Mol Gen Genet (1996) 252:162–168

ORIGINAL PAPER

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Two cold-inducible genes encoding lipid transfer protein LTP4 from barley show differential responses to bacterial pathogens

Received: 7 February 1996 / Accepted: 3 April 1996

Abstract The barley genes *HvLtp4.2* and *HvLtp4.3* both encode the lipid transfer protein LTP4 and are less than 1 kb apart in tail-to-tail orientation. They differ in their non-coding regions from each other and from the gene corresponding to a previously reported *Ltp4* cDNA (now *Ltp4.1*). Southern blot analysis indicated the existence of three or more *Ltp4* genes per haploid genome and showed considerable polymorphism among barley cultivars. We have investigated the transient expression of genes *HvLtp4.2* and *HvLtp4.3* following transformation by particle bombardment, using promoter fusions to the β-glucuronidase reporter sequence. In leaves, activities of the two promoters were of the same order as those of the sucrose synthase (*Ss1*) and cauliflower mosaic virus 35S promoters used as controls. Their expression patterns were similar, except that *Ltp4.2* was more active than *Ltp4.3* in endosperm, and *Ltp4.3* was active in roots, while *Ltp4.2* was not. The promoters of both genes were induced by low temperature, both in winter and spring barley cultivars. Northern blot analysis, using the *Ltp4*-specific probe, indicated that *Xanthomonas campestris* pv. *translucens* induced an increase over basal levels of *Ltp4* mRNA, while *Pseudomonas syringae* pv. *japonica* caused a decrease. The *Ltp4.3-Gus* promoter fusion also responded in opposite ways to these two compatible bacterial pathogens, whereas the *Ltp4.2-Gus* construction did not respond to infection.

Key words Bacterial pathogens · Barley · Lipid transfer proteins · Low temperature · Promoter analysis

Communicated by H. Saedler

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Introduction

Non-specific lipid transfer proteins (LTPs) that stimulate the transfer of various lipids between membranes and artificial vesicles in vitro have been isolated from a wide range of plant sources (see Arondel and Kader 1990; Yamada 1992). A cytoplasmic role has been excluded for LTPs because they are generally secreted (Mundy and Rogers 1986; Sterk et al. 1991) and are associated with the cell wall (Molina et al. 1993; Molina and García-Olmedo 1993; Segura et al. 1993; Thoma et al. 1993; Pyee et al. 1994). Their possible involvement in the secretion or deposition of extracellular lipophilic molecules, such as cutin or wax, is under investigation (Sterk et al. 1991; Meijer et al. 1993; Hendriks et al. 1994; Pyee et al. 1994). Apart from their developmentally regulated expression, *Ltp* genes have been reported to respond to environmental stresses in tomato (Plant et al. 1991; Torres-Schumann et al. 1992; Kahn et al. 1993) and to cold treatment in barley (Dunn et al. 1991, 1994; Hughes et al. 1992; White et al. 1994). Evidence in support of a defense role for LTPs has been recently reviewed (García-Olmedo et al. 1995). These proteins are active against both bacterial and fungal pathogens in vitro (Molina and García-Olmedo 1991; Terras et al. 1992; Molina et al. 1993; Segura et al. 1993), and are distributed over exposed surfaces and in the vascular tissues, at basal concentrations that can be inhibitory for sensitive pathogens (Molina and García-Olmedo 1993; Pyee et al. 1994). Genes encoding these proteins respond to bacterial and fungal infections in a complex manner (Molina and García-Olmedo 1994; García-Olmedo et al. 1995, 1996). Furthermore, transgenic plants expressing LTP show enhanced tolerance to a variety of pathogens (Molina et al., in preparation) and LTP-sensitive mutants of bacterial pathogens have been shown to be avirulent (Titarenko et al., in preparation).

In barley, one aleurone-specific and four ubiquitous LTPs have been purified (Mundy and Rogers 1986; Molina et al. 1993), and several of the corresponding

cDNAs and genes have been cloned (Linnestead et al. 1991; Skriver et al. 1992; Dunn et al. 1991; Molina and García-Olmedo 1993; Gausing 1994; White et al. 1994). Leaf-expressed *Ltp* genes have been shown to be induced by pathogens (Molina and García-Olmedo 1993; García-Olmedo et al. 1995) and although a cold response was observed in the winter barley cv. Igri (Dunn et al. 1991; Hughes et al. 1992; White et al. 1994), such a response was not observed in spring barley cv. Bomi (Molina and García-Olmedo 1993).

We have now characterized two *Ltp* genes, *HvLtp4.2* and *HvLtp4.3*, both of which encode the same mature protein as a previously reported LTP4 cDNA (Molina and García-Olmedo 1993), whose corresponding gene (now *HvLtp4.1*; syn. *gblt4.2* from White et al. 1994) is different from *HvLtp4.2* and *HvLtp4.3*. Gene *HvLtp4.2* is practically identical to that in clone *gblt4.9* (White et al. 1994), whereas *HvLtp4.3* is significantly divergent. We have investigated the activity of these two promoters, using fusions to the β-glucuronidase reporter (GUS) gene and particle bombardment, and have found that while both promoters are induced by cold treatment, only one of them (*HvLtp4.3*) responds to pathogen infection.

Materials and methods

Cloning of *HvLtp4.2* and *HvLtp4.3*

Lambda EMBL3 recombinant clones (1.2×10^6) of a genomic library from *Hordeum vulgare* L. var. NK1558 (spring habit; Clontech), were transferred to Hybond N membranes (Amersham) and hybridized at 65° C with the cDNA probe (Molina and García-Olmedo 1993) following standard procedures (Sambrook et al. 1989). Two of the selected clones (λ*ltp4.11* and λ*ltp4.14*) were purified through two further rounds of screening and restriction mapped. The *Bam*HI fragments hybridizing with the probe were subcloned into pBluescript II KS (Stratagene) and sequenced according to Hattoni and Sakaki (1986).

DNA and RNA hybridization

DNA was isolated essentially as described (Taylor and Powell 1982), restricted with *Bam*HI endonuclease, subjected to electrophoresis in 0.8% agarose, and transferred to Hybond N membranes (Amersham) following standard procedures. Hybridization at 65° C was according to Church and Gilbert (1984).

RNA was purified from frozen tissues by phenol/chloroform extraction, followed by precipitation with 3 M lithium chloride (Lagrimini et al. 1987), and subjected to electrophoresis (7.5 µg each) in 7.5% formaldehyde/agarose gels, which were blotted to Hybond N membranes (Amersham). Hybridization was carried out at 65° C as above. Ethidium bromide (40 µg/ml) was included in the loading buffer to allow visualization under UV light and photography. Equal sample loads were checked by densitometry (INH Image programme, Bio-Rad) of the negatives of the UV photographs. Quantitation of radioactive signals in Northern-blot experiments was carried out by densitometry (INH Image programme, Bio-Rad) of the autoradiographs from three independent filters.

Gene constructions and particle bombardment

Fragments corresponding to the promoters of *HvLtp4.2* (positions -1 to -665 nt) and *HvLtp4.3* (-1 to -755 nt) were fused in-phase to the β-glucuronidase reporter gene (Jefferson 1987) in the pBluescript II KS (Stratagene) vector (Fig. 1A). The indicated region of *Ltp4.2* promoter was amplified by the polymerase chain reaction from a *Bam*HI subclone of the gene (Fig. 1A) and the equivalent region (as determined by sequence alignment) from promoter Ltp4.3 was similarly amplified from a *Sal*I-*Eco*RI subclone of the corresponding gene (Fig. 1A). Gene constructions used as controls have been previously described: *35S*-GUS contained the coding region of the Gus (*uidA*) gene and the 35S cauliflower mosaic virus (CaMV) promoter, subcloned in the pUC19 vector (Diaz and Carbonero 1992); *35SI*-GUS additionally contained intron 1 of the maize *Adh1* gene (Vasil et al. 1991); *UbI*-GUS and *UbI*-LUC contained the maize ubiquitin promoter-exon 1-intron 1, fused either to the GUS or the luciferase reporter genes (Christensen et al. 1992); *Itr1*-GUS carried the endosperm-specific promoter of the barley trypsin inhibitor (Díaz et al. 1993); in *Ss1*-GUS the reporter gene is controlled by the promoter and first intron of barley sucrose synthase gene *Ss1* (Diaz et al. 1995).

Particle bombardment was carried out with a biolistic helium gun device (DuPont PDS-100λ). Gold particles (1.0 µm in size) were coated with DNA as described by Taylor and Vasil (1991). Barley tissues were placed onto a Phytagel-H_2O support (Sigma) in 6-cm diameter dishes and each plate was bombarded once with 0.154 µg gold and 0.350 µg DNA at a distance of 7.5 cm from the macroprojectile stopper shelf. After bombardment, dishes were incubated at 25° C for 2 days. Standard fluorometric GUS assays were performed using 4-methyl-umbelliferyl-β-D-glucuronide (MUG) as substrate (Jefferson 1987) and were quantified with a Heraeus TK 100 fluorimeter. Luciferase assays were done as described by Callis et al. (1987), using a 15-s integration time on a luminometer (Analytical Luminiscence Laboratory, Monolight 2010). Treatment with abscisic acid (10 µM) was carried out by including this hormone in the solid medium where leaves were bombarded and incubated for 24 h.

Infection with bacteria

Five-day-old barley (cv. Bomi) plants grown on vermiculite (22° C day/18° C night; 16 h light) were inoculated by infiltration with the bacteria *Pseudomonas syringae* pv. *japonica*, *Xanthomonas campestris* pv. *translucens* (10 µl of 10^8 cfu/ml in 10 mM $MgCl_2$), or with 10 mM $MgCl_2$ (mock inoculation). After inoculation, leaf samples were taken at times indicated in Fig. 5A and immediately frozen in liquid nitrogen. In the experiments with excised leaves, inoculations were carried out as above just before excision.

Results

Gene, *HvLtp4.2* and *HvLtp4.3*

Two genomic clones, λ*gltp4.11* and λ*gltp4.14*, which hybridized with a probe that consisted of the 3′ untranslated region from the previously reported *Ltp4* cDNA (now *Ltp4.1*; Molina and García-Olmedo 1993), were isolated from a genomic barley library. As shown in Fig. 1A, both clones contained the same two *Ltp* genes, *HvLtp4.2* and *HvLtp4.3*, less than 1 kb apart in a tail-to-tail orientation. Their deduced mature protein sequences were identical and differed from that of the LTP4.1 in a conservative amino acid change (A/V), but

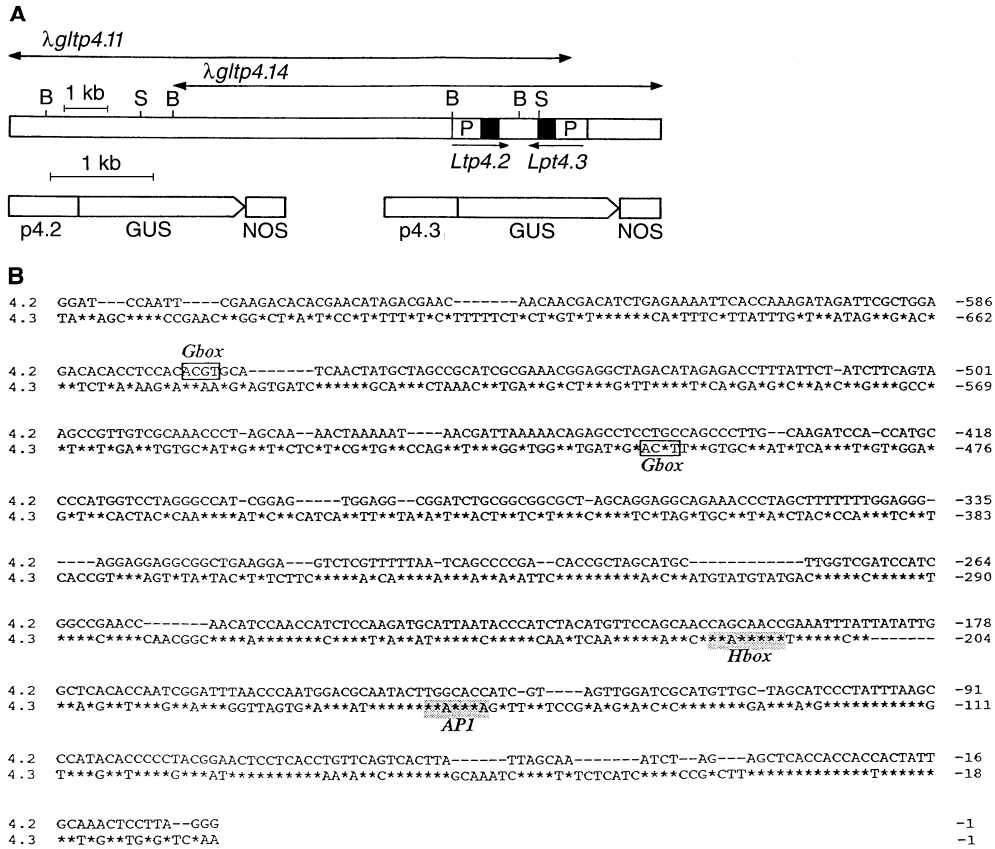

Fig. 1 **A** Schematic representation of genomic clones containing genes *HvLtp4.2* and *HvLtp4.3* and of fusions of their promoters (P4.2 and P4.3) to the *β*-glucuronidase/nopaline synthase (GUS/NOS) reporter gene. Restriction endonuclease sites for *Bam*HI (B) and *Sal*I (S) are indicated. **B** Alignment of the 5′ untranslated sequences of the two *Ltp* genes used in the gene fusions. Numbering is from the ATG translation initiation site; identities and gaps are indicated by *asterisks* and *dashes*, respectively. The following motifs have been highlighted: G-box, consensus ACGT (*Gbox*; Guiltinan et al. 1990; Schindler et al. 1992); H-box, consensus (C/A)A(C/A) C(T/A)A(C/A)C(*Hbox*; Lois et al. 1989; Cramer et al. 1989; Ohl et al. 1990; Loake et al. 1992; Yu et al. 1993); and AP-1, consensus TGACACA (*AP1*; Després et al. 1995). The alignment was constructed out using the CLUSTAL W programme (Thompson et al. 1994), and the SIGNAL SCAN 4.0 programme (Prestridge 1991) was used to search for sequence motifs

nucleotide sequences encoding the signal peptide and the 3′ untranslated regions were distinct from each other. In Fig. 1B, an alignment of the two promoters shows that they are also divergent, specially with respect to key motifs potentially involved in their responses to pathogen infection.

Southern blot analysis with the *Ltp4*-specific probe, which recognized all three *Ltp4* genes, but not the other known *Ltp* genes, detected polymorphic patterns that were consistent with the existence of multiple copies per haploid genome (Fig. 2). Previously reported Northern blot analyses with the same probe must therefore have detected the overall expression of all the *Ltp4* genes (Molina and García-Olmedo 1993).

Gene fusions involving the *HvLtp4.2* and *HvLtp4.3* promoters and the GUS reporter (Fig. 1A) were transiently expressed following particle bombardment in barley leaves and activity levels were similar in both cases, and of the same order as those obtained with the barley sucrose synthase *Ss1* and CaMV 35S promoters used as positive controls (Fig. 3A). Under the same conditions, no significant activity was driven by the endosperm-specific promoter from barley *Itr1* gene, which was used as a negative control. Activities of the two *Ltp* gene constructions found in different tissues, using the *UbI*-LUC chimeric fusion as internal control, were similar to each other and essentially reproduced the expression pattern obtained by Northern blot analysis, except that *Ltp4.2* was more active than *Ltp4.3* in endosperm, and *Ltp4.3* was active in roots, while *Ltp4.2* was not (Fig. 3B).

Responses to low temperature

Experimental conditions were optimized using the *Ss1*-GUS gene fusion, as the sucrose synthase *Ss1* promoter had been previously shown to be cold responsive (Maraña et al. 1990). It was established that an induction

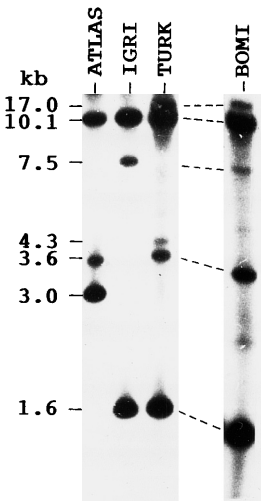

Fig. 2 Southern blot analysis of genomic DNAs (15 μg in each lane) from the indicated barley cultivars digested with the *Bam*HI endonuclease, and hybridized with the *Ltp4*-specific probe that recognizes all known *Ltp4* genes. *Dashed lines* link equivalent bands in two separate blots. Size standards were fragments of λ phage digested with endonucleases *Eco*RI and *Hin*dIII

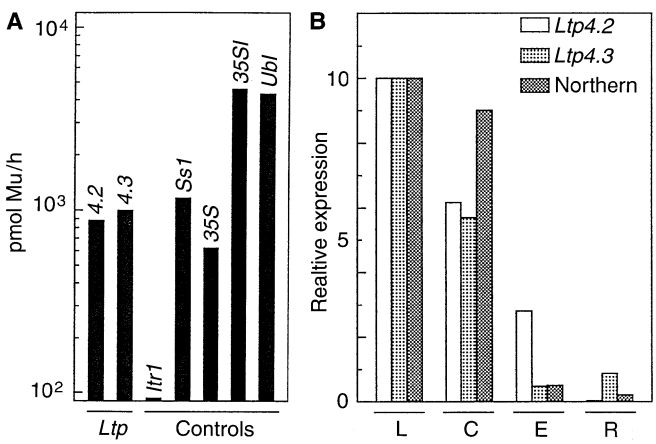

Fig. 3 **A** Expression of GUS fusions to *Ltp4.2* and *Ltp4.3* promoters in barley leaves compared with that of GUS fusions to promoters *Itr1* (negative control), *Ss1*, *35S*, *35SI*, *UbI* (positive controls). Activity is expressed per bombarded leaf and standard error of the mean for triplicate independent bombardments with the same particle/plasmid suspension was <15%. **B** Relative expression of the two *Ltp* GUS fusions in the leaves (L), coleoptile (C), endosperm (E) and roots (R). Northern blot signals were obtained with the *Ltp4*-specific probe (Molina and García-Olmedo 1993) and quantitated by densitometry (INH Image programme, Bio-Rad); in each tissue, GUS activity was assessed with reference to the internal control luciferase activity driven by the *UbI* promoter, which was added in equimolar amounts with respect to the *Ltp* constructions; in all cases, an arbitrary value of 10 was assigned to activity in the leaf

could be clearly detected in excised 5-day-old barley leaves, after a 24 h period of post-bombardment incubation at 4° C, and that the standard error of the mean GUS activity was always <15%, if the same particle/plasmid mixture was used for the samples com-

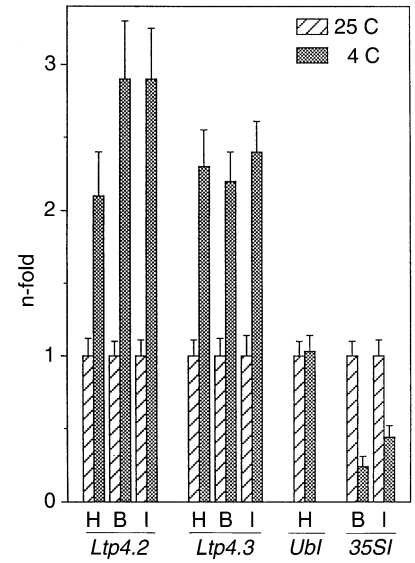

Fig. 4 Responses of *Ltp* promoters to low temperature. Two excised leaves were jointly bombarded in each case with the indicated promoter/GUS fusions and one was incubated at 25° C and the other at 4° C for 24 h and enzyme activity was determined. Barley cultivars used were Himalaya (H), Bomi (B), and Igri (I). Gene fusions using the *UbI* and *35SI* promoters were used for comparison. Experiments were performed three times. Standard errors of the mean are represented by *bars*

pared and the leaves were obtained from the same lot of plants. The two *Ltp* promoters responded to low temperature in a similar manner in all three barley cultivars tested (Fig. 4) and the increase was similar to that previously obtained for the *Ss1* promoter (not shown). Under the same experimental conditions, the activity of the *UbI* promoter was not affected and that of the *35SI* promoter was drastically reduced (Fig. 4). A similar response to that obtained by cold treatment was observed for the two *Ltp* promoters when barley leaves were treated with 10 μM abscisic acid (data not shown).

Differential response to pathogens

To investigate the effects of pathogen infection on the two *Ltp* promoters, screening of responses to different bacterial and fungal pathogens was carried out with 5-day-old plants of barley cv. Bomi, using the *Ltp4*-specific probe in Northern blot analyses. Two bacterial pathogens, *Xanthomonas campestris* pv. *translucens* and *Pseudomonas syringae* pv. *japonica*, which gave a compatible interaction with cv. Bomi, were chosen because of their ability to elicit a rapid increase and a decrease, respectively, of LTP4 mRNA relative to basal levels (Fig. 5A). Excised leaves were inoculated and placed on the solid Phytagel-H_2O support (Fig. 5B). Each set of three leaves was bombarded twice through a plastic window that was placed first at an apical site that included the inoculated areas and then

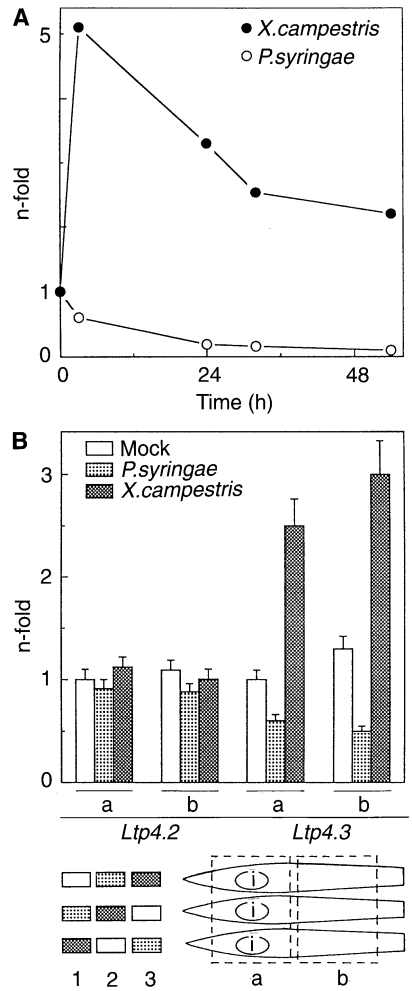

Fig. 5 **A** Responses of *Ltp4* genes to the indicated bacterial pathogens. Northern blot signals were obtained with the *Ltp4*-specific probe (Molina and García-Olmedo 1993) and quantitated by densitometry (INH Image programme, Bio-Rad). **B** Responses of promoter fusions *Ltp4.2* and *Ltp4.3* to the same pathogens. Groups of three leaves were inoculated (i) in the apical half leaf (a) and bombarded both in the apical and in the basal (b) parts of the leaf, using a plastic window in the positions indicated by a discontinuous line. The experiment was repeated three times for each permutation (1–3) of the treatment. GUS activity was determined after 24 h incubation in the Phytagel-H_2O support. The control (mock) value of the apical part was taken as 1 in each experiment. Standard errors of the mean are indicated by *bars*

at a basal site (non-inoculated half-leaves), as illustrated in Fig. 5B, and GUS activity was determined fluorometrically for each bombarded half-leaf. The two *Ltp* promoters responded in a differential manner to the pathogens: whereas the activity of promoter *Ltp4.2* was not affected by the bacterial infections, that of promoter *Ltp4.3* reproduced the responses detected by Northern analysis both in the apical and in the basal half of the leaf: an increase in response to infection by *X. campestris* and a decrease in the case of *P. syringae* (Fig. 5B). These results implied that the response of the *Ltp4.3* promoter to pathogens occurs beyond the infected zone of the leaf.

Discussion

A common characteristic of related genes is that their coding regions have diverged less than their non-coding regions. Genes *HvLtp4.2* and *HvLtp4.3* represent an extreme example, as they encode the same mature protein while their non-coding regions are quite different. However, this divergence does not seem simply to reflect less stringent selective constraints affecting these parts of the genes but to have positive functional relevance, as it has led to alterations in their expression patterns. The above results indicate that both genes characterized here are active (not pseudogenes), and confirmed the previously reported complexity of the *Ltp* gene family (Molina and García-Olmedo 1993; White et al. 1994). Their developmental expression patterns, as judged from the transient expression experiments, were similar – except for minor differences – and in general agreement with the overall LTP4 expression pattern previously observed by Northern blot analysis (Molina and García-Olmedo 1993).

The excised-leaf procedure that has been developed here to study responses to low temperature and to pathogen infection in barley, using the well-established particle bombardment technique, represents a simplification of previous whole-plant approaches and allows the rapid investigation of different promoters in multiple genetic backgrounds. Thus, it has been shown that the two *Ltp* promoters, which have been isolated from a spring barley, are induced by low temperature both in winter (cv. Igri) and in spring (cvs. Bomi and Himalaya) barleys, indicating that the observed response must depend on the promoter characteristics, and is not dependent on the genetic background of the cultivars used in the study or on their spring or winter habit. We have previously observed a non-significant cold-induced increase ($<20\%$) in *Ltp4* mRNA levels in cv. Bomi by Northern blot analysis, under conditions in which the *Ss1* promoter underwent a 20-fold induction (Molina and García-Olmedo 1993), while an induction of *Ltp* genes by low temperature has been reported for cv. Igri (Dunn et al. 1991; Hughes et al. 1992; White et al. 1994). In view of the current results, the difference can be ascribed either to divergence in the *Ltp4* promoters of the two cultivars or to the presence of a highly expressed, non-responsive *Ltp4* gene in cv. Bomi that would mask the induction of other *Ltp4* genes. In contrast, a significant response of *Ltp4* genes to abscisic acid was detected both in winter and spring barley (Hughes et al. 1992; Molina and García-Olmedo 1993).

It has previously been shown that expression of *Ltp* genes can be altered, independently from each other

and from other defense genes, in different plant-pathogen interactions (Molina and García-Olmedo 1993, 1994; García-Olmedo et al. 1995, 1996). We have observed opposite responses in two compatible bacterium-plant interactions by Northern blot analysis, which detects the overall expression of *Ltp4* genes. The observed negative response to *P. syringae* infection has some precedents in the literature, including those of several defense genes in bean (Jakobek et al. 1993) and the *StPth1* gene in potato (Moreno et al. 1994), that have been interpreted as a mechanism by which some pathogens might overcome constitutive defense barriers.

The two *Ltp* promoters analysed here behave differentially with respect to pathogens: responses of promoter *Ltp4.3* mimic the overall *Ltp4* pattern observed by Northern blot analysis, while the activity of promoter *Ltp4.2* is not affected by either pathogen. This is consistent with the overall divergence of these promoters and, in particular, with the observed differences in putative pathogen-response motifs (Fig. 1B). The 5′ untranslated regions of the two *Ltp* genes each contain a copy of an ACGT motif common to several cis-acting elements that bind basic leucine zipper proteins, including those denominated G-box (Schindler et al. 1992) and ABRE (Guiltinan et al. 1990). Perhaps of greater relevance is the presence in gene *HvLtp4.3* of the pathogen-responsive motifs H-box and AP-1 (Fig. 1B). The first motif is present in promoters of phenylpropanoid biosynthetic genes (Cramer et al. 1989; Lois et al. 1989; Ohl et al. 1990; Loake et al. 1992; Yu et al. 1993), which have been shown to display elicitor-inducible and light-inducible footprints in vivo (Lois et al. 1989). The presence of H-box and G-box motifs in the promoter of these genes has been found to be required for elicitor induction (Loake et al. 1992). The AP-1 element (TGACACA) has been described in the pathogenesis-related *PR-10a* gene from potato (Després et al. 1995), where it is involved, together with an E-box (CANNTG), in gene activation by an elicitor of *Phytophthora infestans* and interacts with nuclear factor PBF-1, which requires phosphorylation for activity (Després et al. 1995). Several E-box cores can be identified in the *HvLtp4.3* promoter (not highlighted in Fig. 1B). The variation in pathogen-response motifs among *Ltp* promoters is in agreement with the previously observed plasticity of defense-gene elicitation in plant-pathogen interactions and with the combinatorial nature of plant defense responses (Molina and García-Olmedo 1994; García-Olmedo et al. 1995, 1996).

Acknowledgements Sequencing of DNA by R. Novitzky (CIBA-GEIGY, Research Triangle Park, NC, USA) and technical assistance from D. Lamoneda and J. García are gratefully acknowledged. This work was financed by the DGICYT, Spain (Grants PB92-0325 and PB94-0404).

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