average total lesion area (necrosis plus chlorosis) per inoculation was measured and a reduction to 38% of non-transformed control values ( $P < 0.001$ ) was again observed in the transformants (Figure 5).

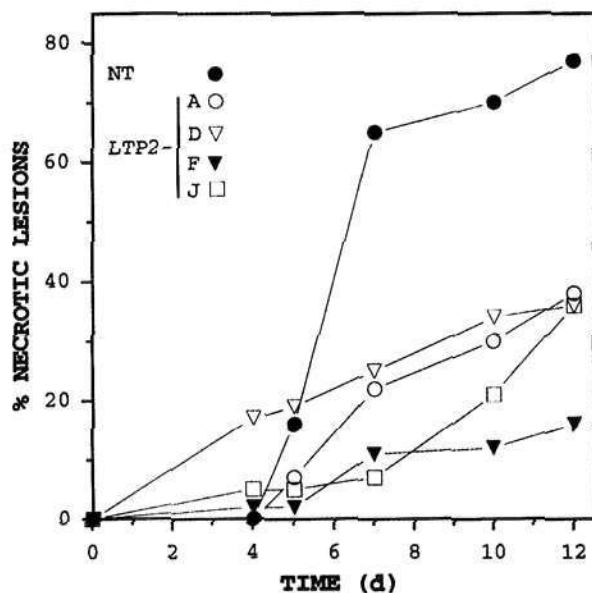
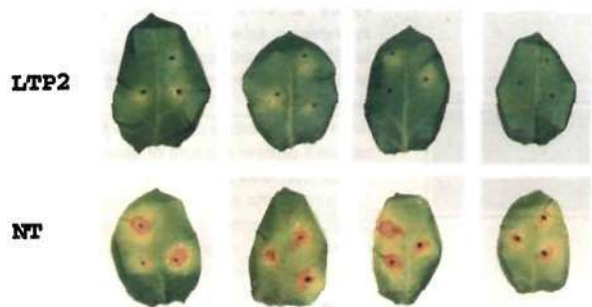
#### Evaluation of LTP2 transgenic Arabidopsis

The protective effect of LTP2 was also investigated in transgenic *Arabidopsis* using the virulent, LTP2-sensitive bacterium *P. syringae* pv. *tomato* DC3000 and following the same criteria as for the tobacco/*P. syringae* pv. *tabaci* 153 experiments described above. The results, which are summarized in Figures 6 and 7, show clear decreases in symptoms in the LTP2 transgenic plants compared with the non-transformed controls: lower percentages of necrotic lesions (22–38% versus 76%), reduced necrotic area per necrotic lesion (53–67% of control), and smaller total (necrotic plus chlorotic) lesion area per inoculation (43% of control;  $P < 0.001$ ).

#### Discussion

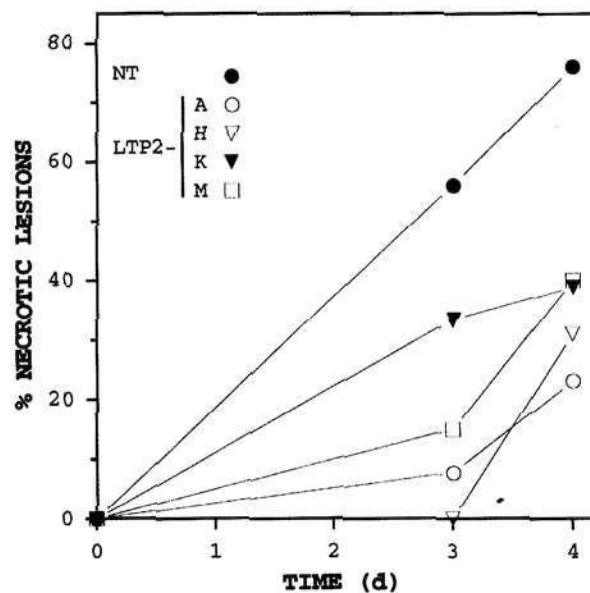
It has been shown that there are considerable differences in sensitivity to LTPs among different pathogenic bacterial and fungal species – or even among strains or isolates of the same species – and that differences in sensitivity towards LTP variants against a given pathogen are comparatively small (Molina and García-Olmedo, 1991; Molina *et al.*, 1993b; Segura *et al.*, 1993). The mechanism of action





**Figure 4.** Challenge of transgenic tobacco plants with *P. syringae* pv. *tabaci* 153. Representative lesions caused by the bacteria 12 days after inoculation in non-transformed (NT) and LTP2-transformed leaves (LTP2-) are shown. Percentages of necrotic lesions were scored at the times indicated. At least 42 inoculation points and 14 plants were treated for each transformant or control. This experiment is one of three performed which gave similar results.

of LTPs is not yet well understood, but it seems to be different from those of other plant defense proteins, such as thionins or defensins, as judged from the differences in activity spectra (Molina and García-Olmedo, 1994). Based on these observations, transgenic expression of an LTP-coding sequence under a strong, constitutive promoter should not essentially change the specificity of the LTP defense barrier already in existence in the recipient plant, but would reinforce and extend it by increasing basal LTP levels in epidermal regions and by leading to significant LTP accumulation in places with little or no endogenous LTPs. Tissue-print expression analysis of the transgenic plants and the increased antibiotic activity of their cell-wall protein extracts reported here indicate that this was the case. This pattern of expression would enhance resistance to pathogens by interfering with their penetration, propagation and movement inside the plant. In the case of bacteria used in this study, the pattern of transgenic expression

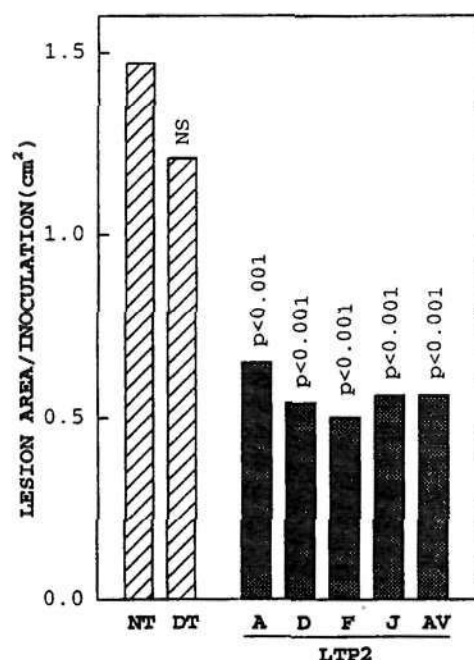


**Figure 6.** Challenge of transgenic *Arabidopsis* plants with *P. syringae* pv. *tomato* DC3000. Representative lesions caused by the bacteria 4 days after inoculation in non-transformed plants (NT) and LTP2 transformants (LTP2-) are shown. Percentages of necrotic lesions were scored at the times indicated. At least 15 inoculation points and 15 plants were treated for each transformant or control. This experiment is one of three performed with similar results.

would not only interfere with growth on the leaf surface but also prevent progress through stomata and small lesions. The results of the analysis of bacterial growth showed that the propagation of bacteria on leaves of the transgenic plants was retarded with respect to that on non-transformed plants.

Evidence has been presented indicating that externally added, purified LTP2 is effective in preventing the symptoms caused by inoculation of the bacterial pathogen on the leaf surface. This does not exclude the possibility that correlation between *in vitro* and *in planta* LTP inhibitory properties might not be found for other pathogens because of possible differences in their growth characteristics in liquid media compared with plant tissues. Such differences have been shown, for example, in the case of thionins (Molina *et al.*, 1993a).

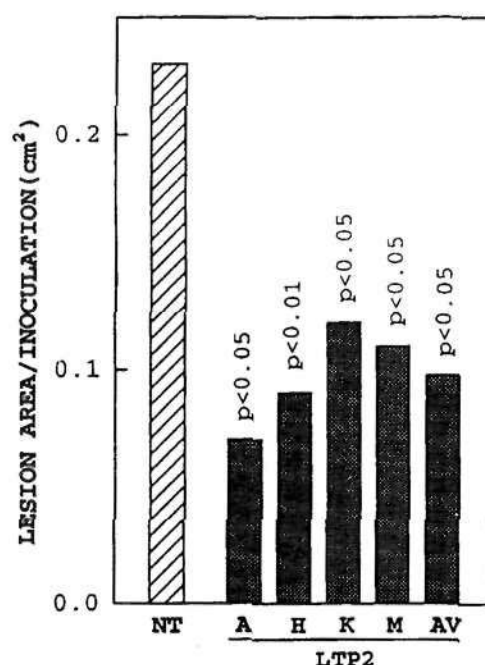
The reductions of *P. syringae* symptoms caused by LTP2



**Figure 5.** Total lesion area (chlorosis plus necrosis) per inoculation caused in tobacco plants by *P. syringae* pv. *tabaci* 153 in the experiment described in Figure 4. Significance was calculated by the pairwise *t* test. NT, non transformed; DT, transformed with a partially-deleted LTP2 cDNA; (A, D, F, J), independent transformants; AV, average for transformed plants.

overexpression in tobacco and *Arabidopsis* are similar and of the same order of those observed for other pathogens in transgenic plants overexpressing other plant antibiotic proteins (Alexander *et al.*, 1993; Carmona *et al.*, 1993; Jach *et al.*, 1995; Lin *et al.*, 1995; Liu *et al.*, 1994; Logemann *et al.*, 1992; Terras *et al.*, 1995). This evidence further supports the assignment of a defensive role to LTPs and is in line with recent observations indicating that LTP-sensitive mutants of *P. solanacearum* are avirulent (Titarenko, E., López-Solanilla, E., García-Olmedo, F. and Rodríguez-Palenzuela, P., unpublished results). The role of LTPs as active components of both constitutive (Molina and García-Olmedo, 1993; Molina *et al.*, 1993b; Segura *et al.*, 1993) and inducible (García-Olmedo *et al.*, 1995; Molina and García-Olmedo, 1993, 1994) defense barriers does not exclude the possibility that some or all of the members of this protein family might have other functions in response to various stresses (Hughes *et al.*, 1992; Plant *et al.*, 1991; Torres-Schumann *et al.*, 1992), during development (Stern *et al.*, 1991; Tchang *et al.*, 1988), or during the course of normal metabolism (Arondel and Kader, 1990; Yamada, 1992).

As recently reviewed (Lamb *et al.*, 1992), strategies to engineer plants against pathogens are based on the manipulation of either (i) regulatory mechanisms (i.e. signal perception and transduction), (ii) multi-gene defense mechanisms (i.e. biosynthesis of phytoalexins), or



**Figure 7.** Total lesion area (necrotic plus chlorotic) per inoculation caused in *Arabidopsis* plants by *P. syringae* pv. *tomato* DC3000 in the experiment described in Figure 6. Significance was calculated by the pairwise *t* test. NT, non transformed; (A, H, K, M), independent transformants; AV, average for transformed plants.

(iii) single-gene defense mechanisms. Our results demonstrate the potential use of *Ltp* genes in the context of the latter strategy. Other single proteins from plants as well as from non-plant sources (Lamb *et al.*, 1992; Shah *et al.*, 1995) have been shown to significantly enhance tolerance to pathogens. Gene combinations involving more than one of these proteins can be used in practice both to take advantage of synergistic effects and to create more polyvalent barriers (Jach *et al.*, 1995; Leah *et al.*, 1991; Mauch *et al.*, 1988; Terras *et al.*, 1993; Zhu *et al.*, 1994). In this context, it should be noted that synergism of LTPs with thionins has been demonstrated *in vitro* (Molina *et al.*, 1993b).

## Experimental procedures

### Transgenic plants

A previously described LTP2 cDNA clone from barley containing the LTP2 signal peptide and mature protein-coding regions (Molina and García-Olmedo, 1993) was subcloned into the *EcoRI* site of pCGN1716 (Comai *et al.*, 1990) between the enhanced 35S promoter and the *tm/3'* terminator (Figure 1). The cassette containing the cDNA in a sense orientation was then subcloned into the *XbaI* site of pCIB200, a binary vector containing the neomycin phosphotransferase II gene that confers kanamycin resistance, resulting in plasmid pCIB200LTP2. A gene construction including a 3' partially deleted LTP2 cDNA clone was generated in a similar manner. The plasmids were transformed into *Agrobacterium tumefaciens* strain CIB542 (Hood *et al.*, 1996) according to the

procedure of Wen-jun and Forde (1989). Leaf disks of *Nicotiana tabacum* cv. Xanthi were inoculated with the *A. tumefaciens* strains and selected on kanamycin (Horsch *et al.*, 1985). A single shoot ( $T_1$  generation) was regenerated from each leaf disk and grown in soil until seed set. Seeds ( $T_2$  generation) resulting from self-pollination of the regenerated transformants were scored for antibiotic resistance on MS medium (Sigma, St Louis, MO) containing 100 mg l<sup>-1</sup> of kanamycin. Those giving a 3:1 (resistant: susceptible) segregation for the kanamycin marker were checked for expression by the Northern and Western blot techniques. Homozygous lines were identified by screening ten kanamycin-resistant  $T_2$  progeny from each independent transformant ( $T_3$  generation) for non-segregation of the marker. Roots of 10–14-day-old *A. thaliana* ecotype Dijon O plants were inoculated with *A. tumefaciens* and selected on kanamycin (25 mg l<sup>-1</sup>) as described by Huang and Ma, (1992).  $T_3$  homozygous lines were selected as above.

### Analysis of RNA and protein

Total RNA (10 µg) was extracted from leaves of tobacco or *Arabidopsis*, subjected to electrophoresis on 1.5% formaldehyde/agarose gels, blotted to Hybond N membranes (Amersham, UK) and hybridized at 65°C with a specific barley LTP2 probe as previously described (Molina and García-Olmedo, 1993). Proteins were extracted from leaves (150 mg fresh weight) of tobacco or *Arabidopsis* with three volumes of sample buffer, subjected to electrophoresis on 12% SDS-polyacrylamide gels (Laemmli, 1970), and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA) according to Towbin *et al.* (1979). Western blots and tissue prints were carried out as previously described (Molina and García-Olmedo, 1993).

### Tests with pathogens

Transformants and non-transformed controls were grown in sterile soil in multi-well plastic containers that were kept in a growth chamber (Heraeus, Balingen, Germany) at 75% relative humidity, 25°C, 16 h (day) and 65% relative humidity, 23°C, 8 h (night) for tobacco, and at 75% relative humidity, 23°C, 12 h (day) and 65% relative humidity, 21°C, 12 h (night) for *Arabidopsis*. Tobacco inoculation with *P. syringae* pv. *tabaci* 153 (15 µl,  $2 \times 10^5$  c.f.u. ml<sup>-1</sup> in 10 mM MgCl<sub>2</sub>) was carried out by infiltration into the abaxial side of intact leaves from 12-week-old plants with a plastic syringe without a needle, and inoculation points were labeled by ink marks on the upper surface (Figure 4). Five-week-old *Arabidopsis* plants were similarly inoculated with the bacteria *P. syringae* pv. *tomato* DC3000 (15 µl  $5 \times 10^5$  c.f.u. ml<sup>-1</sup> in 10 mM MgCl<sub>2</sub>). After inoculation, plants were kept at 85% (day) and 75% (night) relative humidity.

Barley LTP2 protein was purified as previously described (Molina *et al.*, 1993b) and co-infiltrated with *P. syringae* pv. *tabaci* (200 c.f.u. per inoculation) in the abaxial side of the leaves of 12-week-old non-transformed tobacco, using a plastic syringe without a needle; symptoms were evaluated 12 days after infiltration. Cell-wall extracts containing LTPs were obtained from tobacco leaves, and used for *in vitro* inhibition tests as described by Molina *et al.* (1993b). Bacterial growth in leaves was monitored by harvesting and homogenizing at each stage three leaves which had been previously infiltrated with 750 c.f.u. of the bacterium. Appropriate 10-fold dilutions were plated in nutrient broth agar (Difco, Detroit, MI) containing the selection antibiotic rifampycin (100 µg ml<sup>-1</sup>) and colonies were counted.

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