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Inducibility by pathogen attack and developmental regulation of the rice *Ltp1* gene

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Abstract

Using a genomic clone encoding a rice lipid transfer protein, LTP1, we analysed the activity of the 5' region of the *Ltp1* gene in transgenic rice (*Oryza sativa* L.) during plant development and under pathogen attack. The –1176/+13, –556/+13 and –284/+13 regions of the promoter were fused upstream from the *uidA* reporter gene and *nos* 3' polyadenylation signal, resulting in the p Δ 1176Gus, p Δ 556Gus and p Δ 284Gus constructs which were transferred to rice by microprojectile bombardment. Histochemical and fluorometric GUS assays and *in situ* detection of *uidA* transcripts in transgenic homozygous lines harbouring the p Δ 1176Gus construct demonstrated that the *Ltp1* promoter is preferentially active in aerial vegetative and reproductive organs and that both specificity and level of expression are regulated during organ development. In leaf sheath, GUS activity which is initially strictly localized in the epidermis of growing tissue, becomes restricted to the vascular system in mature tissues. In expanded leaf blade, expression of the *uidA* gene was restricted to the cutting level suggesting inducibility by wounding. Strong activity was detected in lemma and palea, sterile glumes, and immature anther walls and microspores but not in female reproductive organs. No GUS activity was detected during seed embryo maturation whereas the *uidA* gene was strongly expressed at early stages of somatic embryogenesis in scutellum tissue. The *Ltp1* transcripts were found to strongly accumulate in response to inoculation with the fungal agent of the blast disease, *Magnaporthe grisea*, in two rice cultivars exhibiting compatible or incompatible host-pathogen interactions. Analysis of p Δ 1176Gus leaf samples inoculated with the blast fungus demonstrated that the *Ltp1* promoter is induced in all cell types of tissues surrounding the lesion and notably in stomata guard cells. In plants harbouring the *Ltp1* promoter deletion construct p Δ 556Gus, activity was solely detected in the vascular system of mature leaves whereas no *uidA* gene expression was observed in p Δ 284Gus plants. These observations are consistent with the proposed role of LTP1 in strengthening of structural barriers and organ protection against mechanical disruption and pathogen attack.

Introduction

Plant lipid transfer proteins (LTPs) are small, soluble basic proteins of 7–9 kDa consisting of 91–

95 amino acids, which may represent up to 4% of total soluble proteins. They have been purified from many species (Kader, 1996 for a review). The biological function of LTPs is still controversial although

the first one was purified more than 15 years ago: *in vitro* demonstration of their broad lipid specificity and of their ability to catalyze the transfer or exchange of lipids first suggested that LTPs may be involved in intracellular lipid transfer. However, the presence of a signal peptide (Tchang *et al.*, 1988) and immunological evidence of extracellular localization of LTPs (Thoma *et al.*, 1993), accumulation of *Ltp* transcripts in epidermal layers revealed by *in situ* hybridization (Fleming *et al.*, 1992; Thoma *et al.*, 1993, 1994) and epidermal localization of GUS activity in transgenic *Arabidopsis* and tobacco plants expressing an *Ltp1* promoter-*uidA* fusion (Thoma *et al.*, 1994; Canevascini *et al.*, 1996) appear incompatible with a general role in lipid redistribution. It was suggested that LTPs are involved in cutin formation by transporting cutin monomers through the extracellular matrix (Koltunow *et al.*, 1990; Serk *et al.*, 1991; Sossountzov *et al.*, 1991; Kalla *et al.*, 1994; Thoma *et al.*, 1994). Beyond this passive role in the formation of structural barriers, demonstrated antibacterial and antifungal activities of LTP homologues (Terras *et al.*, 1992; Molina and Garcia-Olmedo, 1993; Cammue *et al.*, 1995; Dubreil *et al.*, 1998) and inducibility of some *Ltp* genes by pathogen attack (Molina and Garcia Olmedo, 1993) rather suggest an active role in plant defence. Demonstration of enhanced tolerance to bacterial pathogens by transgenic expression of a barley *Ltp* gene supports this hypothesis (Molina and Garcia Olmedo, 1997).

To date, there are at least 178 sequence accessions in GenBank corresponding to full-length cDNA or genes encoding LTP in many plant species as well as many EST. All the full-length sequences reveal the presence of N-terminal extensions of 26–27 amino acids in the deduced proteins that have characteristics of signal targeting the peptide to the cell wall or outside of the cell. Based on sequence comparisons, there are two groups of LTP protein, LTP1 and LTP2. Most *Ltp* genes encode group 1 proteins, show strong sequence homology and have an intron in the 3' part of their coding sequence whereas group 2 genes are intronless.

Lipid transfer proteins in plants are generally encoded by multigenic families with a number of members initially estimated between 2 and 7 (Kader, 1996). However, recent results demonstrated that this number is much higher. In *Arabidopsis*, at least 15 genes were identified (Arondel *et al.*, 2000). In rice, we first isolated and sequenced an *Ltp1* gene through screening of an *Oryza sativa* L. (cv. IR 36) genomic library, using

as a probe a cDNA encoding a maize LTP (Vignols *et al.*, 1994). We later demonstrated that *Ltp1* genes in cv. Nipponbare belong to a complex multigene family of at least 7 members grouped into 3, possibly 4, classes each encompassing 2–4 genes and showing a differential regulation (Vignols *et al.*, 1997). The rice *Ltp1* gene initially isolated, and used in this study, belongs to class I. In other species, the various members also generally exhibit differential expression patterns, characterized by a strong tissue specificity and inducibility by various external stresses such as salt, ABA, drought, cold treatment or pathogen infection. This suggests that plants are equipped with a large variety of *Ltp* genes which serve similar functions but can be specifically turned on in different parts of the plants and in response to a variety of stimuli. We also isolated a rice (cv. IR 36) *Ltp2* gene which seems to be single-copy as estimated by stringent southern blot (Garcia-Garrido *et al.*, 1998).

So far, the expression of only two *Ltp 1* genes has been analyzed using a *uidA* reporter gene: the *Arabidopsis Ltp1* (Thoma *et al.*, 1994) and tobacco *NtLtp1* (Canevascini *et al.*, 1996). These two genes have been studied in homologous systems. Both studies confirmed the strong preferential expression of *Ltp1* genes in aerial vegetative and floral organs and in epidermal tissues. Expression of a barley *Ltp2*-type promoter has been analysed in transgenic rice (Kalla *et al.*, 1994) and the promoter of rice *Ltp2* was analysed by biolistic transfer to maize embryos (Garcia-Garrido *et al.*, 1998).

We have analysed hereafter the sequence of the 5' region of the *Ltp1* gene to identify motifs that could be involved in tissue and developmental specificity as well as in inducibility by external stresses. To determine whether the expression of the rice *Ltp1* gene is enhanced by pathogen attack, we monitored the time course of transcript accumulation over 6 days after inoculation with the fungal agent of the blast disease, *Magnaporthe grisea*, in compatible and incompatible cultivar/strain interactions. We further investigated the tissue and developmental specificity of the *Ltp1* gene through histochemical and fluorometric GUS assays and *in situ* hybridization of *uidA* mRNA in transgenic rice lines harbouring a translational fusion between the putative full-length promoter region and the *uidA* reporter gene. This plant material was also used to determine the cells expressing the *Ltp1* gene during a somatic embryogenesis process in scutellum cells and after inoculation with *M. grisea*. As a first insight into the organization of promoter regions involved in tissue

and developmental specificity, two deletion mutant-*uidA* reporter gene fusions were transferred to rice and analysed.

Materials and methods

Sequence analysis

The *Ltp1* gene was isolated as a 3.4 kb phage DNA fragment. This fragment was subcloned and only the sequence corresponding to the gene region was initially reported (Vignols *et al.*, 1994). The adjacent region containing the promoter was sequenced by the dideoxy nucleotide method (Sanger *et al.*, 1977) and analysed by means of the TFSEARCH and PlantCARE databases. Both are available via the Internet at <http://www.genome.ad.jp/SIT/> and <http://sphinx.rug.ac.be:8080/PlantCARE/>, respectively.

Inoculation of rice with *Magnaporthe grisea*

To investigate the inducibility of the *Ltp1* gene by pathogen attack, we used the *japonica* rice cultivars IRAT216 (IDSA6) and Maratelli and *Magnaporthe grisea* isolates CD142 and PH 14. IRAT 216 exhibits a compatible (moderately resistant) and incompatible (hypersensitive) response with CD142 and PH14, respectively, whereas Maratelli exhibits a compatible (susceptible) response to both isolates. For each cultivar × isolate treatment, one tray of 75 seedlings at the 4–5 leaf stage each was inoculated by spraying 30 ml of an aqueous suspension of spores (50 000 spores/ml) supplemented with 0.5% w/v gelatin. Controls were mock inoculated. A new set of two trays was inoculated every day for a week to assess the kinetics of infection. Leaf samples were then collected individually on the inoculated and control trays and immediately frozen in liquid nitrogen for RNA isolation. In these experimental conditions symptoms appear at day 3.

To localize the leaf cells in which the *Ltp1* promoter is induced by *Magnaporthe grisea* infection, we used T₃ progeny plants of the homozygous transgenic lines (cv. Taipei 309) 3, 30, 64 and 65, C-24 and C+8 harbouring the pΔ1176Gus, pΔ1262Gus and pUGCI constructs respectively (see below). Thirty seedlings were inoculated by spraying with 15 ml of an aqueous suspension of 50 000 spores/ml of strain CD128-supplemented with 0.5% w/v gelatin. Taipei 309 is the variety most amenable to transformation in our hands and is susceptible to *M. grisea* strain CD128.

Thirty seedlings of line 64 were also inoculated with the gelatin solution devoid of spores. Samples of the last expanded leaf, known to be more susceptible to infection and to exhibit the strongest symptoms, were collected from 5 seedlings per line every day for 6 days and subjected to GUS histochemical assay (see below).

RNA extraction and northern blot analyses

Tissue samples were stored frozen at –80 °C until extraction. RNA was prepared and analyzed as previously described (Garcia-Garrido *et al.*, 1998). The *Ltp1* probe corresponds to the 3'-untranslated sequence of the gene and is gene-specific (Vignols *et al.*, 1997). Probes derived from other *Ltp1* rice genes, but belonging to class II, were also prepared in similar conditions.

Constructs

The –1176/+13, –556/+13 and –284/+13 fragments were first isolated from the promoter region of the *Ltp1* gene contained in the pBBO4A subclone of a IR36 genomic library (Vignols *et al.*, 1994), by digestion with *Bam*HI/*Pvu*II, *Hind*III/*Pvu*II and *Xba*I/*Pvu*II, respectively. The fragments were then inserted into the pGUS3 plasmid, a pUC18 containing the *uidA*-coding sequence and the polyadenylation signal of the nopaline synthase gene of the pBi101.1 vector (Bevan *et al.*, 1983), at the corresponding *Bam*HI/*Sma*I, *Hind*III/*Sma*I and *Xba*I/*Sma*I sites, respectively. The resulting constructs were designated as pΔ1176Gus, pΔ556Gus and pΔ284Gus (Figure 1) and sequenced to check that the reporter gene was inserted as a translational fusion. A fourth construct was obtained by inserting a –1176/+84 fragment into the pGUS1 plasmid with a deletion of 2 nucleotides between the *Sma*I site and the first ATG codon of the *uidA* gene. The resulting construct referred as pΔ1262Gus was used as a negative control in transformation experiments.

The pUGCI construct (Chair *et al.*, 1996), was obtained by cloning the *Hind*III fragment of pAHC25 (Christensen and Quail, 1996) containing the promoter region and first exon and intron of the maize ubiquitin gene fused to the *uidA* gene and *nos* 3' terminator into pUC19. This fragment is known to exhibit a near-constitutive activity in transgenic rice (Cornejo *et al.*, 1993), and was used as a positive control in transformation experiments, notably to assess GUS substrate penetration in transgenic rice organs.

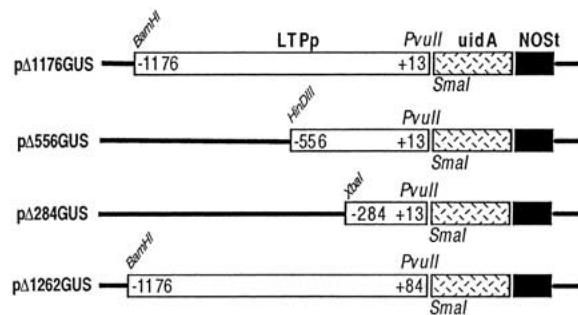


Figure 1. Schematic representation of deletions produced in the rice *Ltp1* gene promoter and strategy for cloning into the pUC18 vectors used in microprojectile bombardment-mediated transformation of rice. A 2 kb fragment (hatched box) corresponding to the 1.8 kb *uidA*-coding region and ended by the 0.21 kb *nos* gene terminator (black box) was first inserted into the *EcoRI* restriction site of pUC18 polylinker such that the 5' end of *uidA* gene is placed next to the *SmaI* restriction site. Three different sizes (–1176 bp, –556 bp, –284 bp) of the *Ltp1* gene promoter (white box) were isolated by taking advantage of *Bam*HI, *Hind*III and *Xba*I restriction sites in the 5' region (at –1176, –556 and –284 bp, respectively) and *Pvu*II located at +13 bp in the *Ltp1* gene-coding region. The corresponding fragments were further cloned between *Bam*HI, *Hind*III, or *Xba*I, and *Sma*I restriction sites of the *uidA* gene-containing pUC18. A fourth construct (pΔ1262GUS) carrying another *Bam*HI-*Pvu*II fragment (–1176 to +84) and originated from a partial *Pvu*II digestion was used as a negative control since the fusion with *uidA* gene produces in this case a wrong *uidA* ORF. Sizes are indicated in bp.

Production of transgenic rice plants

The five constructs were introduced into mature embryo scutellum-derived embryogenic calluses of the *japonica* cultivar of rice (*Oryza sativa* L.) Taipei 309 by microprojectile bombardment according to the procedure described in Chen *et al.* (1998), with the PDS1000/He particle gun device (BioRad, USA). Each construct was mixed in a 4:1 molar ratio with the 5.1 kb pILTAB227 plasmid consisting of the CaMV 35S promoter with a duplicated enhancer sequence (e35S) controlling the hygromycin phosphotransferase gene and followed by the *nos* 3' terminator (Kindly supplied by Dr C. Fauquet, ILTAB, La Jolla, CA). Mixed plasmid DNA (5, 10 and 15 μ g) was used to coat 3, 6 or 9 mg of 1 μ m diameter gold particles for duplicate bombardments of two, three and six plates, respectively. Each plate contained ca. 100 embryogenic callus pieces, according to the construct (Table 1). Procedures for selecting transgenic rice plants based on resistance to hygromycin were those of Chen *et al.* (1998). The number of bombarded calluses generating shoot-forming, hygromycin-resistant cell lines is shown in Table 1.

Analysis of transformants

Total genomic DNA was extracted from leaf tissue of transgenic and control plants using a modified CTAB method (Hoisington, 1992). DNA (5 μ g) was digested with appropriate restriction endonucleases and DNA fragments were separated on 0.8% agarose gels, and transferred onto nylon membranes (Hybond N+, Amersham). A 1.8 kb fragment of the *uidA* coding sequence served as template for synthesizing [α - 32 P]-labelled probes by random priming. After hybridization, the membrane was washed twice (2 \times SSC, 1% SDS and 0.1 \times SSC, 0.1% SDS, both at 65 $^{\circ}$ C for 10 min) and analysed by autoradiography. All the regenerated plants were characterized by Southern blot analysis to assess copy number (single cut) and integrity (release of the promoter-*gusA*-*nos* 3' gene cassette) of the inserted construct. Primary transformants harbouring few (2 or 3) insertions and at least one intact gene cassette were kept per construct for further evaluation (data not shown).

GUS histochemical and fluorometrical assays

Four transgenic lines harbouring pΔ1176Gus (3, 30, 64 and 65), pΔ556Gus (8, 14, 17 and 25) and pΔ284Gus (5, 14, 16 and 24) were selected for a histochemical survey of *uidA* gene expression in various organs over plant development. Homozygous T₃ lines C-24 and C+8 harbouring the pΔ1262Gus and pUGCI constructs were used in histochemical and fluorometric analyses as negative and positive controls, respectively, notably to avoid artefacts related to poor penetration of the GUS substrate, despite repeated vacuum infiltrations. Histochemical assay and fluorometric quantification of *uidA* gene expression in transgenic tissue were conducted as described by Jefferson (1987). For fluorimetric assays of plant tissues, the concentration of protein extracted in the GUS assay buffer was determined according to Bradford (1976) using a Multiskan (Labsystems) spectrophotometer. GUS specific activity (hydrolysis of 4-methylumbelliferone glucuronide per mg protein per hour) was determined at 460 nm with a Fluoroscan (Labsystems) apparatus.

Localization of *uidA* gene expression by *in situ* hybridization

After the histochemical assay, specimens for histology were washed in 200 mM phosphate buffer pH 7.2

Table 1. Results of microprojectile-mediated transformation of embryogenic calluses of rice cv. Taipei309 with fusions between the *uidA* gene and *Ltp1* gene promoter regions.

Construct	Number of bombarded calluses	Number of shoot-forming hygromycin-resistant lines (transformation efficiency)	Frequency of T ₀ plants exhibiting GUS staining in response to histochemical assay
pΔ1176Gus	560	80 (14.3)	23.7
pΔ556Gus	320	42 (13.1)	21.0
pΔ284Gus	320	32 (10.0)	0
pΔ1262Gus	320	28 (8.7)	0
pUGCI	240	42 (17.5)	54.0

then fixed in 2% paraformaldehyde, 1% glutaraldehyde, 200 mM phosphate buffer pH 7.2 for 24 h and cleared through a graded ethanol series. Sections (3 μm) of GUS-stained material were prepared from 2-hydroxyethylmethacrylate (Historesin Technovit 7100 Kulzer)-embedded tissue with an LKB microtome. Cell wall was counterstained in purple by periodic acid Schiff reaction (Gabe, 1968).

Biotine-labelled DNA probes were prepared according to procedures recommended by Bioprime DNA labelling system (Life Technologies Ref. 18094-011). Tissues were fixed according to Luther and Bloch (1989) and embedded in methylbutylmethacrylate resin according to the procedure of Kronenberger *et al.* (1993). Embedded tissue was cut at 3 μm thickness on an LKB microtome.

Dehydrated tissues were treated with 0.5 mg/ml proteinase K, washed in a proteinase K buffer for 10 min, and then in PBS buffer for 2 min. Prehybridization was performed at 37 °C for 2 h in 50% formamide, 10% dextran sulfate, 50% Denhardt's solution, 20× SSC, 250 ng/μl blocking tRNA, 20% SDS). The probe was then hybridized on the sections for 14 h at 37 °C in the same solution containing the DNA probe at a concentration of 2 ng/μl. Sections were subsequently washed in a blocking buffer (125 mM Tris-HCl pH 7.5, 140 mM NaCl and blocking reagent (Boehringer Mannheim, 1096-176). Revelation was performed with 1 μg/ml streptavidine-alkaline phosphatase in Tris-HCl buffer pH 9.55 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂) for 30 min. Slides were rinsed with the blocking buffer (100 mM Tris-HCl pH 8, 100 mM EDTA pH 8) then dehydrated and mounted in Moviol. Controls included treatments on untransformed plant sections and those proposed by the kit supplier.

Results

Sequence analysis of the *Ltp1* gene promoter region

In previous work, a genomic clone containing the rice *Ltp1* gene was isolated and analysed (Vignols *et al.*, 1994). The transcription initiation site (TIS) had been determined by primer extension and located at 123 bp upstream from the ATG start codon. In the present work, 1176 bp located upstream from the *Ltp1* gene ATG start codon were cloned separately for further analyses and are presented in Figure 2.

Inspection of the *Ltp1* promoter region identified sequences presenting similarity to the eukaryotic TATA and CAAT box consensus. Two AT-rich stretches, ⁻²⁴¹TATATAA⁻²³⁵ and ⁻²⁰⁴TAATTAT⁻¹⁹⁷, are located 72 bp and 109 bp upstream from the TIS, respectively, the TATATAA stretch being the most frequently used in plant promoters (Rombauts *et al.*, 1999). A putative CCAAT box at -288 was found 50 bp upstream of the TATATAA stretch.

Using TFSEARCH and PlantCARE databases, we further scanned the 1176 bp genomic sequence for additional promoter elements and compared the results with those from other plant *Ltp* gene promoters. Although a number of such *cis* elements were found in the sequence reported in Figure 2 as well as in some of the other *Ltp1* genes (Kalla *et al.*, 1994; Molina *et al.*, 1996), their biological significance is not clear and, at this stage, they could only be used as information for designing constructions. The RY element has been described as a seed specific element (Baumlein *et al.*, 1992). The TCA element is found in the promoter region of many genes induced by wounding or pathogen attacks (Goldsbrough *et al.*, 1993). ARE and ABRE are auxin- and ABA-responsive elements respectively (Guiltingan *et al.* 1990).


GGATCCAAACATCATGAATGCGCTCAACTACGTGCATGTTCCAAGTGTAACAACATGCATG -1117
BamHI (A1176.Gus) ABRE-like RY element
 CTAGCTGCTACAAACGACTAGCTCTGTTAGGCCGCTGCTAATCATTTTCTCATCACTAAT -1057
 ARE-box TCA-box
 ACTAACACTGTGTGACTAGCTAGCTAAACCAGCCCTAACCAACCAACCACCTGATTCAAT -997
 TGAATTGAATTGAATAACATTAGACGCACCGACGACTAAGTGCAAGTCCCAACCCCTCCACC -937
 TATAATGATGTCTATATATAGCATTAACATAAATTATCATGTAGGACTTTTAACTTATATG -877
 ACACATATATTAATTAATAAGGAGAGTGAAGAGAGGAAGAACTGGATCTCATGCAAGACA -817
 CAGCTTCAACACGAGAATCTATGCACCTAGACACTATCAAGTTTGTGTTTGGGAGAGAATA -757
 GTGTCTTTATAATAGTTGAAGAATAAATATGATTGGTAGAGAAGAGAGATGATGTATTTA -697
 TTAATGCCCACTTTAAGAAATCATGGGTTGTGGAGTGTAGTTTCTATGTGATGTCTTAT -637
 TGACATGACACTATAGACACTGCTTATGGACATTATGGGTTGGAACGCCCCTAAGAACGG -577
 TGCCTAAACACTAGCACTCCAAAGCTTAATTACACTATGTGTACGCCAATTAGAAAATGTC -517
 HindIII (A556.Gus)
 ATGAAACATTTCTATAAAACAAATTAGATATTTACTCATAATATATTATACCTACTGGAAA -457
 GTCTCATACTCAAATTTAATATATTTTAGCTGTAACATAAAGTGAAAAAACCCGACAGTT -397
 TTAAGGGTAAAAATGTCAGGATTTTGTCTTTTTTTATGGCTAAAATATAATGAAACTAAA -337
 GATAAGATTTTCACATGTAAGTGTAAACTATTGGAAGTATGTGTCCAATTTTCTAGAA -277
 I-box XbaI (A284.Gus)
 CTTTTGTGACATTTGCTAATTTGATATATCAGGGTGTGTATATAAGAAAAGTTTGTGTGT -217
 ATAGAATATGTTCTCTAATTTATAGGTGGACTATTCGCGTAGTAGCTACATTTGACTTGG -157
 ABRE-like 
 TCGATGCATCGGCACAGATGAAATAATAGCTATTTAAAGCCCCCTACATTCGCGGAATT -97
 CTCATCGATCGATCTCACCTGCAGCAGCAAACGAGCACCACACCAGCAGCAGCAGCAA -37
 GTCGATCGATCGTCAGCACACACGACCAAGATCGAG **ATG** GCC CGT GCA CAG CTG +18

Figure 2. Nucleotide sequence of the promoter region of the *Oryza sativa* *Ltp1* gene. Numbering is relative to the first base upstream from the ATG codon, which is indicated in bold letters. The transcription start site determined by Vignols *et al.* (1994) is indicated by a bent arrow. Motifs with significant similarity to previously identified *cis*-acting elements are underlined. These include seed-specific element (RY element, Baumlein *et al.*, 1992), a TCA element (Goldsbrough *et al.*, 1993), an auxin-responsive element (ARE, Nagao *et al.*, 1993), a putative ABA-responsive element (ABRE-like, Mundy *et al.*, 1990) and a light-responsive element (I-box, Rose *et al.*, 1999). The TATA and CAAT boxes are double-underlined. The *Bam*HI, *Hind*III and *Xba*I restriction sites (plus corresponding names of deleted construct) are underlined.

Tissue and developmental specificity of the *Ltp1* gene

Ltp1 gene expression during development has been studied by RNA blot hybridization (Vignols *et al.*, 1994, 1997) and these data guided the analysis of transgenic plants. Histochemical assays conducted on shoot sections of young T₀ plantlets regenerated from embryogenic calluses bombarded with the pΔ1176Gus plasmid allowed detection of GUS staining in only 23.7% of the plants before transfer to the greenhouse (Table 1). This frequency appeared

far lower than observed among plantlets regenerated from calluses treated with the positive control pUGCI construct and generally observed in T₀ plants co-transformed with promoter-*uidA* fusions in transgenic rice in our laboratory (unpublished results). Four pΔ1176Gus transformants (3, 30, 64 and 65) were selected on the basis of simple integration patterns and conducted to the homozygous status together with positive pUGCI and negative pΔ1262Gus control lines.

Table 2. GUS-specific activities (pmol 4-MU h⁻¹ μg⁻¹) in protein extracts of sheath (S) and blade (B) tissue of the second to the sixth leaf formed after the incomplete leaf (see Figure 3A) of progeny seedlings at the 6-leaf stage of T₃ homozygous transgenic lines harbouring the *uidA* gene controlled by 5' regions of the rice *Ltp1* gene. The mean ± SD value observed on 5 progeny plants is given. The three highest GUS activities recorded in each line appear in bold.

Construct	Line	Leaf rank									
		2		3		4		5		6	
		S	B	S	B	S	B	S	B	S	B
pΔ1176Gus	30	84 ± 24	12 ± 12	90 ± 30	24 ± 8.0	204 ± 66	36 ± 18	258 ± 138	108 ± 36	24 ± 18	
	64	66 ± 12	18 ± 6.0	84 ± 18	18 ± 6.0	84 ± 18	36 ± 18	138 ± 24	114 ± 30	30 ± 18	
pΔ556Gus	8	216 ± 114	6.0 ± 1.8	210 ± 54	36 ± 6.0	84 ± 30	54 ± 12	18 ± 12	48 ± 6.0	6 ± 3.6	
	17	24 ± 6.0	1.2 ± 1.2	48 ± 6.0	6.0 ± 3.6	18 ± 12	12 ± 4.2	6.0 ± 6.0	12 ± 6.0	3.6 ± 1.8	
pΔ1262Gus	24	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
pUGCI	8	348 ± 18	294 ± 6.0	390 ± 54	342 ± 48	528 ± 150	300 ± 78	552 ± 114	330 ± 138	1296 ± 258	

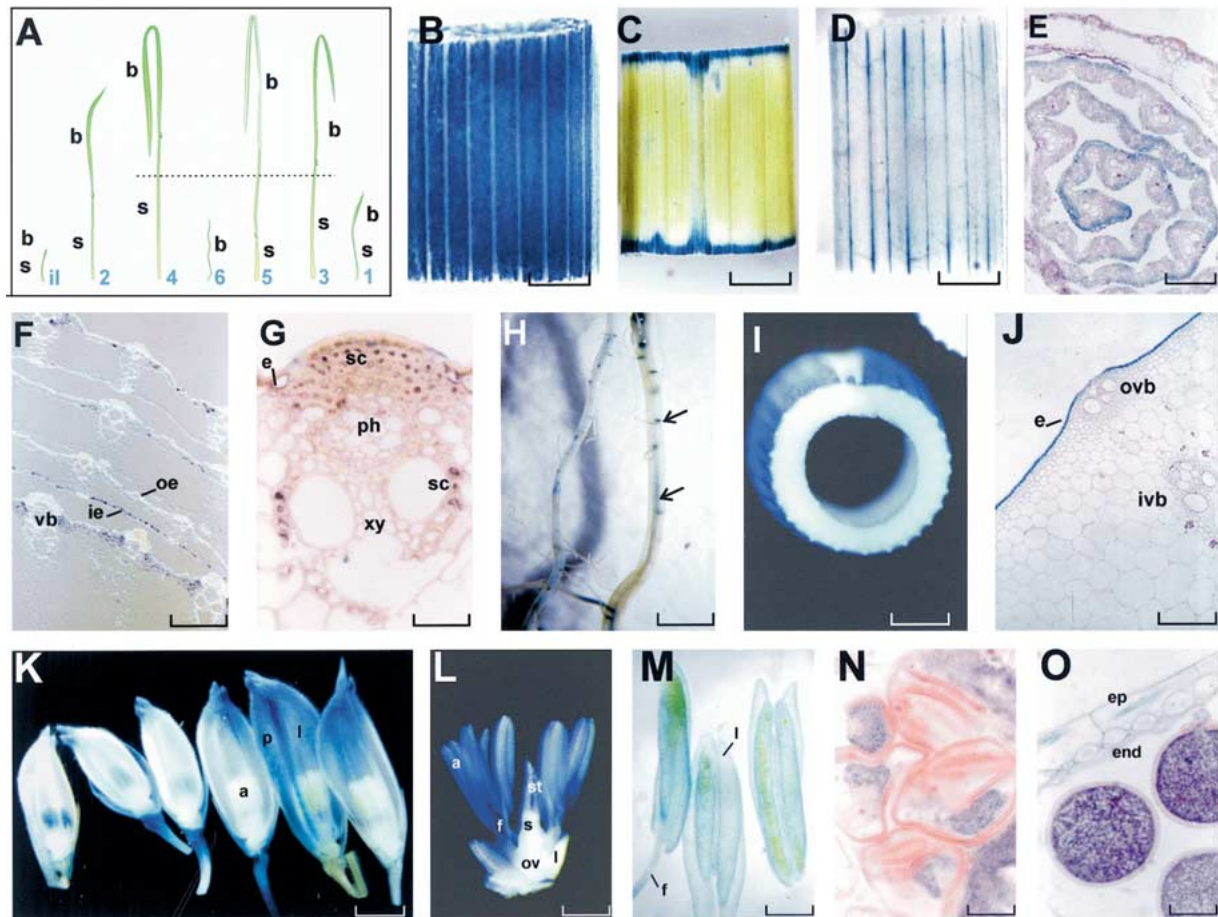


Figure 3. Developmental regulation and tissue-specificity of the activity of the promoter of the *Ltp1* gene in transgenic rice plants harbouring the p Δ 1176Gus construct. **A.** Schematic representation of the developmental status of leaf blade (b) and sheath (s) in T₃ pUGCI and p Δ Gus1262 control and p Δ 1176Gus transgenic seedlings (cv. Taipei309) used for fluorometric quantitation of GUS activity (leaves are numbered according to their age; il, incomplete leaf; 1, oldest first complete leaf; 6, immature innermost rolled leaf. Level of histological sections E and F is represented by a dotted line). **B–D.** Localization of GUS-specific activity in sheath section of leaf 4 (B), blade section of leaf 4 (C) and sheath section of leaf 2 (D) (bar = 1 mm). **E.** Transversal section of a young shoot 3 cm above the shoot apex (bar = 180 μ m). **F.** Localization of *uidA* gene transcripts in a transversal histological section of a young shoot through *in situ* hybridization (vb, vascular bundle; ie, inner epidermis; oe, outer epidermis). Bar = 180 μ m. **G.** Detail of a vascular bundle of a mature leaf sheath showing accumulation of *uidA* gene transcripts in sclerenchyma (sc) and xylema (xy) cells (ph, phloem; e, epidermis). Bar = 18 μ m. **H–O.** Histochemical localization of GUS-specific activity in actively growing roots of a 7–8 leaf stage plant (H; arrows point sites of formation of lateral roots; bar = 3 mm), a segment of ante-penultimate pith internode of a heading plant (I, bar = 1 mm), a transversal histological internode section (J; e, outer epidermis; ovb, outer vascular bundle; ivb, inner vascular bundle; bar = 150 μ m), spikelets of increasing maturity of an emerging panicle (K; l, lemma; p, palea; a, anthers; bar = 3 mm), excised androcae and gynaeae of an immature flower (early microspore stage) (L; a, anther; f, filament; ov, ovary; st, style; st, stigmata; l, lodicule; bar = 0.5 mm), dehiscent anthers at anthesis (M; l, anther loculus; f, filament; bar = 0.5 mm), a longitudinal histological section of an anther loculus at the uninucleated microspore stage (N; bar = 18 μ m), a longitudinal histological section of an anther loculus at the mature pollen stage (O; ep, epidermis; end, endothecium; bar = 18 μ m).

Tissue and developmental specificity of the *Ltp1* gene was investigated by localization and quantification of GUS activity in leaf samples of young homozygous seedlings harbouring the p Δ 1176Gus construct at the 6 leaf stage. Naturally occurring maturity gradients along and among the leaves of the graminaceous plant provide an experimental system to monitor de-

velopmental changes in gene expression during leaf tissue differentiation and maturation. Leaf sections 5 mm wide were regularly collected along the leaves from base to top starting with the innermost rolled to the older and not senescent leaf with a well differentiated blade which are the 6th and 2nd leaf respectively formed after the first incomplete leaf of the seedling

(Figure 3A). Systematic staining of one sample every centimetre revealed striking changes in both localization and intensity of staining according to the position of the sample along the leaf and of the rank of the leaf.

The actively growing innermost rolled leaf blade showed a faint staining and GUS activity in p Δ 1176Gus plants whereas the leaf of the same rank of the positive control showed the strongest staining and highest activity among leaves tested (Table 2). In p Δ 1176Gus plants, the base of the surrounding young developing sheath of the 5th leaf was faintly stained whereas older sheath and non-emerged blade tissues exhibited very strong activity localized in the epidermal cell layer situated between the vascular bundles (Figure 3B). In the emerged part the leaf blade, strong GUS staining was observed at the epidermis of mid-rib and at the cutting of the expanded tissue (Figure 3C). The same changes in staining patterns were observed along the 4th leaf with strongest staining observed in epidermis and in stomatal guard cells along the vascular bundles of the leaf sheath. For the 2nd and 3rd leaves, a declining and finally barely detectable GUS signal was observed in the epidermis while GUS activity becomes prominent in the vascular bundles (Figure 3D). Consistently, the highest GUS activities were observed in leaves 4 and 5 (Table 2) yet remaining far below the level observed in the positive control.

Tissue specificity was further examined by light microscopy of transversal sections of GUS-stained shoot samples. Blue indigo precipitates were observed in inner and outer epidermal cells of growing leaf sheath whereas in mature leaf sheaths, low GUS activity products were found, solely in stomata guard cells and in vascular bundles (Figure 3E). Transversal sections in expanded leaf blade samples allowed detecting of accumulation of blue crystal precipitates in all cell types of the tissues adjacent to the cutting site whereas more distal, uninjured tissues did not contain any GUS assay product.

We further conducted *in situ* examination of the *uidA* gene expression. The GUS assay might be prone to artefacts due to diffusion of intermediate products of the reaction, as well as to misinterpretation related to the stability of the GUS enzyme in cells which can accumulate even through residual expression levels. Furthermore, *in situ* hybridization may reveal very transient developmental changes in promoter activity. In our materials, *Ltp1* transcripts were finely localized in inner and/or outer epidermis of growing leaves and

in sclerenchyma cells of vascular bundles of mature leaves (Figures 3F and G).

Activity of the Ltp1 gene promoter in other organs

Although no GUS staining was observed in primary seminal and secondary roots of 3–7-day old seedlings of lines harbouring the p Δ 1176Gus construct, analysis of the root system of plantlets at the 5–6 leaf stage allowed us to detect GUS staining in the vascular system of the differentiated roots, which appeared more intense at the lateral root emission sites (Figure 3H). Consistent GUS-specific activity ranging from 6.0 ± 3.0 to 15.0 ± 12.0 pmol 4-MU h⁻¹ μ g⁻¹ were detected in root protein extracts of the four p Δ 1176Gus transgenic lines under investigation (positive control: 1986 ± 396 pmol 4-MU h⁻¹ μ g⁻¹).

Strong staining limited to the outer epidermis was observed in the first two internodes beneath the apical meristem of booting plants (Figure 3I and J). In reproductive organs, blue staining was detected at the lemma and palea, sterile glumes and in anther walls containing the microspores (Figure 3K–N). Histological sections in immature and mature anthers permitted detection of blue indigo crystal precipitates (Figure 3N and O). GUS-specific activity (ranging from 0.3 to 60 pmol 4-MU h⁻¹ μ g⁻¹ vs. 0.0 and 960 pmol 4-MU h⁻¹ μ g⁻¹ in pollen of negative and positive control plants respectively) was detectable by fluorometric assays of protein extracts of shedding pollen grains in the four p Δ 1176Gus events. Conversely, no staining was observed in the female part of the flower (ovary, style, stigmata, ovary) whatever the developmental stage investigated. Intense GUS activity was also found at the shoot tip level (Figure 4A).

Activity of the Ltp1 gene promoter during embryogenesis

Analysis of longitudinal sections of developing seeds of plants of transgenic lines harbouring the p Δ 1176Gus construct from 5 to 30 days after anthesis did not allow detection of any GUS activity in the tegument, endosperm and embryo. In young germinating seedlings strong staining was observed in the mesocotyl whereas faint GUS activity was detected in the scutellum (Figure 4B).

As excision of embryos at earlier stage of seed development proved to be difficult, we investigated whether the *Ltp1* gene is expressed during somatic embryogenesis by inoculating a callus induction medium with aseptic seeds of the p Δ 1176Gus line 64 and of the

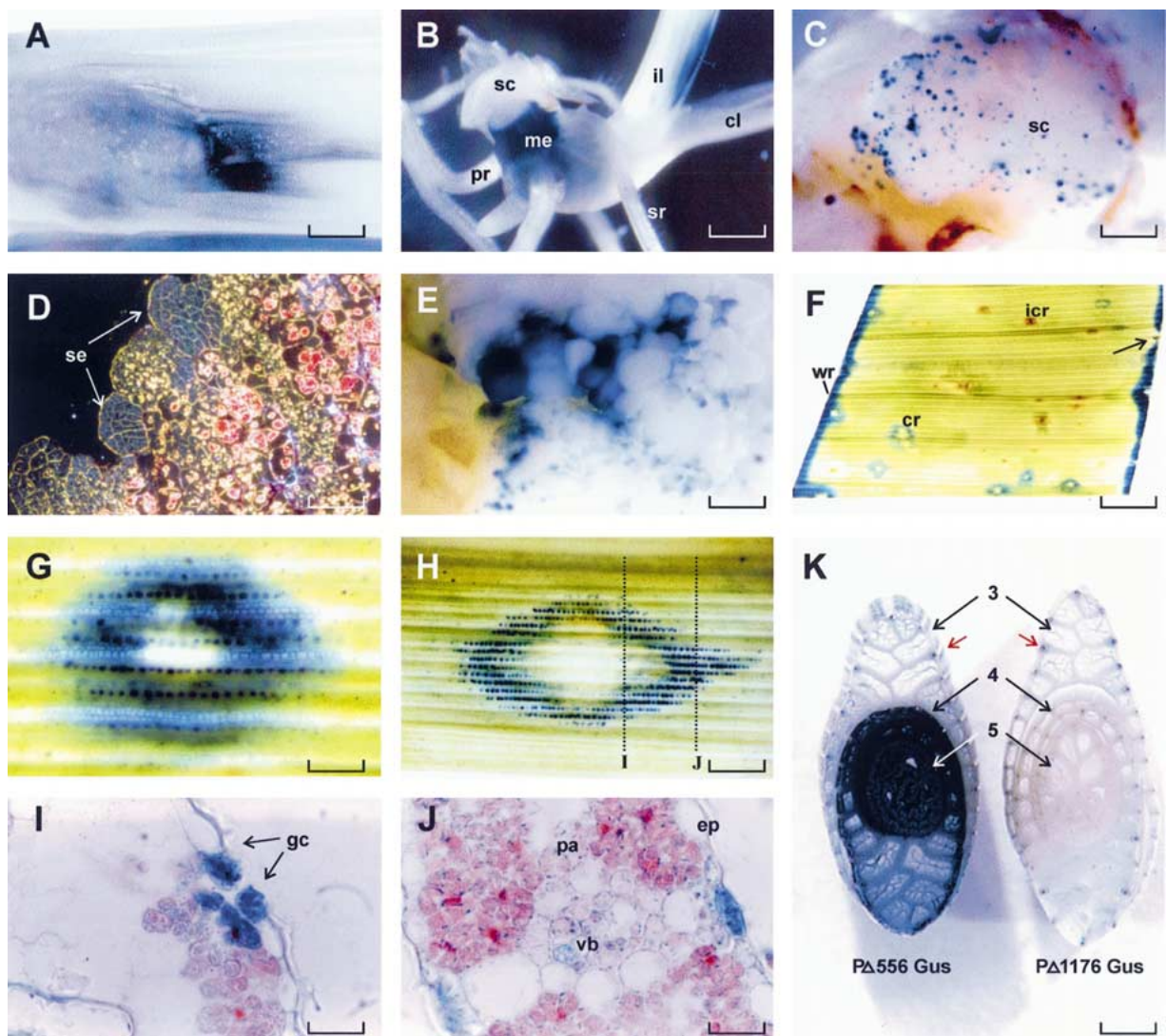


Figure 4. Activity of the the promoter of the *Ltp1* gene during embryogenesis and in response to fungal attack. A–J. Localization of GUS-specific activity in transgenic rice plants of cv. Taipei 309 harbouring the pΔ1176 Gus construct in a longitudinal section of the shoot basis of a seedling at the 5–6 leaf stage (A; bar = 1 mm), in a 7-day old seedling (B; sc, scutellum; me, mesocotyl; pr, primary root; sr, secondary root; cl, coleoptile; il, first incomplete leaf; bar = 2 mm), in developing embryo scutellum-derived primary callus (C–E) formed on callus induction medium 7 days after inoculation (C; bar = 0.5 mm), 7 days after inoculation after histological section and dark-field observation (GUS precipitates appear here in pink) (D; bar = 18 μ m) and 14 days after inoculation (E; bar = 0.25 mm), in blade segments of the last ligulated and expanded leaf of seedlings inoculated with *Magnaporthe grisea* strain CD128 (F–J) 4 days after inoculation (F; icr, necrotic lesion resulting from an incompatible reaction; cr, developed lesion resulting from a compatible reaction; wr, wound response; arrow points to the absence of GUS activity related to wr at the icr level; bar = 1 mm), 4 days after inoculation (G; detail of a cr lesion; bar = 0.25 mm), 6 days after inoculation (H; bar = 1 mm), in a transverse section of the discoloured region of a cr lesion, 6 days after inoculation (I; gc, stomata guard cells; note disorganization of the internal tissues; bar = 18 μ m), in a transverse section of the coloured region of a cr lesion, 6 days after inoculation (J; ep, epidermis; vb, vascular bundle; pa, parenchyma; bar = 18 μ m). K. Comparative localisations of GUS-specific activity in segments of young shoots of 5–6 leaf stage seedlings harbouring the pΔ1176Gus or the pΔ556Gus construct, 3 cm above the shoot apex (3, 4, 5: leaf rank; see Figure 3A, red arrows point to GUS activity in vascular bundles; bar = 1 mm).

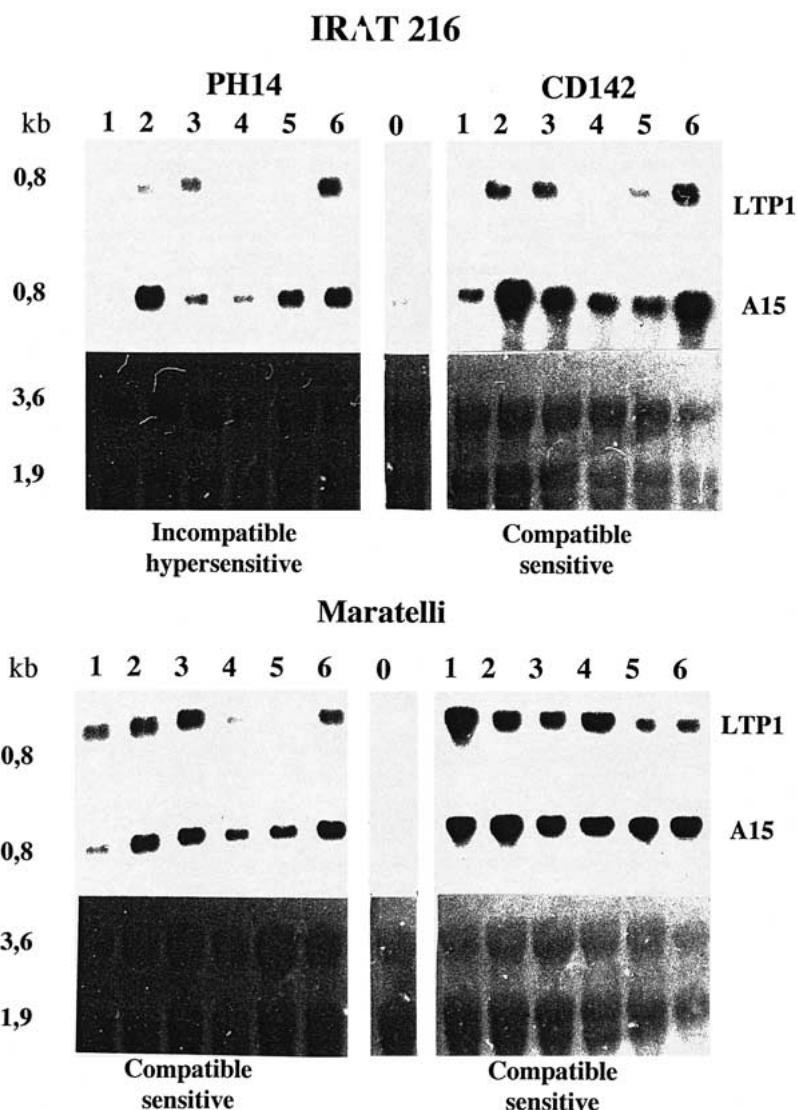


Figure 5. Time course of *Ltp1* mRNA accumulation in Maratelli and IRAT216 seedlings, 0–6 days after inoculation with *Magnaporthe grisea* strains PH14 and CD142. The RNA gel blots were probed with randomly primed ³²P-labelled PCR fragments corresponding to the 3'-untranslated region of the *Ltp1* gene and of the A15 cDNA clone, another class II of rice *Ltp1* gene (Vignols *et al.*, 1997) (upper panels). The RNA gel blots were re-probed with a wheat ribosomal RNA gene (PTA71) to determine the relative amount of RNA in each lane (lowest panel).

positive and negative controls. Histochemical assays, performed 5, 15 and 28 days after inoculation, led to heterogeneous staining of the primary callus at its various stages of formation from the scutellum tissue. At day 5, activity was localized to small round-shaped protuberances at the scutellum surface which were in fact globular somatic embryos formed from the reactive epidermal cell layer (Figure 4C). Dark-field microscopy observations of callus sections confirmed that crystal products of the GUS reaction (seen here in

pink) were localized to cells of globular somatic embryos separated from the rest of the developing callus by a thickening of the mid lamella of the cell walls (Figure 4D). Activity tended to decline in developed primary calluses and secondary calluses, which exhibited a faint and diffuse superficial staining (Figure 4E). A contrasting homogeneous dark blue staining was observed in comparable samples collected throughout the same callus developmental stages of the positive

control (not shown). Young regenerating p Δ 1176Gus plantlets were deeply stained in blue.

Inducibility of the Ltp1 gene upon inoculation with Magnaporthe grisea

Several barley *Ltp1*-type genes have been shown to be induced by pathogenic fungi and some LTP proteins exhibit *in vitro* antifungal and antibacterial activity. We therefore assayed the response of this rice *Ltp1* gene to inoculation with *M. grisea*, the agent of rice blast. We used two rice varieties, Maratelli and IRAT 216, which have been previously well characterized for their reaction spectrum against a range of *M. grisea* isolates, and two strains of *M. grisea*, PH14 and CD142. Whereas Maratelli is sensitive to both strains, IRAT216 is sensitive to and compatible with CD142 and resistant and incompatible with PH14. *Ltp1* gene expression was examined over a week (infection symptoms are apparent after 3 days) with a gene-specific probe (Figure 5), as well as with two related probes A14 and A15 (Vignols *et al.*, 1997) corresponding to other *Ltp1* genes belonging to class II (A14, which is not shown, gives an identical pattern to A15) which do not cross-hybridize with *Ltp1*. In all situations, the *Ltp1* mRNA and other *Ltp1* (A15 and A14) were detectable at a very low level in control leaves but rapidly induced one or two days after inoculation. Induction of *Ltp1* appears slower in the case of the incompatible reaction between IRAT 216 and PH14, being at its maximum at day 3. In contrast, the corresponding gene was already induced at day 1 in Maratelli. A lower level of expression was regularly observed at day 4 and 5, but the reason for this reduced expression is not clear. Other probes corresponding to the class III *Ltp1* genes (Vignols *et al.*, 1997) or to the *Ltp2* gene (Garcia-Garrido *et al.*, 1998) were assayed on the same filters but did not reveal any induction (not shown), suggesting that amongst the multiple rice *Ltp* genes, only a subset belonging to classes I and II *Ltp1* respond to *M. grisea*. Attempts to detect an increase in *Ltp1* protein content was not fully conclusive due to the lack of specificity of the antibodies and high background.

Localization of leaf cells expressing the p Δ 1176Gus construct in response to infection by Magnaporthe grisea

We have shown above that transcription of the *Ltp1* gene is strongly enhanced by fungal attack. As a

complementary approach, transgenic plants harbouring the p Δ 1176Gus construct offer the opportunity to localize precisely the leaf cells in which the *Ltp1* gene is induced by the development of *M. grisea* infection. As transformation of Maratelli and IRAT216 was not sufficiently routine in our hands for introducing p Δ 1176Gus and positive and negative control constructs, experiments were carried out with the available transgenic lines of cv. Taipei 309. Blade sections 1 cm wide of the last-emerged leaf of progeny seedlings of 4 transgenic lines harbouring the p Δ 1176Gus were subjected to histochemical GUS assay from the day of spraying with spores of the isolate CD128 to 6 days after inoculation. Positive (pUGCI C+8) and negative (p Δ 1262Gus C-24) control lines were included in the trial. p Δ 1176Gus samples sprayed with an aqueous solution of gelatin exhibited only a blue staining in response to cutting, as mentioned above, which was rather stable throughout the experiment. In contrast, the whole leaf surface of samples of the positive controls were more or less homogeneously stained. In inoculated p Δ 1176Gus samples, however, the staining in response to cutting tended to decline throughout the week of sample collection as the blast symptoms developed and leaf senescence occurred.

In inoculated lines, the first symptoms were detectable 3–4 days after inoculation; they comprise small necrotic lesions resulting from hypersensitive reactions and developed lesions resulting from a compatible interaction (Figure 4F). GUS staining was only observed around the latter infection sites which exhibited a ring of diffuse staining of inner tissues around the discoloured area and a stronger staining in certain epidermal cells (those located along the vascular bundles and the stomatal guard cells. At day 4, a lesion appeared as a 0.5–1.0 mm ring of blue punctuations which could be seen on both sides of the leaf (Figure 4G). Less developed reactions concerning a smaller number of cells and only one leaf face where the spore was deposited were also observed. Six days after inoculation, the blue punctuated ring may have attained 3–5 mm diameter (Figure 4H). Light microscopy observations of histochemically assayed samples permitted detection of blue crystal precipitates in stomatal guard cells in the discoloration zone where the tissues are totally disorganized by fungal development (Figure 4I). In contrast, all cell types, including vascular and parenchymatous tissues, abundantly accumulated products of the GUS reaction in

apparently intact tissues surrounding the discoloration zone (Figure 4J).

No GUS activity was observed in inoculated samples of the negative control p Δ 1262Gus, indicating that the GUS activity cannot be ascribed to fungal hyphae. Samples of the positive control pUGCI exhibited a uniform deep staining at the whole lesion level whereas the rest of the leaf tissue was more lightly but rather uniformly stained, as mentioned above.

Analysis of Ltp1 gene promoter activity in rice deletion mutants

Shoot sections of 22% of the young T₀ plantlets regenerated from calluses bombarded with the p Δ 556Gus constructs expressed the *uidA* gene (Table 1). Staining intensity in p Δ 556Gus plants was generally very faint and irregular except in transgenic lines 8 and 17, and always limited to vascular bundles of the sheath. Histochemical and fluorometric assays were systematically performed on samples collected along leaves of 6-leaf-staged T₃ plants of the transgenic lines 8 and 17. These assays confirmed that staining was limited to the vascular bundles of blade and sheath tissues of the second and third emerging leaves (Figure 4K), where the highest GUS activities were also observed (Table 2). No GUS staining was observed at the cutting site of leaf blade samples. On the other hand, higher GUS-specific activities – comparable to the level attained in p Δ 1176Gus plants – were observed in root protein extracts of the p Δ 556Gus 8 (24.0 ± 12.0 pmol 4-MU h⁻¹ μ g⁻¹) and 17 (24.0 ± 6.0 pmol 4-MU h⁻¹ μ g⁻¹) transgenic lines. No GUS staining was observed in pith internode and reproductive organs. This suggests that the rice plants harbouring the p Δ 556Gus construct do not express the *uidA* gene in epidermis and pollen as well as in response to wounding whereas they retain expression in vascular bundles of leaf and root systems. Vascular-specific expression along the leaf appeared spatially deregulated since it was also observed in its blade part contrasting with the pattern seen in mature leaves of p Δ 1176Gus plants. Conversely, observation of leaf blade of p Δ 556Gus plants of lines 8 and 17 inoculated with spores of *M. grisea* following the same protocol as described above for p Δ 1176Gus plants did not allow detection of any GUS activity at the lesion level (not shown).

None of the T₀ plantlets regenerated from calluses bombarded with the p Δ 284Gus construct exhibited GUS activity using both histochemical and fluorometric assays. This suggests that the 284 bp fragment of

the *Ltp1* gene promoter is too short to confer gene activity.

Discussion

Plant lipid transfer proteins are known to be members of multigenic families. One approach to better understand the function of the various isoforms in plants is to analyse their pattern of expression. With northern blot experiments on rice tissues, at least seven genes encoding LTP1 proteins belonging to 3 subclasses were shown to be submitted to specific regulation depending on the tissue and culture conditions (Vignols *et al.*, 1997). Using a genomic clone containing a rice *Ltp1* gene (Vignols *et al.*, 1994), we isolated a 1176 bp fragment containing 5' sequences and fused this promoter region to the *uidA* reporter gene in order to obtain and analyse transgenic rice plants for *Ltp1* gene expression.

The promoter of Ltp1 gene is preferentially active in aerial organs

We observed a relatively low frequency of plants exhibiting a detectable GUS specific activity among p Δ 1176Gus primary transformants. Nevertheless, we were able to select 4 independent transgenic lines on the basis of integration pattern and stability of expression across generations to conduct histochemical and fluorometric assays. This can be considered a minimum to avoid artefacts related to an influence of the transcriptional activity of the site of transgene integration on its expression. The four selected lines exhibited consistent staining patterns, which makes observations reliable. The low frequency of p Δ 1176Gus plants expressing the *uidA* gene may be due to analysis of a promoter of a gene member of a multigenic family in a homologous host. The expression of the various members could be strictly coordinated at the transcriptional level which may favour *trans*-inactivation of an additional promoter sequence carried in the genome by the transgene. Silencing of the fusion between the barley aleurone-specific *Ltp2* promoter and the *uidA* gene has been studied in detail in transgenic rice (Morino *et al.*, 1999).

Preferential activity of the *Ltp1* promoter in aerial organs is consistent with *Ltp1* transcript accumulation observed in young rice plantlets (Vignols *et al.*, 1994) and confirms reports on genes encoding spinach (Bernhard *et al.*, 1991), maize (Sossountzov *et al.*,

1991), barley (Molina and Garcia Olmedo, 1993), carrot (Sterk *et al.*, 1991), tobacco (Fleming *et al.*, 1992), *Arabidopsis* (Thoma *et al.*, 1993) and broccoli (Pyee and Kollatukudy, 1995) LTPs. The *Ltp1* gene promoter activity in aerial organs, and particularly in leaves, is also consistent with the presence of an I-box, an element shown to be involved in leaf-specific gene expression and light responsiveness (Rose *et al.*, 1999). The plants originating from transformation events with the p Δ 556Gus construct, which still contains the I-box element, also exhibit a specific expression in aerial green tissues, although slightly different from that observed in p Δ 1176Gus-transformed lines. This suggests that the I-box may not be the only element that controls *Ltp1* gene expression in aerial tissues.

In most species, no *Ltp* transcripts were detected in roots, in contrast to rice roots which accumulate *Ltp1* mRNAs, although at a low level (Vignols *et al.*, 1994). The detection of GUS activity only in secondary roots having produced lateral roots in our p Δ 1176Gus material definitively establishes that this gene is expressed in some regions of the root system. We have shown that GUS-specific activity declines progressively in leaf tissues as the organ matures among leaves of increasing rank. Similarly, abundance of *NtLtp1* transcripts was found to decrease with leaf aging (Fleming *et al.*, 1992).

A strong activity of the *Ltp1* promoter was observed in glumes and young male reproductive organs. High levels of expression in inflorescences were generally observed with *Ltp1* genes expressed in foliar organs of dicot species (Koltunow *et al.*, 1990; Sterk *et al.*, 1991; Fleming *et al.*, 1992; Foster *et al.*, 1992; Thoma *et al.*, 1994; Pyee and Kollatukudy, 1995). However, there is no information on monocot inflorescences. We detected the most intense staining patterns for the rice *Ltp1* gene in immature anther walls and microspores of the p Δ 1176Gus plants suggesting that LTP1 could participate in cell wall strengthening in these tissues.

The Ltp1 promoter is mainly active in epidermal cell layers

Histochemical assay of GUS activity and *in situ* hybridization of *uidA* mRNA in leaves of p Δ 1176Gus plants demonstrated that the tissue specificity of the *Ltp1* gene is under developmental control. Preferential localization at the epidermis level in leaf sheath and non-emerged leaf blade is consistent with previous observations with other *Ltp1* genes using immunolocal-

ization, *in situ* hybridization on analysis of transgenic plants expressing a promoter *uidA* gene fusion (Kader, 1996). The *Arabidopsis Ltp1* transcripts accumulate in the protodermis of embryos and the epidermis of the meristem, primary and secondary leaves of young seedlings and floral meristem, nectaries and female reproductive organs (Thoma *et al.*, 1993). Analyses of transgenic tobacco plants expressing the *uidA* gene controlled by either *NtLtp1* (Canevascini *et al.*, 1996) or *wax9D* (Pyee and Kollatukudy, 1995) demonstrated localization of GUS activity in the epidermis of aerial organs, with additional localization in the epidermis of the root system for the *NtLtp1* gene.

Stronger staining observed in certain epidermal cell types such as stomatal guard cells has also been observed in plants harbouring a fusion of the *uidA* gene with the *Arabidopsis* (Thoma *et al.*, 1994) or tobacco (Canevascini *et al.*, 1995) *Ltp1* promoters. Other epidermal cell types may exhibit preferential expression of *Ltp* genes such as trichomes or the modified trichomes that are cotton fibre cells (Ma *et al.*, 1995). A recent analysis of the promoter of the cotton *Ltp6* gene fused to the *uidA* reporter gene in transgenic tobacco confirmed that it directed expression only in trichomes in the heterologous host (Hsu *et al.*, 1999).

Localization of *uidA* transcripts by *in situ* hybridization has shown that they are abundant in the leaf inner and/or outer epidermal cell layer depending on the developmental stage. In young internodes GUS-specific activity was found to be limited to the outer epidermis. Prominent accumulation of *Ltp1* transcripts in outer epidermis has also been observed in maize coleoptile (Sossountzov *et al.*, 1991) and young barley leaves (Molina and Garcia-Olmedo, 1993). This indicates that *Ltp1* genes are preferentially expressed in the epidermal face which is more likely to be prone to a physical disruption.

The tissue specificity of the activity of the Ltp1 gene is developmentally regulated

We have shown that, while the epidermal activity of the *Ltp1* promoter seems prominent in sheath of developing leaves which are surrounded by mature leaf sheaths, promoter activity became limited to the vascular bundles and precisely in sclerenchyma cells in mature leaves of higher rank. This may be correlated with cuticle deposition and sclerenchyma cell wall thickening during leaf development. Accumulation of *Ltp1* transcripts in the vascular bundles has also been observed in maize (Sossountzov *et al.*, 1991) and

barley (Molina and Garcia-Olmedo, 1993; Gausing 1994). A shift from epidermal to vascular specificity of *Ltp1* gene expression as organs mature has also been reported in *Arabidopsis* (Thoma *et al.*, 1994) and barley (Gausing, 1994). The epidermal or vascular specificity of the rice *Ltp1* promoter found in the sheaths is not observed in the expanded leaf blades. In the latter tissues, the *Ltp1* promoter appears active only in cells adjacent to the cutting site but in all cell types, including parenchyma cells and other cells of the vascular bundles. This localized staining pattern is not due to a weaker substrate penetration in inner tissues since it is neither expanded by treatments enhancing tissue permeability – which otherwise allow homogeneous staining of the blade surface in positive control samples – nor observed in leaf sheath samples. This putative local wound inducibility has not been reported in other *Ltp* genes. However, it might be an explanation of the few discrepancies observed between histological localizations of *Ltp* transcripts and final products of the GUS assay when parallel *in situ* hybridization and analysis of promoter-*uidA* gene fusions in transgenic plants were conducted (Thoma *et al.*, 1994; Canevascini *et al.*, 1996).

The Ltp1 promoter is active at an early stage of somatic embryogenesis

Although the *Ltp1* 1176 bp promoter sequence contains a RY-element known to be involved in seed-specific expression in many genes (Bäumlein *et al.*, 1992), we failed to detect expression of the *uidA* gene controlled by the *Ltp1* promoter during seed development. Other *Ltp* genes expressed in vegetative organs in other species are generally also expressed at early stages of zygotic (Sossountzov *et al.*, 1991; Sterk *et al.*, 1991; Molina and Garcia-Olmedo, 1993; Thoma *et al.*, 1994) and somatic (Sterk *et al.*, 1991; Toonen *et al.*, 1997) embryo development. On the other hand, we observed intense GUS staining at an early stage of somatic embryo development from scutellum cells of mature seed embryo of a line harbouring the p Δ 1176Gus construct, corresponding to the globular stage (Jones and Rost, 1989). This may suggest that the rice *Ltp1* gene is also transiently but strongly expressed at a very early stage of zygotic embryogenesis which was not studied here.

Expression of the Ltp1 gene is enhanced after inoculation with Magnaporthe grisea

This paper reports the first evidence that a rice *Ltp1* gene is induced by the rice pathogen *Magnaporthe grisea*. Although various host \times pathogen race combinations were tested there are no striking differences, except in the kinetics of expression, between induction in a compatible and an incompatible interaction, suggesting that the response of this *Ltp1* gene is not specific. It is noteworthy that the induction, when analysed with a reporter gene, is essentially restricted to the lesion region, suggesting that LTP 1 protein is involved in the establishment of a physical barrier to the fungus rather than in a systemic response. An obvious application of this observation is to couple the *Ltp1* promoter with a more potent antifungal protein to engineer plant resistance to fungi. Because it is also induced by wounding such a promoter could also be used to engineer resistance to chewing insects and their larvae.

Beyond its contribution to organ protection, rice LTP1 may have an active role of defence against fungal pathogens

Localization of both crystal products of the GUS assay and *Ltp1* transcripts (Vignols *et al.*, 1994) in transgenic rice harbouring the p Δ 1176Gus construct, in addition to the proposed general role of LTPs in transport of lipophilic materials outside the cell, suggest that rice LTP1 participates in aerial organ protection and strengthening of the vascular system. It should be noted that this elaboration of structural barriers occurs in those parts of the plant which are most vulnerable to physical disruption or pathogen attack. The local inducibility in response to mechanical wounding and fungal hyphal development in the expanded blade tissue also suggests that participation of the *Ltp1* gene in plant protection is re-inducible. We have shown here that accumulation of rice *Ltp1* transcripts is strongly induced after inoculation by *M. grisea*. This finding was also illustrated by the observation of inoculated leaves of transgenic lines harbouring the p Δ 1176Gus construct. The GUS staining patterns surrounding blast lesions were different from those resulting from simple mechanical wounding, as GUS expression occurs in all cells of undisrupted tissues and in stomatal guard cells. These data are in agreement with the presence of a TCA-related sequence, that has been previously shown to be involved in response to

pathogen attack (Goldsbrough *et al.*, 1993). Interestingly, this element is located in the distal part of the *Ltp1* gene promoter. Its absence in the p Δ 556Gus and p Δ 284Gus constructs could explain the absence of response to pathogen attack by the plants harbouring these constructs.

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