ORIGINAL ARTICLE

The *Triticum aestivum* non-specific lipid transfer protein (*TaLtp*) gene family: comparative promoter activity of six *TaLtp* genes in transgenic rice

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Abstract Plant non-specific lipid transfer proteins (nsLTPs) are encoded by a multigene family and support physiological functions, which remain unclear. We adapted an efficient ligation-mediated polymerase chain reaction (LM-PCR) procedure that enabled isolation of 22 novel *Triticum aestivum nsLtp* (*TaLtp*) genes encoding types 1 and 2 nsLTPs. A phylogenetic tree clustered the wheat nsLTPs into ten subfamilies comprising 1–7 members. We also studied the activity of four type 1 and two type 2 *TaLtp* gene promoters in transgenic rice using the β-Glucuronidase reporter gene. The activities of the six promoters displayed both overlapping and distinct features in rice. In vegetative organs, these promoters were active in leaves and root vascular tissues while no β-Glucuronidase (GUS) activity was detected in stems. In flowers, the GUS activity driven by the *TaLtp7.2a*, *TaLtp9.1a*, *TaLtp9.2d*, and *TaLtp9.3e* gene promoters was associated with vascular tissues in glumes and in the extremities of anther filaments whereas only the *TaLtp9.4a* gene promoter was active in anther epidermal cells. In developing grains, GUS activity and GUS immunolocalization data evidenced complex patterns of activity of the *TaLtp7.1a*, *TaLtp9.2d*, and *TaLtp9.4a* gene promoters in embryo scutellum and in the grain epicarp cell layer. In contrast, GUS activity driven by the *TaLtp7.2a*, *TaLtp9.1a*, and *TaLtp9.3e* promoters was restricted to the vascular bundle of the embryo scutellum. This diversity of *TaLtp* gene promoter activity supports the hypothesis that the encoded TaLTPs possess distinct functions in planta.

Abbreviations

dpa	Day post-anthesis
EST	Expressed sequence tag
GUS	β-Glucuronidase
LM-PCR	Ligation mediated PCR
MATAB	Mixed alkyl trimethyl ammonium bromide
nsLTP	Non-specific lipid transfer protein
nsLtp	Non-specific lipid transfer protein gene
SAR	Systemic acquired resistance
TaLtp	*Triticum aestivum* non-specific lipid transfer protein gene
uidA	β-Glucuronidase gene

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Introduction

Non-specific lipid transfer proteins (nsLTPs) are cysteine-rich proteins that are found throughout the plant kingdom. NsLTPs were originally defined by their capacity to transfer various lipid compounds between lipid bilayers in vitro (Kader et al. 1984), but are currently more often defined by sequence homology than on a functional basis. NsLTPs are encoded by a multigene family and can be classified in three types according to their primary structure (Boutrot et al. 2005). The

most detailed characterization has been made of types 1 and 2 nsLTPs, which are 9 and 7 kDa basic proteins, respectively. Type 3 nsLTPs also display a 7 kDa molecular mass and all type 3 non-specific lipid transfer protein (*nsLtp*) genes show anther-specific expression (Lauga et al. 2000). NsLTPs belong to the eight-cysteine motif protein superfamily (José-Estanyol et al. 2004). These cysteine residues are involved in four disulfide bonds that stabilize the nsLTP tertiary structure. This structure is characterized by a hydrophobic cavity whose size plasticity allows the in vitro loading of a great variety of lipid compounds (Sy et al. 2003).

The existence of multiple *nsLtp* genes in many plant genomes was first revealed by Southern blot and then by analyzing expressed sequence tag (EST) databases. The number of *nsLtp* genes identified so far ranges from six in pepper (Liu et al. 2006) to 15 in *Arabidopsis* (Arondel et al. 2000). However, the recent analysis of the complete rice genome indicates the presence of 53 *nsLtp* genes (F. Boutrot et al. unpublished data).

The different members of several *nsLtp* gene families exhibit a wide range of expression profiles during plant development. For instance, RT-PCR and northern blot analysis revealed distinct regulations of expression in peach flowers (Botton et al. 2002) and in *Arabidopsis* and pepper plants (Arondel et al. 2000; Jung et al. 2003). Differences were also found at the protein level since lipid transfer activity differed between nsLTPs from grapevine (Coutos-Thévenot et al. 1993) and from wheat (de Lamotte, INRA, Montpellier, personal communication).

The *nsLtp* gene transcripts and corresponding proteins are mainly localized in epidermal cell layers such as the leaf and embryo epidermis or the fruit pericarp (for review see Kader 1996). Transcripts are also associated with the vascular system (Buhtz et al. 2004) or localized in anthers, mainly in tapetum cells (Vrinten et al. 1999). At the subcellular level, nsLTPs are mainly found in apoplastic spaces since they are synthesized as preproteins with a signal peptide that directs the mature protein to the secretory pathway (Kader 1996).

The physiological function of nsLTPs is not clear; however, the widely reported localization of nsLTPs in epidermal cell layers and the secretion of these proteins in cell walls support the hypothesis that they are involved in the deposition of cutin monomers that form the cuticle of leaf epidermal cells (Sterk et al. 1991). This hypothesis is further supported by positive correlations established between nsLTP accumulation and conditions that stimulate cuticle synthesis as drought (Cameron et al. 2006b). Several *nsLtp* genes were also preferentially expressed in the outer epidermal layer of fleshy fruits (Kader 1996; Botton et al. 2002; Liu et al.

2006). Because of the in vitro antibacterial and antifungal activities of certain nsLTPs (Cammue et al. 1995; Dubreil et al. 1998) and the induction of expression of many *nsLtp* genes in response to biotic infections (Molina and García-Olmedo 1993; Guiderdoni et al. 2002; Jung et al. 2003; Lu et al. 2005), plant nsLTPs are thought to be involved in plant defense mechanisms. NsLTPs could therefore play an indirect role in plant defense by establishing a mechanical barrier of cutin, and a direct role thanks to their intrinsic antibiotic properties and their preferential localization in epidermal cell layers.

Evidence for nsLTP biological activity was provided by Maldonado et al. (2002). Following plant-pathogen interaction, these authors showed that the *Arabidopsis dir1* mutant displays a normal local phenotype but fails to develop systemic acquired resistance (SAR). The *DIR1* gene encodes a putative nsLTP, which is thought to be involved in long-distance signaling (Maldonado et al. 2002). The localization of the *DIR1* transcripts in phloem companion cells (Ivashikina et al. 2003) strongly supports the hypothesis that the SAR-associated molecule signal is transported through the phloem sieve tubes (van Bel and Gaupels 2004). With less than 24% sequence identity to *Arabidopsis* nsLTPs, the DIR1 protein is not phylogenetically distributed within the three nsLTPs types usually reported (F. Boutrot et al. unpublished data). Moreover, the strong phenotype associated with the disruption of the *DIR1* gene indicates that the corresponding protein supports a physiological function that cannot be assigned to other nsLTPs. Biological activity was also demonstrated in a tobacco type 1 nsLTP1 by treating stem sections with recombinant nsLTP1 complexed with jasmonic acid. This treatment enhanced the resistance of tobacco against *Phytophthora parasitica*, which was not observed with application of nsLTP1 or jasmonic acid alone (Buhot et al. 2004). Several other physiological functions have been proposed for nsLTPs which are widely thought to play a central role in adaptation to abiotic stresses such as desiccation (Cameron et al. 2006b), cold or salinity (Jung et al. 2003). NsLTPs could also be involved in programed cell death since they could protect the living cells from the effects of proteolytic activities triggered during this cell process (Eklund and Edqvist 2003). NsLTPs are also thought to possess a physiological function in male reproductive tissues where they could be involved in the deposition of material in the developing pollen wall (Foster et al. 1992); however their precise physiological function in pollen remains to be elucidated.

In wheat, most knowledge on nsLTPs is related to their protein structure and technological properties

(for review see Marion et al. 2003). Transcript accumulation of only a few *Triticum aestivum nsLtp* genes has been monitored during grain development (Altenbach and Kothari 2004; Boutrot et al. 2005), in crown of wheat subjected to cold (Gaudet et al. 2003) or fungal infection (Lu et al. 2005), and in plants of a wheat-rye translocation line exposed to different biotic and abiotic stress (Jang et al. 2004, 2005).

Taken together these data strongly suggest that different nsLTPs are involved in distinct biological functions. In this context, the identification of multiple wheat *nsLtp* genes and the characterization of their expression during plant development could provide new insight into nsLTP function. In the present study, we report the isolation and characterization of 22 putative *T. aestivum* genes belonging to the *nsLtp* gene family. Six genes belonging to different subfamilies were further analyzed due to the display of diverse patterns of gene transcript accumulation in developing seeds (Boutrot et al. 2005). We compared the tissue-specificity and developmental regulation of these six *T. aestivum* non-specific lipid transfer protein gene (*TaLtp*) promoters in transgenic rice using the β-Glucuronidase reporter gene (*uidA*). β-Glucuronidase (GUS) activity was analyzed by fluorometric and histochemical assays, and by immunolocalization.

Materials and methods

Plant material

Field-grown wheat (*T. aestivum* L. 'Apache'; INRA, experimental station Melgueil, 34130 Mauguio, France) leaves were used for genomic DNA extraction. Mature seeds of the japonica rice *Oryza sativa* L. 'Nipponbare' (kindly supplied by Dr. M. Yano, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan) and *O. sativa* 'Zhongzuo321' (kindly supplied by Dr. Z.L. Chen, Beijing University, Beijing, China) were used for transformation. Histochemical and immunological assays were performed using *O. sativa* 'Nipponbare' and *O. sativa* 'Zhongzuo321' T_0 transgenic plants, T_1 seeds from progeny, and T_1 seedlings. GUS activities were determined using T_2 transgenic plants (*O. sativa* 'Nipponbare').

Genomic-library screening and cDNA isolation

The *TaLtp9.2c* gene was isolated from a genomic library of *T. aestivum* 'Chinese spring', constructed into the λFixII vector (Stratagene, La Jolla, CA, USA), kindly provided by Dr. C. Hartmann (Université Paris VII, Paris, France). Following standard protocols, this library was screened with a *nsLtp* gene fragment amplified from the pTd6.48 cDNA (Boutrot et al. 2005).

The cDNA clones pTaD2-2, pTa268, pTa232-5, and pTa232-6 were isolated by PCR using plasmid DNA prepared from an aliquot of a 4-day post-anthesis (dpa) *T. aestivum* seed cDNA library as template. PCRs were done with *nsLTP* specific antisense-strand primers and the universal T7 promoter as sense-strand primer as described by Boutrot et al. (2005).

Cloning of *T. aestivum nsLtp* gene promoters by genome walking

A genome walking procedure, the ligation mediated PCR (LM-PCR), was used to clone wheat *nsLtp* genes. The protocol of Siebert et al. (1995) modified by Dr. E. Bourgeois (personal communication) to allow one-step genomic DNA digestion and adaptor ligation was adapted for wheat. Briefly, 500 ng of wheat genomic DNA were digested overnight with 5 U of restriction enzyme (Promega, Madison, WI, USA) and ligated to adaptors (1.6 μM) with 5 U of T4 DNA ligase (Promega) in a total volume of 10 μl in 1× ligation buffer. Nine blunt-end restriction enzymes *Bal*I, *Dra*I, *Eco*RV, *Hin*dII, *Nae*I, *Pvu*II, *Sca*I, *Ssp*I, and *Stu*I were used independently. The adaptor consisted of an upper primer (5′-CACTGAATCTTGCTGACTAGGTCTG GGGAGGT-3′) and a lower primer (5′-O_4P-ACCTC CCCAGAC-NH_2-3′) synthesized by MWG Biotech (Courtaboeuf, France). Two rounds of PCR were then performed on digested-ligated DNAs. The primary PCR reactions contained 1 μM of the adaptor-related primer (5′-CTGAATCTTGCTGACT-3′), 0.2 μM of a *nsLtp* gene-specific primer, 12.5 μM of each dNTP, 1 mM $MgCl_2$, 1 U *Taq* DNA polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA, USA) and 50 ng digested-ligated DNA in a volume of 20 μl in 1× *Taq* DNA polymerase buffer. Touchdown PCRs were performed with an initial denaturation stage at 94°C for 3 min, 14 cycles at 94°C for 30 s, 61°C for 45 s (−0.5°C per cycle), 72°C for 2.5 min; and 20 cycles at 94°C for 30 s, 54°C for 45 s, 72°C for 2.5 min; with a final extension at 72°C for 5 min. The second PCRs were performed using 1 μl of the primary PCR product (diluted 1/50) in a reaction mixture containing 0.2 μM of the nested adaptor-related primer (5′-ATCTTGCTGACT AGGT-3′), 0.2 μM of a nested *nsLtp* gene-specific primer, 12.5 μM of each dNTP, 1.5 mM $MgCl_2$, 2 U *Taq* DNA polymerase in a total volume of 50 μl in 1× *Taq* DNA polymerase buffer. PCR cycles were as described above, but with 25 additional cycles instead of 20. All *nsLtp* gene-specific primers (Eurogentec, Liege,

Belgium) were 18–20 nucleotides long with an annealing temperature above 56°C. The secondary PCR products were analyzed on a 1.5% agarose/EtBr gel, cloned into pGEM-T Easy Vector System I (Promega) and recombinant plasmids were introduced into *Escherichia coli* JM109. Sequencing was performed on an ABI Prism 373 DNA sequencer (Applied Biosystems).

Wheat *nsLtp* genes obtained from sequence alignment of successive genome walks were confirmed by PCR using wheat genomic DNA as template and primers designed in the most distant 5′ and 3′ sequences of the amplified fragments. All the primers (Eurogentec) were 18–20 nucleotides long with an annealing temperature of above 56°C. Reactions were performed in a final volume of 25 μl containing 100 ng of genomic DNA, 1.6 μM of each primer, 100 μM of each dNTP, 1.9 U of *Pfu* DNA polymerase (Stratagene), 1× *Pfu* DNA polymerase reaction buffer and 10% (v/v) glycerol. Initial template denaturation was at 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, annealing temperature for 30 s, 72°C for 2 min, with a final extension at 72°C for 7 min. The PCR products were cloned and then sequenced as described above.

Binary vector constructs and rice transformation

The promoter regions of six wheat *nsLtp* genes, *TaLtp7.1a* (−745 to +3), *TaLtp7.2a* (−1,437 to +3), *TaLtp9.1a* (−1,390 to +3), *TaLtp9.2d* (−1,257 to +3), *TaLtp9.3e* (−824 to +3), and *TaLtp9.4a* (−843 to +3) were PCR-amplified from genomic DNA and subcloned into the pGEM-T Easy vector (Promega). PCR primers generated an *Eco*RI or *Sal*I site at their 5′ end and a *Pst*I, *Sal*I or *Hin*dIII site at their 3′ end (Table 1). The cloned promoter sequences were digested with *Eco*RI and *Pst*I (*TaLtp7.1a*, *TaLtp7.2a*, *TaLtp9.1a*, and *TaLtp9.2d*), *Eco*RI and *Sal*I (*TaLtp9.3e*) or *Sal*I and *Hin*dIII (*TaLtp9.4a*) and ligated to the corresponding digested pCAMBIA1381Xa binary vectors (R. Jefferson, CAMBIA, Canberra, Australia). Selectable marker genes allowed hygromycin resistance in plants and kanamycin

resistance in bacteria. Each of the resulting binary vectors was then introduced into *Agrobacterium tumefaciens* EHA105 strain (Hood et al. 1993) by electroporation. DNA digestion, ligation and electroporation were carried out following standard protocols (Sambrook et al. 1989). *Agrobacterium*-mediated transformation of embryogenic calli derived from mature rice seed embryos and plant regeneration were performed as described by Sallaud et al. (2003). The pCAMBIA1381Xa binary vector containing a promoter-less *uidA* reporter gene was used as negative control (Xa plants). Transgenic T_0 rice plants harboring a *CaMV35S::uidA* construct (kindly provided by J. Petit, Cirad, Montpellier, France) were used as positive controls.

Molecular analysis of transgenic T_0 rice plants

T_0 transgenic rice plants presenting a single T-DNA insertion were identified by Southern blot analysis. Genomic DNA was extracted from 40 mg of fresh 3-week-old rice leaves using 320 μl of mixed alkyl trimethyl ammonium bromide (MATAB) buffer [100 mM Tris–HCl, pH 8.0, 1.5 M NaCl, 20 mM EDTA, 2% (w/v) MATAB, 1% (w/v) PEG 6000, 0.5% (w/v) Na_2SO_2] prewarmed at 72°C. The mix was incubated for 2 h at 72°C, cooled to room temperature, extracted with 360 μl of chloroform: isoamyl alcohol (24:1, v/v) and then, DNA was isopropanol precipitated. Approximately 5 μg of *Hin*dIII-digested DNA were separated on a 0.8% agarose gel and blotted onto Hybond-N$^+$ membranes (Amersham Biosciences, Piscataway, NJ, USA). The coding sequences of *uidA* and *hptII* were radiolabeled with [α-^{32}P]-dCTP using random primer labeling (Amersham Biosciences) and used as probes. Hybridization signals were detected by autoradiography of X-Ray films exposed to membranes at −80°C.

GUS activity

Two hundred milligrams of 4-week-old leaves from T_2 rice plants were ground to powder using liquid nitrogen.

Table 1 Wheat *nsLtp*-specific primers used for promoter amplification

nsLtp gene[a]	5′-forward primer-3′	5′-reverse primer-3′	Amplicon size (bp)
TaLtp7.1a	CGG*AATTC*ACTCCATATCCTTGTACGAG	AAAA*CTGCAGC***CAT**GGTTCACGCTCTCG	767
TaLtp7.2a	*GAATTC*CAGCCGAGCATTC	AA*CTGCAG*T**CAT**GGCCGTC	1,454
TaLtp9.1a	CGG*AATTC*AATGGCTCAGTAGATGACAAG	AAAA*CTGCAGC***CAT**GATCACTTCTTGGTCTG	1,412
TaLtp9.2d	*GAATTC*CCAGGCATGCATG	*CTGCAGA***CAT**CTCGATCAAG	1,251
TaLtp9.3e	CGG*AATTC*AGTGCAGATCG	*GTCGACA***CAT**CGAGGATC	842
TaLtp9.4a	*GTCGAC*TGAGAGCCTATAG	G*AAGCTT***CAT**CGATCGGTAG	859

Introduced restriction sites are shown in italics and translation initiation codons in bold

[a] Accession numbers are given in Table 2

Proteins were extracted with 50 mM phosphate buffer, pH 7.0, containing 10 mM EDTA, 0.1% sodium laurylsarcosine and 0.1% Triton X-100. Homogenates were cleared by centrifugation and the resultant supernatants were assayed for total protein (BCA protein assay kit, Pierce, Rockford, IL, USA) and GUS activity (Jefferson et al. 1987). The reaction product 4-methylumbelliferone was measured fluorometrically (Fluoroskan II Ascent, Thermo LabSystems, Helsinki, Finland).

Histochemical GUS assay

Histochemical experiments were performed according to Jefferson et al. (1987). For each construct, expression in the rice root system, flowers, and seeds was carried out in four independent transgenic lines. To monitor expression in leaves, depending on the number of regenerated lines 4–11 independent transgenic lines were analyzed. Rice seeds were longitudinally half-sectioned prior to immersion in X-Gluc reaction buffer and then fixed in 200 mM phosphate buffer (pH 7.0) containing 1% (v/v) acrolein, 2% (w/v) glutaraldehyde, and 50 mM caffeine for 30 min at room temperature. The other tissues were fixed in 200 mM phosphate buffer (pH 7.0) containing 2% (w/v) paraformaldehyde, 1% (w/v) glutaraldehyde, and 50 mM caffeine. Chlorophyll was excluded by soaking rice tissues for several hours in 70% ethanol. Tissue observations were performed with a Leica MZFLIII binocular microscope (Leica Microsystems, Heerbrugg, Switzerland). Roots, stems and leaves were mounted in a 4% (w/v) agar block, and transversal vibratome sections 30–40 μm thick were cut. Flowers and grains were dehydrated and embedded prior to longitudinal microtome sectioning. Dehydration was performed in a graded ethanol series (70–95%, v/v). Grains were finally dehydrated in an additional butanol bath. Flowers were impregnated in a technovit 7100 resin (Kulzer, Wehrheim, Germany)-ethanol (1:1, v/v) mixture for 72 h while a resin-butanol (1:1, v/v) mixture was used for grains. Tissues were then embedded in molds with resin and polymerized for 2 h by adding the polymerization agent to the resin. Sections 3–5 μm thick were cut with a microtome. Bright- or dark-field light microscopic observations were performed with a Leica DM RXA fluorescence microscope (Leica Microsystems).

Immunological assay

Rice seeds were harvested, longitudinally sectioned with a razor blade and immediately fixed for 8 h in 100 mM Pipes buffer, pH 7.0, containing 1.6% (w/v) paraformaldehyde, 10 mM sodium *m*-periodate, 375 mM lysine, and 1 mM DTT. After several rinses with 100 mM Pipes buffer containing 100 mM glycine and 1 mM DTT, the fixed samples were dehydrated in a graded ethanol series (70–95%, v/v) containing 1 mM DTT, in butanol supplemented with 10 mM DTT and in a butanol-methacrylate series (3:1, 1:1, 1:3, v/v). Tissues were impregnated four times with 100% methacrylate containing 10 mM DTT and 0.4% (v/v) benzoil ethyl ether to enable polymerization. Sections were incubated in the final bath for 12 h at 4°C. Dissolved oxygen was displaced by bubbling gaseous nitrogen through the methacrylate mixture. The samples were then transferred to molds and covered with parafilm to limit contact with oxygen. The molds were placed 28 cm under an 8 W UV light source for 15 h at 4°C. For immunodetection, methacrylate sections (4 μm) were fixed on silanized slides (Dako Cytomation, Carpinteria, CA, USA) and the resin removed with acetone. The sections were rehydrated with a graded ethanol series from 100 to 50%, soaked in phosphate-buffered saline (PBS) for 10 min, incubated for 8 min in PBS containing 0.1% (w/v) trypsin (type XI, Sigma, St. Louis, MO, USA) and washed for 3 min with PBS. The reaction was stopped by washing with PBS containing 0.05% (w/v) trypsin inhibitor (type II-S, Sigma). The sections were washed two times (5 min each) with PBS, incubated for 1 h in PBS containing 1% (w/v) blocking agent (Roche, Basel, Switzerland), washed three times (10 min each) with PBS and, incubated for 15 h in PBS with 1% blocking agent and a 1:500 dilution of the monoclonal antibody raised against GUS (anti-β-glucuronidase rabbit IgG (H+L) fraction, Molecular Probes, Leiden, The Netherlands). The sections were washed with PBS three times (5 min each) and incubated for 90 min in PBS containing alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma) at a dilution of 1:500. After being washed three times (5 min each) in PBS buffer, the sections were incubated for 15 min with NBT/BCIP (*p*-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyphosphate-toluidine salt, Chemicon International, Temecula, CA, USA) containing 5 mM levamisole. After incubation with the secondary antibody, the reaction was stopped by washing with distilled water and 95% ethanol and the sections were stained for 2 min in a ruthenium red (0.005%, w/v) solution. Bright-field light microscopic observations were performed with a Leica DM RXA fluorescence microscope (Leica Microsystems).

Sequence alignments and phylogenetic analysis

A systematic search was performed by examining the EMBL (srs.ebi.ac.uk) and Entrez (www.ncbi.nlm.nih.gov/entrez) databases for wheat *nsLtp* cDNAs, genes

and proteins. Proteins were analyzed for the presence of a signal peptide using SignalP v3.0 (Bendtsen et al. 2004). Deduced primary structures of mature nsLTPs were aligned using the ClustalW v1.83 program (Thompson et al. 1994). The relationship between nsLTPs was investigated with the Phylip v3.6a3 package (Felsenstein 2005) using neighbor-joining analysis. Support for nodes was estimated by the bootstrap procedure, using 1,000 re-samplings of the data. The unrooted phylogenetic tree was graphically displayed using the Treeview v1.6.6 program (Page 1996).

Statistical analysis

The hemizygous or homozygous state of T_2 plants was determined by segregation analysis (Chi-square test, $P < 0.05$) for hygromycin resistance resulting from expression of the *hptII* selectable gene. For each construct, the GUS activity was measured from two independent T_2 transgenic lines analyzed in three replicates. Data are presented as the mean value ± SD of the GUS activity per *uidA* copy (one for hemizygous or two for homozygous plants). The data were analyzed by ANOVA and means were compared for level of significance ($P < 0.05$) by Bonferroni's multiple comparison test using GraphPad Prism v4.00 (GraphPad Software, San Diego, CA, USA).

Results

Identification of *Triticum aestivum* non-specific lipid transfer protein genes

In this study we isolated 22 *T. aestivum* genes encoding putative nsLTPs whose belonging to the nsLTP family was based solely on sequence homology (Table 2). The *TaLtp9.2c* gene was isolated by screening the *T. aestivum* genomic library whereas the *TaLtp9.2d* and *TaLtp9.5b* genes were amplified by PCR using specific primers designed from the *TaLtp9.2c* gene and the pLTP2 cDNA, respectively. All other wheat *nsLtp* genes were isolated using a genome walking approach, taking advantage of sequence information from cDNA clones to design specific primers. Thirty-two walks resulted in the amplification of genomic DNA fragments that included part of the coding sequence and/or promoter and led to the identification of 19 wheat *nsLtp* genes. Since the gene-specific primers were not as specific as expected and revealed complementarities with closely related genes, some DNA fragments belonging to different members of an *nsLtp* gene subfamily were amplified. In addition, because successive

walks were often necessary, the contiguity of fragments resulting from sequential PCRs was confirmed to avoid the creation of chimera genes. To this end, a final PCR was carried out using wheat genomic DNA as template and primers designed in the 5′ and 3′ extremities of the identified sequences, and all the *TaLtp* genes were fully sequenced. The 22 *TaLtp* gene sequences contained full-length ORF and the length of 5′ untranslated regions ranged from 9 (*TaLtp7.1c*) to 2,856 bp (*TaLtp9.5a*). In the time course of this study we also isolated four new cDNAs from an orientated 4-dpa *T. aestivum* seed cDNA library whose sequences correspond to *TaLtp9.3d*, *TaLtp9.8a*, *TaLtp9.7d*, and *TaLtp9.7e* genes. All the nucleotide sequence data were deposited in the EMBL database under accession numbers AJ852536 to AJ852561.

The previously characterized wheat *TaLtp9.5a* gene, cDNAs encoding nsLTPs and purified proteins were also included in Table 2. Of the 23 wheat *nsLtp* genes reported, corresponding cDNA clones were identified for nine of them indicating that these genes were transcribed at least in developing seeds (*TaLtp7.2a*, *TaLtp9.3d*, *TaLtp9.3f*, *TaLtp9.4a*, *TaLtp9.4b*, *TaLtp9.6a*, and *TaLtp9.7d*) or leaves (*TaLtp9.5a* and *TaLtp9.5b*). In contrast, no corresponding genes had been identified for seven cDNA clones isolated from developing seeds (pTa4.90, pTd4.90, pTd6.48, pTd6.48, pTaD2, and pTa232) or infected leaves (pLTP3) cDNA libraries and two proteins (TaLTP9.8a and TaLTP9.8b) isolated from wheat embryos.

In the absence of chromosome assignment and given the increasing number of wheat *nsLtp* genes described, we recently proposed a temporary nomenclature based on the phylogenetic classification of nsLTP sequences (Boutrot et al. 2005). To establish a more rigorous nomenclature, the following criteria were added: nsLTP mature proteins whose sequence identity is over 30% constitute a type and, within a type nsLTPs whose sequence identity is over 75% constitute a protein subfamily. Based on these criteria, the *TaLtp9.5a* gene (Boutrot et al. 2005) was reclassified as *TaLtp9.3f*. To date, we have identified two subfamilies within type 2 wheat nsLTPs and eight within type 1. Subfamilies were represented by 1–7 members.

Sequence features of *TaLtp* genes

In order to characterize the exon/intron organization of the 23 *TaLtp* genes reported in Table 2, genomic DNA sequences were compared to those of cDNAs when available or deduced from sequence alignments. This analysis indicated that no type 2 *TaLtp* genes contain introns. This also held true for four type 1 *TaLtp*

Table 2 Characteristics and references of wheat *nsLtp* genes, cDNA clones and proteins

nsLtp gene	cDNA	Protein	Accession number		Reference
			nsLtp gene	cDNA or protein	
Type-2					
	pTd18	TdLTP7.1a		AJ297768	Monnet et al. (2001)
		TdLTP7.1b		n.a.	Monnet (1990)
TaLtp7.1a		TaLTP7.1a[a]	AJ852549		
TaLtp7.1b		TaLTP7.1b	AJ852550		
TaLtp7.1c		TaLTP7.1c[b]	AJ852554		
TaLtp7.1d		TaLTP7.1d	AJ852551		
TaLtp7.1e		TaLTP7.1e	AJ852552		
TaLtp7.2a	pTa176	TaLTP7.2a	AJ852553	AJ784895	Boutrot et al. (2005)
Type-1					
	pTd4.90	TdLTP9.1a		X63669	Dieryck et al. (1992)
	pTa4.90	TaLTP9.1b		AJ784902	Boutrot et al. (2005)
TaLtp9.1a		TaLTP9.1a[c]	AJ852536	AF551849	
	pTd6.48	TdLTP9.2a		AJ784903	Boutrot et al. (2005)
	pTa6.48	TaLTP9.2b		AJ784901	Boutrot et al. (2005)
TaLtp9.2c		TaLTP9.2c	AJ852555		
TaLtp9.2d		TaLTP9.2d	AJ852556		
	pTaD2	TaLTP9.3a		AJ784899	Boutrot et al. (2005)
TaLtp9.3b		TaLTP9.3b	AJ852537		
TaLtp9.3c		TaLTP9.3c	AJ852538		
TaLtp9.3d	pTaD2-2	TaLTP9.3d	AJ852539	AJ852558	
TaLtp9.3e		TaLTP9.3e	AJ852540		
TaLtp9.3f[d]	pTa260	TaLTP9.3f	AJ852542	AJ784896	Boutrot et al. (2005)
TaLtp9.3g		TaLTP9.3g	AJ852543		
TaLtp9.4a	pTa8.3	TaLTP9.4a	AJ852541	AJ784900	Boutrot et al. (2005)
TaLtp9.4b	pTa268	TaLTP9.4b	AJ852548	AJ852561	
	pLTP3	TaLTP9.4c[e]		AY226580	Jang et al. (2005)
TaLtp9.5a	pLTP1	TaLTP9.5a[f]	AY566607	AF302788	Jang et al. (2002); (2004)
TaLtp9.5b	pLTP2	TaLTP9.5b[g]	AJ852557	AF334185	Jang et al. (2002)
TaLtp9.6a	pTa360	TaLTP9.6a	AJ852544	AJ784897	Boutrot et al. (2005)
	pTa232	TaLTP9.7a		AJ784898	Boutrot et al. (2005)
TaLtp9.7b		TaLTP9.7b	AJ852545		
TaLtp9.7c		TaLTP9.7c	AJ852546		
TaLtp9.7d	pTa232-6	TaLTP9.7d	AJ852547	AJ852560	
	pTa232-5	TaLTP9.7e		AJ852559	
		TaLTP9.8a[h]		AAB32995	Neumann et al. (1994)
		TaLTP9.8b[i]		AAB32996	Neumann et al. (1994)

Sequences isolated in this work are highlighted in bold italics. n.a. not available

[a] Formerly LTP2G (Douliez et al. 2001)

[b] Formerly LTP2P (Douliez et al. 2001)

[c] Formerly LTP1500 (GenBank annotation report)

[d] Formerly *TaLtp9.5a* (Boutrot et al. 2005)

[e] Formerly TaLTP3 (Jang et al. 2005)

[f] Formerly TaLTP1 (Jang et al. 2002)

[g] Formerly TaLTP2 (Jang et al. 2002)

[h] Formerly WBP1A (Neumann et al. 1994)

[i] Formerly WBP1B (Neumann et al. 1994)

genes which are the two members of the *TaLtp9.2* gene subfamily and the two members of the *TaLtp9.5* gene subfamily. A unique intron whose size ranged from 91 to 444 bp was identified in all the other characterized type 1 *TaLtp* genes (Table 3). All the identified intron donor sites are located at a highly conserved position 7 bp upstream of the stop codon except the *TaLtp9.1a*

gene whose intron donor site is positioned 4 bp upstream of the stop codon. All introns have canonical splice signals, GU in 5′ and AG in 3′ (Hebsgaard et al. 1996) and are in phase 2 with respect to the ORF.

The *TaLtp9.3d* gene presents the particularity of harboring a *Stowaway* foldback element in its intron sequence (Fig. 1). This element belongs to the family

Table 3 Wheat type-1 *nsLtp* genes: sequences of donor and acceptor sites of the unique intron

nsLtp gene[a]	Donor site	Intron size (bp)	Acceptor site
TaLtp9.1a	GCAG/gtgat	90	tgcag/GGTG
TaLtp9.2c		No intron	
TaLtp9.2d		No intron	
TaLtp9.3b	ACAC/gtact	125	tgcag/CCTT
TaLtp9.3c	ACAA/gtaca	121	tgcag/CCTT
TaLtp9.3d	ATAA/gtaca	175	tgcag/CATT
TaLtp9.3e	ACAA/gtaca	121	tgcag/CCTT
TaLtp9.3f	ACAA/gtaca	122	tgcag/CCTT
TaLtp9.3g	ACAC/gtaca	129	tgtag/CCTT
TaLtp9.4a	ACAC/gtatg	440	tgcag/TGTC
TaLtp9.4b	ACAA/gtatg	444	tgcag/TGTC
TaLtp9.5a		No intron	
TaLtp9.5b		No intron	
TaLtp9.6a	ACAA/gtaag	110	tgcag/GATA
TaLtp9.7b	ACTC/gtacg	91	tgcag/GATC
TaLtp9.7c	ACTC/gtacg	116	tgcag/GATC
TaLtp9.7d	ACTC/gtacg	119	tgcag/GATC

[a] Accession numbers are given in Table 2

of the miniature inverted-repeat transposable elements (MITE) which are less than 400 bp long, flanked by a 2–3 bp target site (TA(A)), terminated by an inverted repeat CTCCCTCC motif and present no coding activity (Petersen and Seberg 2000). The 59 bp long element present in the *TaLtp9.3d* gene intron is shorter than previously described *Stowaway* elements (Petersen and Seberg 2000). The terminal inverted motifs are perfectly repeated within the first 6 bp and flanked by the typical TA target site at one end and its duplicated copy at the opposite extremity. The sequence alignment of *TaLtp9.3* gene introns at the level of the *Stowaway* element shows that only the *TaLtp9.3d* gene harbors this transposable element. However, the five other *TaLtp9.3* genes present footprints of the *Stowaway* element. The presence of the two nucleotides (AG) from the 3′ terminal inverted repeat of the *Stowaway* element and the duplicated copy of the TA recognition site support the hypothesis that a *Stowaway* element was excised from these intron sequences.

The cloned 5′ upstream region of wheat *TaLtp* genes ranged from 9 to 2,856 bp. Within *TaLtp* gene subfamilies, alignments of available promoter sequences revealed high homology in the 400 bp

upstream of the translation initiation codon (data not shown). The largest sequence deletions were observed within promoters of the *TaLtp7.1* subfamily with 32–69 bp deletion polymorphism at different positions. Conversely, with only 31 mutations, the *TaLtp9.2c* (1,269 bp) and *TaLtp9.2d* (1,257 bp) promoter regions displayed the highest conservation. Comparison of the other *TaLtp* promoter regions revealed a few single mutations or small deletions. Nevertheless, some of these modifications could be as crucial as large sequence deletions for the regulation of gene expression.

Phylogeny of TaLTPs

The alignment of the 35 wheat nsLTP sequences described in Table 2 is presented in Fig. 2. NsLTPs are synthesized as preproteins and present an N-terminal sequence (24–36 amino acids) consistent with a secretory signal peptide as defined by SignalP program analysis. Mature type 2 TaLTPs whose molecular mass ranged from 6,971 to 7,046 kDa contain 67 amino acids whereas type 1 TaLTPs contain 90–94 amino acids and have a molecular mass ranging from 8,672 to 9,609 kDa. Wheat nsLTPs displayed the features of plant nsLTPs including a pattern of eight-cysteine in a conserved position and typical amino acids between these cysteine residues.

A cladogram of the *T. aestivum* mature nsLTPs is presented in Fig. 3. The TdLTP7.1b sequence from *Triticum durum* was also taken into account since no identical mature protein was deduced from the *T. aestivum* genes characterized to date. Because *TaLtp7.1d* and *TaLtp7.1e* genes encoded the same preprotein, and *TaLtp9.7b*, *TaLtp9.7c*, and *TaLtp9.7d* genes the same mature protein, only the TaLTP7.1d and TaLTP9.7b sequences were taken into account in the phylogenetic analysis. The phylogenic tree is presented as an unrooted cladogram since all plant nsLTPs described so far belong to the type 1 or 2 nsLTPs (or with a less extent to the type 3) and could not be used as outgroup to root the paralogous wheat sequences. The robustness of internal branches was estimated by 1,000 bootstrap resamplings. As determined by sequence identity


```
TaLTp9.3b  370  TGAAAGATTATATTC--------TA----------------------------------------------------AGTACTCATTGGCCATTTA
TaLTp9.3c  366  TGAAAGATTATATTA--------TA----------------------------------------------------AGTACTCGTTAGCCATTTA
TaLTp9.3d  366  TGAAAGAATATGTTC--------TACTCCCTGCATAACGAAATGTATGAGCGTTTAGACCACTACGCTCCGTATAGAGGGAGTACTCATTAGCCATTTA
TaLTp9.3e  366  TGAAAGATTATATTC--------TA----------------------------------------------------AGTACTCGTTAGCCATTTA
TaLTp9.3f  360  TGAAATATTATATTT--------TA----------------------------------------------------AGTACTCATTAGCCATTTA
TaLTp9.3g  360  TGAAAGATTATATTTATATAATTCTA----------------------------------------------------AGTACTCATTGGCTATTAA
```

Fig. 1 Sequence alignment of six *TaLtp9.3* genes in the region of the *Stowaway* element identified in the *TaLtp9.3d* gene intron. The *Stowaway* terminal inverted motifs are underlined and the flanking TA target site and its duplicated copy are in *bold*. Highly conserved nucleotides are *gray boxed*. The nucleotide sequences are numbered with respect to the first base of the initiation codon. Accession numbers of sequences are given in Table 2

```
TdLTP7.1a    MAGMMKKQ--VVTALMLALVVLAAAPGG--ARAAC--QASQLAVCASAILSGAK--PSGECCG---NLR-------AQQGCFCQYAKDPTYGQYIRSPHARDTLTSCGLAVP------HC    67
TdLTP7.1b           AC--QASQLAVCASAILSGAK--PSGECCG---NLR-------AQQGCFCQYAKDPTYGQYIRSPHARDTLQSCGLAVP------HC    67
TaLTP7.1a    MAGMMKKQ--VVTALMLALVVLAAAPGG--ARAAC--QASQLAVCASAILSGAK--PSGECCG---NLR-------AQQGCFCQYAKDPTYGQYIRSPHARDTLTSCGLAVP------HC    67
TaLTP7.1b    MASMGKKQKQVAATLMLALVVLAAAPGG--ARAAC--QASQLAVCASAILSGAK--PSGECCG---NLR-------AQQGCFCQYAKDPNYGQYIRSPHARDTLHSCGLAVP------HC    67
TaLTP7.1c    MASMGKKQ--VVAALMLVLVVLAAAPGG--AHAAC--QASQLAVCASAILSGAK--PSGECCG---NLR-------AQQPCFCQYAKDPTYGQYIRSPHARDTLQSCGLAVP------HC    67
TaLTP7.1d    MAGMGKQQ--VVAALMLALVVLAAAPGG--AHAAC--RASQLAVCASAILSGAK--PSGECCG---NLR-------AQQGCFCQYAKDPTYGQYIRSPHARDTLTSCGLAVP------HC    67
TaLTP7.1e    MAGMGKQQ--VVAALMLALVVLAAAPGG--AHAAC--RASQLAVCASAILSGAK--PSGECCG---NLR-------AQQGCFCQYAKDPTYGQYIRSPHARDTLTSCGLAVP------HC    67

TaLTP7.2a    MP-MRKEV--LLVAMMLALVV--AAPGG--ARAAC--EVGQLTVCMPAITTGAK--PSGACCG---NLR-------AQQASFCQYAKDPSLARYITSPHARETLVSCGLAVP------HC    67

TdLTP9.1a       AQVML--MAVALVLMLAAVPRAA----VAIDCGHVDSLVRPCLSYVQGGPG--PSGQCCDGVKNLHNQARSQSDRQSACNCLKGIARGI-HNLNEDNARSIPPKCGVNLPYTISLNIDCSRV    90
TaLTP9.1a    MARAQVML--MAVALVLMLAAVPRAA----VAIDCGHVDSLVRPCLSYVQGGPG--PSGQCCDGVKNLHNQARSQSDRQSACNCLKGIARGI-HNLNEDNARSIPPKCGVNLPYTISLNIDCSRV    90
TaLTP9.1b    MARAQVML--MAVALVLMLAAVPRAA----VAIDCGHVDSLVRPCLSYVQGGPG--PSGQCCDGVKNLHNQARSQSDRQSACNCLRGIARGI-HNLNEDNARGTPLKCGVNLPYTISLNIDCSRV    90

TdLTP9.2a        AVVAAMLLAVTE----AAVSCGQVSSALSPCISYARGNGA-SPSAACCSGVRSLASSARSTADKQAACKCIKSAAAGL----NAGKAAGIPTKCGVSIPYAISSSVDCSKIR    90
TaLTP9.2b    MARSAVAQ--VVLVAVVVLLAVTE----AAVSCGQVSSALSPCISYARGNGA-SPSAACCSGVRSLASSARSTADKQAACKCIKSAAAGL----NAGKAAGIPTKCGVSIPYAISSSVDCSKIR    90
TaLTP9.2c    MARSAVAQ--VVLVAVVVLLAVTE----AAVSCGQVSSALSPCISYARGNGA-SPSAACCSGVRSLASSARSTADKQAACKCIKSAAAGL----NAGKAAGIPTKCGVSVPYAISSSVDCSKIR    90
TaLTP9.2d    MARSAVAQ--VVLVAVVVLLAVTE----AAVSCGQVSSALSPCISYARGNGA-SPSAACCSGVRSLASSARSTADKQAVCKCIKSAAAGL----NAGKAAGIPTKCGVSVPYAISSSVDCSKIH    90

TaLTP9.3a    MARVALLA--VFTVLAALAVAEMASG-----AVTCGDVTSAIAPCMSYATGQAS-SPSAGCCSGVRTLNGKASTSADRQAARCLKNLAGSF-NGISMGNAANIPGKCGVSVSFPINNSVNCNNLH    93
TaLTP9.3b    MARVALLA--VFAVLAALAVAEMASG-----AVTCGDVTSAIAPCMSYATGQAS-SPSAGCCSGVRTLNGKASTSADRQAARCLKNLAGSF-KGISMGNVANIPGECGVSVSFPINNVNCNNLH    93
TaLTP9.3c    MARVALLV--VFTVLATLAVAEMASG-----AVTCGDVTSAIAPCMSYATGQAS-SPSAGCCSGVRTLNGKASTSADRQAARCLKNLAGSF-NGISMGNAANIPGKCGVSVSFPINNNVNCNNLH    93
TaLTP9.3d    MARMALLA--VFAVLAALAVAEMASG-----AVTCGDVTSAIAPCMSYATGKAS-APSAGCCSGVRTLNGKASTSADRQAARCLKNLAGSF-KGISMGNAATIPGKCGVSVSFPINTNVNCNNIH    93
TaLTP9.3e    MARVALLA--VFSVLAALAVAEMASG-----AVTCGDVTSAIAPCMSYATGQAS-SPSAGCCSGVRTLNGKASTSADRQAARCLKNLAGSF-NGISMGNAANIPGKCGVSVSFPINNVNCNNLH    93
TaLTP9.3f    MARVALVA--VFAVLAALAVADMASG-----AVTCGDVTSAVAPCMSYASGKAS-APSGACCSGVRTLNAKASTPADRKAACNCLKNLAGS---GISMGNAANIPGKCGVSVSIPISTKTNCNNLH    91
TaLTP9.3g    MARVALLA--VFAVLALAVAEMASG-----AVTCGDVTSAVAPCMSYATGQTS-APSAGCCSGVRTLNAKASTSADRQAARCLKLAGS---GISMGNAANIPGKCGVSVSFPINTKVDCNTLH    91

TaLTP9.4a    MARLNSKA--VVAAVVLAAVVLMMAGREASAALSCGQVDSKLAPCVAYVTGRAS-SISKECCSGVQGLNGMARSSSDRKIAARCLKSLATSI-KSINMGKVSGVPGKCGVSVPPFISMSTNCDTVN    93
TaLTP9.4b    MARLNSKA--VVSAVVLAAVVLMMAGREAT-ALSCGQVDSKLAPCVSYVTGKAP-SISKECCSGVQGLNGLARSSPDRKIAARCLKSLATSI-KSINMDKVSGVPGKCGVSVPPFISMSTNCNNVN    93
TaLTP9.4c    MARLNSKA--VAAAVVLAAVVLMMAGREASAALSCGQVDSKLAPCVAYVTGRAS-SISKECCSGVQGLNGLARSSPDRKIAARCLKSLATSI-KSINMGKVSGVPGKCGVSVPPFISMSTNCNNVN    93

TaLTP9.5a    MARTAATK--LVLVALVAAMLLVASD----AAISCGQVNSALASCVSYAKGSGA-SPPGACCSGVRRLAGLARSTADKQAARCIKSAAGGL----NPGKAASIPSKCGVSIPYSISASVDCSKIH    90
TaLTP9.5b    MARTAATK--LVLVALVAAMILAASD----AAISCGQVSSALTPCVAYAKGSGT-SPSGACCSGVRKLAGLARSTADKQATCRCLKSVAGGL----NPNKAAGIPSKCGVSVPYTISASVDCSKIH    90

TaLTP9.6a    MAPSTRRVNAILVVG--LLLAAAMAAVAADAAEAEAAAEVSCGDAVSALIPCGSFLVGAVAGAPSESCCRGAQGLRRMAGTPGARRALCRCLEQSGPSF--GVLPDRARQLPALCKLGISIPVSPHTDCDKIQ    93

TaLTP9.7a    MAAPRGAA--LVLAMVLAAMLVAPPATVH--AISCSTVYSTLMPCPQYVQQGGS--PARGCCTGIQNLLAEANNSPDRRTICGCLKNVANGASGGPYITRAAALPSKCNVALPYKISPSVDCNSIH    92
TaLTP9.7b    MAAPRGAA--LVLAMVLAAMVVAPPATVH--AISCSTVYSTLMPCLQYVQQGGS--PARGCCTGIQNLLAEANNSPDRRTICGCLKNVANGASGGPYITRAAALPSKCNVALPYKISPSVDCNSIH    92
TaLTP9.7c    MAAPRGAA--VVLAMVLAAMVVAPPATVHA-AISCSTVYSTLMPCLQYVQQGGS--PARGCCTGIQNLLAEANNSPDRRTICGCLKNVANGASGGPYITRAAALPSKCNVALPYKISPSVDCNSIH    92
TaLTP9.7d    MAAPRGAA--LVLAMVLAAMLVAPPATVH--AISCSTVYSTLMPCLQYVQQGGS--PARGCCTGIQNLLAEANNSPDRRTICGCLKNVANGASGGPYITRAAALPSKCNVALPYKISPSVDCNSIH    92
TaLTP9.7e    MAAPRGAA--LVLAMVLAAMLVAPPATVH--AISCSTVYSTLMPCLQYVQQGGS--PARGCCTGIQNLLVEANNSPDRRTICGCLENVANGASGGPYITRAAALPSKCNVALPYKISPSVDCNSIH    92

TaLTP9.8a           AVANCGQVVSYLAPCISYAMGRVS-VPGGGCCSGVRGLNAAAATPADRKTTCTCLKQQASGM-GGIKPNLVAGIPGKCGVNIPYAISPRTDCSKVR    94
TaLTP9.8b           AVANCGQVVSYLAPCISYAMGRVS-APGGGCCSGVRGLNAAAATPADRKTTCTCLKQQASGI-GGIKPNLVAGIPGKCGVNIPYAISQGTDCSKVR    94
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Fig. 2 Alignment of wheat nsLTPs. Amino acid sequences were deduced from cDNAs, genes, or indexed in the EMBL protein database. Alignment was performed using the multiple sequence alignment program ClustalW (Thompson et al. 1994). The predicted signal peptides are in *italics*, the conserved cysteine residues are *black boxed*, and for each subfamily highly conserved amino acids are *gray boxed*. Gaps (*dashes*) were introduced to optimize alignment. Amino acid residues are numbered with respect to the N-terminal of mature nsLTPs. Accession numbers of sequences are given in Table 2

calculations, the phylogenetic representation confirmed that the wheat nsLTPs were distributed within two types, which correspond to the types 1 and 2 nsLTPs usually reported, and ten subfamilies, which were represented by 1–7 proteins. Comparison of all the amino acid sequences revealed at the most 23.4% identity between types 1 (TaLTP9.3e) and 2 (TaLTP7.2a) nsLTPs. As expected from the comparison of the deduced amino acid sequences, the seven TaLTP9.3 sequences (76.3–97.8% identity) appeared to be widely distributed within their subfamily whereas the sequences from other multigene subfamilies were more closely related. Only the TaLTP9.2 and TaLTP9.5 subfamilies appeared to be related in the phylogenetic tree. This subfamily dichotomy was caused by the criteria retained for the wheat nsLTP nomenclature but was also confirmed by noticeable features notably among the unexploited signal peptide sequences.

Transformation and analysis of rice plants

Six wheat *nsLtp* genes were chosen to compare the spatial and temporal activity of their promoter in transgenic rice using the *uidA* reporter gene. Their selection was based on their belonging to different subfamilies and displaying diverse patterns of gene transcript accumulation in developing seeds monitored by RT-PCR (Boutrot et al. 2005). Promoter sequences of *TaLtp9.1a* (1,390 bp), *TaLtp9.2d* (1,235 bp), *TaLtp9.3e* (824 bp), and *TaLtp9.4a* (843 bp) type 1 *nsLtp* genes, and *TaLtp7.1a* (745 bp) and *TaLtp7.2a* (1,437 bp) type 2 *nsLtp* genes were amplified by PCR and cloned into the pCAMBIA 1381Xa vector resulting in *nsLtp* promoter::*uidA* gene transcriptional fusions. These constructs were introduced into *O. sativa* 'Zhongzuo321' and *O. sativa* 'Nipponbare' via *Agrobacterium tumefaciens* cocultivation. For each construct, 4–20 independent hygromycin-resistant lines were regenerated. Using *O. sativa* 'Zhongzuo321', we obtained nine (*TaLtp7.1a::uidA*), ten (*TaLtp9.1a::uidA* and promoterless construct), 11 (*TaLtp9.4a::uidA*), 15 (*TaLtp7.2a::uidA* and *TaLtp9.3e::uidA*), and 20 (*TaLtp9.2d::uidA*) independent lines. Using *O. sativa* 'Nipponbare', we regenerated four (*TaLtp9.4a::uidA* and promoterless construct), 11 (*TaLtp9.3e::uidA*), 13 (*TaLtp9.1a::uidA* and *TaLtp9.2d::uidA*), 15 (*TaLtp7.2a::uidA*), and 20 (*TaLtp7.1a::uidA*) independent lines. While the biological basis of variations is not understood, we observed that these variations are
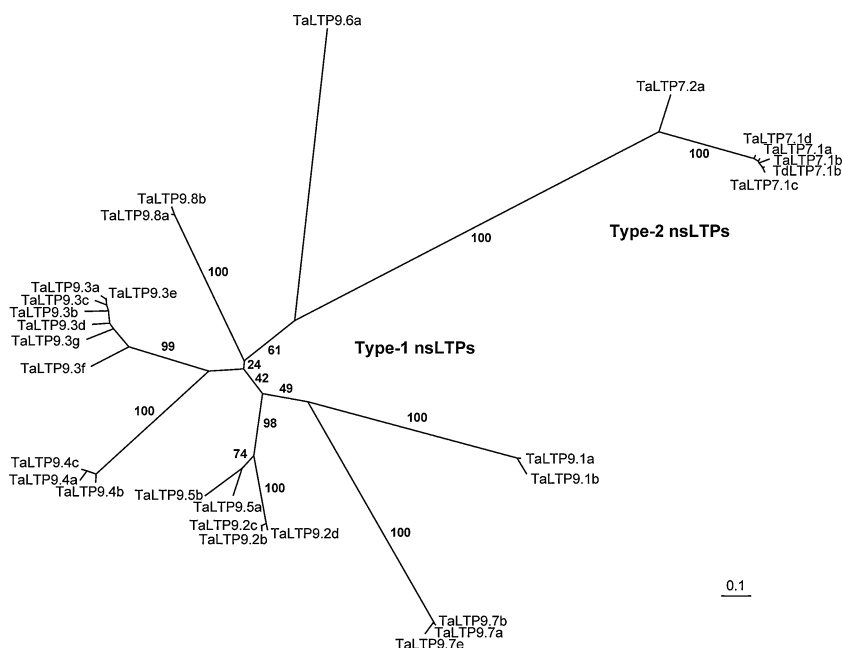
due to differences in transformation efficiency (18.6–80.0% depending on construct and rice cultivars) as well as differences in ability to develop secondary calli from primary calli (34.5–70.9% depending on rice cultivars). A total of 656 T_0 transgenic plants was analyzed to determine the transgene copy number. Southern blot analysis of *Hin*dIII-restricted genomic DNA hybridized with the *uidA* or *hptII* probe indicated that a mean of 1.98 T-DNA copies was inserted in the genome of *O. sativa* 'Zhongzuo321' and 36.58% of plants contained a single copy of the transgene. For the *O. sativa* 'Nipponbare' these values were 2.03 and 38.14%, respectively. For further analysis, only single copy transgenic lines were propagated and grown in the greenhouse until seed maturity.

Analysis of *TaLtp* gene promoter activities in transgenic rice plants

In order to gain better insight into the potential role of nsLTPs, we studied the promoter activity of six wheat *nsLtp* genes in transgenic rice plants by monitoring the expression of the *uidA* reporter gene. For each *TaLtp* promoter, we analyzed the localization of GUS activity in all organs throughout the life cycle of the transgenic rice plants. No GUS activity was detected in the transgenic plants harboring the promoterless reporter gene (Xa) or in the untransformed rice plants (wt) (data not shown). No major variation in the *uidA* expression pattern was observed between plants of the *O. sativa* 'Nipponbare' and *O. sativa* 'Zhongzuo321' cultivars transformed with the same construct.

All six TaLtp gene promoters are active in rice leaves

All the *TaLtp* promoters studied led to expression of the *uidA* reporter gene in the youngest (F7) leaves as illustrated for the *TaLtp9.1a* promoter (Fig. 4a). This was corroborated by analysis of GUS activity in the third to seventh leaf blades of 7-leaf seedlings (Fig. 4b). However, we noticed rather wide variability in GUS activity between replicates of the same experiment. Since plants segregate for the *TaLtp promoter::uidA* constructions, zygosity of the plants was estimated by segregation of hygromycin resistance in their progeny seedlings. Taking into account the ratio of the GUS activity to the transgene copy number rather than raw GUS activity data dramatically reduced the variability between replicates. This analysis highlighted a gene dosage effect, which was relevant for all these single T-DNA copy independent transformation events. As several studies have demonstrated additive transgene expression between homozygous and hemizygous progeny (James et al. 2002), the GUS activity was presented as the ratio of the GUS activity to the transgene copy number (Fig. 4b). While the transgenic plants harboring the *35S::uidA* construct (positive control) presented stable GUS activity in the series of leaf blades (16.2 ± 1.3 pmol [4-MU (μg protein)$^{-1}$ min^{-1}] per transgene copy, data not shown), in untransformed rice plants (wt) and control transgenic plants (Xa) the level of GUS activity was insignificant. The highest and lowest levels of GUS activity were directed by the *TaLtp9.1a* and *TaLtp9.2d* promoters, respectively. There was a statistically
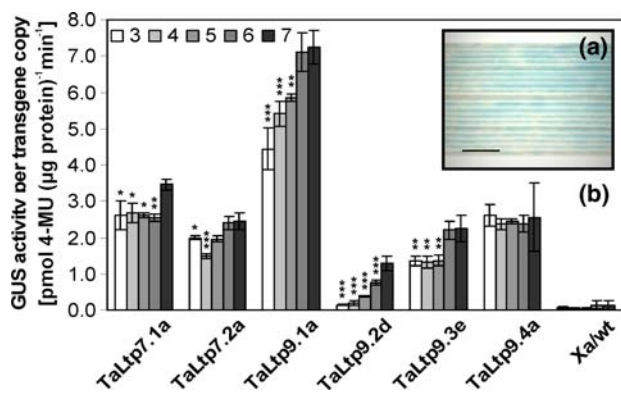
Fig. 4 β-Glucuronidase activity in leaf blades of T_2 transgenic rice carrying different *TaLtp::uidA* constructs. Transgenic rice plants (*O. sativa*. 'Nipponbare') were grown in greenhouse for 4 weeks until they reached the seventh leaf stage. **a** Histochemical analysis of GUS activity in the F7 leaf blade of *TaLtp9.1::uidA* plant. **b** GUS activity driven by six *TaLtp* promoters and negative control (Xa/wt) measured in the five subsequent younger leaves (F7–F3). GUS activity is expressed as [pmol 4-MU (μg protein)$^{-1}$ min^{-1}] per transgene copy. Two independent transgenic lines were assayed for each construct and each was performed in triplicate. The values are mean ± SD ($n = 6$). Values significantly different from the younger leaf (F7) in the same group according to ANOVA test are indicated by asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). *Xa* Promoterless *uidA* plants, *wt* Untransformed rice plants. *Bar*, 1 mm (**a**)

significant decrease in GUS activity as a function of leaf rank from the youngest to the oldest leaves. This held true for all the *TaLtp* promoters studied except the *TaLtp9.4a*, which directed stable GUS activity in blades irrespective of leaf rank ($P > 0.05$, multiple comparison of all means). The most dramatic decrease was found in plants harboring the *TaLtp9.2d::uidA* fusion where no GUS activity was observed in the oldest leaves. In adult transgenic rice plants, aging but still growing leaves presented GUS activity that became restricted to the vascular bundles and finally disappeared in the oldest leaves before any external indications of senescence (data not shown).

All six TaLtp gene promoters are active in rice roots

Six-day-old T_1 plantlets displayed GUS activity in the vascular system of the coleoptile (data not shown) and in the root system. All the *TaLtp* promoters directed *uidA* gene expression in the root system, in which GUS activity was detected in crown roots (data not shown), lateral roots (Fig. 5a) and the distal part of the seminal root (Fig. 5c) as illustrated for the *TaLtp7.1a::uidA* construct. At the branching of lateral roots, transversal sections showed GUS activity in the endodermis of the seminal root but not in the central stele (Fig. 5b). In seminal roots the GUS activity was restricted to the central stele cells and mainly associated with vascular

tissues (Fig. 5d). In contrast, three distinct profiles were observed in the lateral roots. The most frequent, observed in transgenic rice plants carrying the *TaLtp7.1a::uidA*, *TaLtp7.2a::uidA*, *TaLtp9.1a::uidA*, and *TaLtp9.4a::uidA* constructs, was characterized by strong GUS activity in the vascular tissues and weaker activity in the parenchyma tissues and in the root apical meristem as illustrated for the *TaLtp7.1a::uidA* construct (Fig. 5e). The second profile, driven by the *TaLtp9.2d* promoter, was similar to the latter but GUS staining was weaker and no staining was observed in the root apical meristem (Fig. 5f). Finally, the third profile was observed in *TaLtp9.3e::uidA* transgenic plants in which GUS staining was restricted to the vascular tissues and decreased from the proximal to the distal part of the lateral roots (Fig. 5g).

TaLtp gene promoters drive different GUS patterns in rice florets

While no GUS activity was observed in florets carrying the *TaLtp7.1a::uidA* construct (data not shown), two types of GUS activity profiles were observed for the five other *TaLtp::uidA* constructs (Fig. 6). The *TaLtp7.2a*, *TaLtp9.1a*, *TaLtp9.2d*, and *TaLtp9.3e* gene promoters drove similar patterns of *uidA* gene expression in flowers before anthesis. As illustrated for the *TaLtp7.2a::uidA* construct, GUS activity was restricted to the extremities of the stamen filaments (Fig. 6a) and to the vascular bundles of the palea and lemma (Fig. 6e, f). Closer examination of glumellas and stamen filaments showed that GUS staining was localized in cells in the immediate vicinity of vessel elements (Fig. 6g, h). The *TaLtp9.4a* promoter directed *uidA* gene expression in all glumella tissues and was the only *TaLtp* gene promoter to drive *uidA* gene expression in anthers (Fig. 6b). Longitudinal sections revealed that GUS activity was also present in styles and in anthers, where it was restricted to the epidermal cells (Fig. 6c, d).

TaLtp gene promoters drive complex patterns of GUS activity and GUS localization during rice grain development

The *uidA* reporter gene expression driven by the wheat *nsLtp* promoters was studied during grain development from 4 to 30 dpa (Fig. 7). Histochemical analysis of GUS activity was supplemented by immunolocalization of the GUS protein. This analysis was carried out with plant materials fixed immediately after sampling. Depending on the *nsLtp* promoters, two distinct *uidA* gene expression profiles were observed. The first was observed in the spikelets of transgenic rice plants
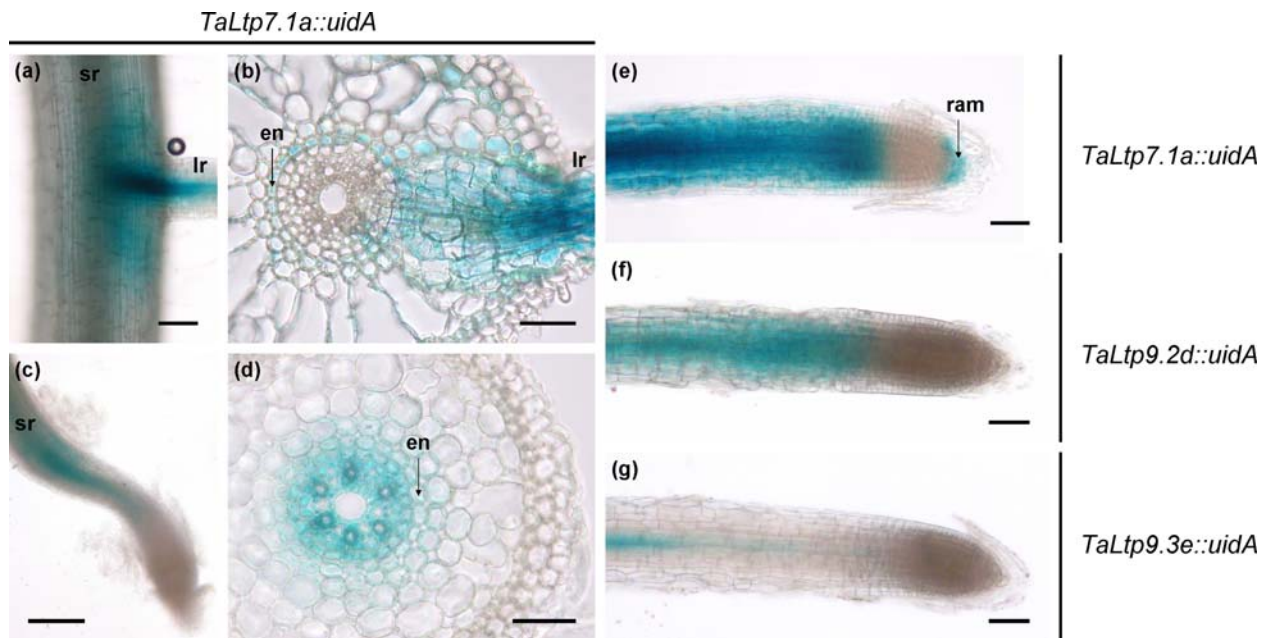
Fig. 5 Histochemical localization of GUS activity under the control of *TaLtp* promoters in the root system of T₁ transgenic rice plants. Six-day-old plantlets (*O. sativa* 'Zhongzuo321') were obtained by germinating T₁ transgenic seeds on moist paper. **a–d** Profile of GUS activity in the seminal root of *TaLtp7.1a::uidA* transgenic rice plantlets. **a** Binocular microscope observation and **b** corresponding transversal vibratome section of the seminal root at the branching of a lateral root. **c** Binocular microscope observation and **d** corresponding transversal vibratome section of the distal region of the seminal root. **e–g** Binocular microscope observation in the distal region of lateral roots of **e** *TaLtp7.1a::uidA*, **f** *TaLtp9.2d::uidA* and **g** *TaLtp9.3e::uidA* plantlets. *en* Endodermis, *lr* Lateral root, *ram* Root apical meristem, *sr* Seminal root. *Bars*, 200 μm (**a**, **c**), 50 μm (**b**, **d**, **e–g**)

carrying the *TaLtp7.1a::uidA*, *TaLtp9.2d::uidA*, and *TaLtp9.4a::uidA* constructs as illustrated for the *TaLtp7.1a::uidA* plants (Fig. 7a–l). At 4 dpa, GUS staining was detected in the entire immature grain (Fig. 7a), with remarkable intensity in the developing endosperm as shown in the longitudinal section in Fig. 7e. This was confirmed by immunolocalization of GUS in *TaLtp9.4a::uidA* seeds (data not shown). In contrast, in *TaLtp7.1a::uidA* and *TaLtp9.2d::uidA* seeds the GUS protein was only detected in the epicarp layer and to a lesser extent in the cell layers surrounding the testa (aleurone layer, nucellar epidermis, and integument) (Fig. 7i). No immunolocalization data was collected on embryos as these were not included in the samples observed at this stage. At 10 dpa, GUS activity was no longer observed in the central endosperm but was still present in all its surrounding layers and in the embryo (Fig. 7b–f). The immunolocalization signal was restricted to the epicarp, the cell layers surrounding the testa, and the epidermal cells of the embryo coleorhiza (Fig. 7j). GUS proteins were also faintly detected in the embryo vascular system (data not shown). From 20 to 30 dpa, *uidA* gene expression was restricted to the embryo (Fig. 7c, d, g, h). At 20 dpa, GUS activity and immunolocalization indicated that the *TaLtp7.1a*, *TaLtp9.2d*, and *TaLtp9.4a* promoters drove the

reporter gene expression in the embryo epidermal cells (Fig. 7g) and in the embryo vascular system (Fig. 7k). At 30 dpa, GUS activity and immunolocalization signal were almost indiscernible and only faint staining was observed in the epidermal cells located in the basal part of embryo (Fig. 7h, l).

The second GUS activity profile was observed in the grains carrying the *TaLtp7.2a::uidA*, *TaLtp9.1a::uidA*, and *TaLtp9.3e::uidA* constructs. *TaLtp* promoter activity was restricted to the mid-maturation to late-maturation stages of grain development as illustrated for the *TaLtp9.1a::uidA* plants (Fig. 7m–t). While no GUS activity was detected at 4 dpa (Fig. 7m, q), intense GUS activity restricted to the scutellum vascular bundle and the adjacent endosperm cells appeared at 10 dpa (Fig. 7n, r). This pattern was conserved throughout grain development but GUS activity decreased and was very weak in 20 and 30-dpa-old rice grains (Fig. 7o, p, s, t).

Discussion

Complexity of the wheat *nsLtp* gene family

The identification of *nsLtp* genes and the analysis of their expression patterns during plant development are
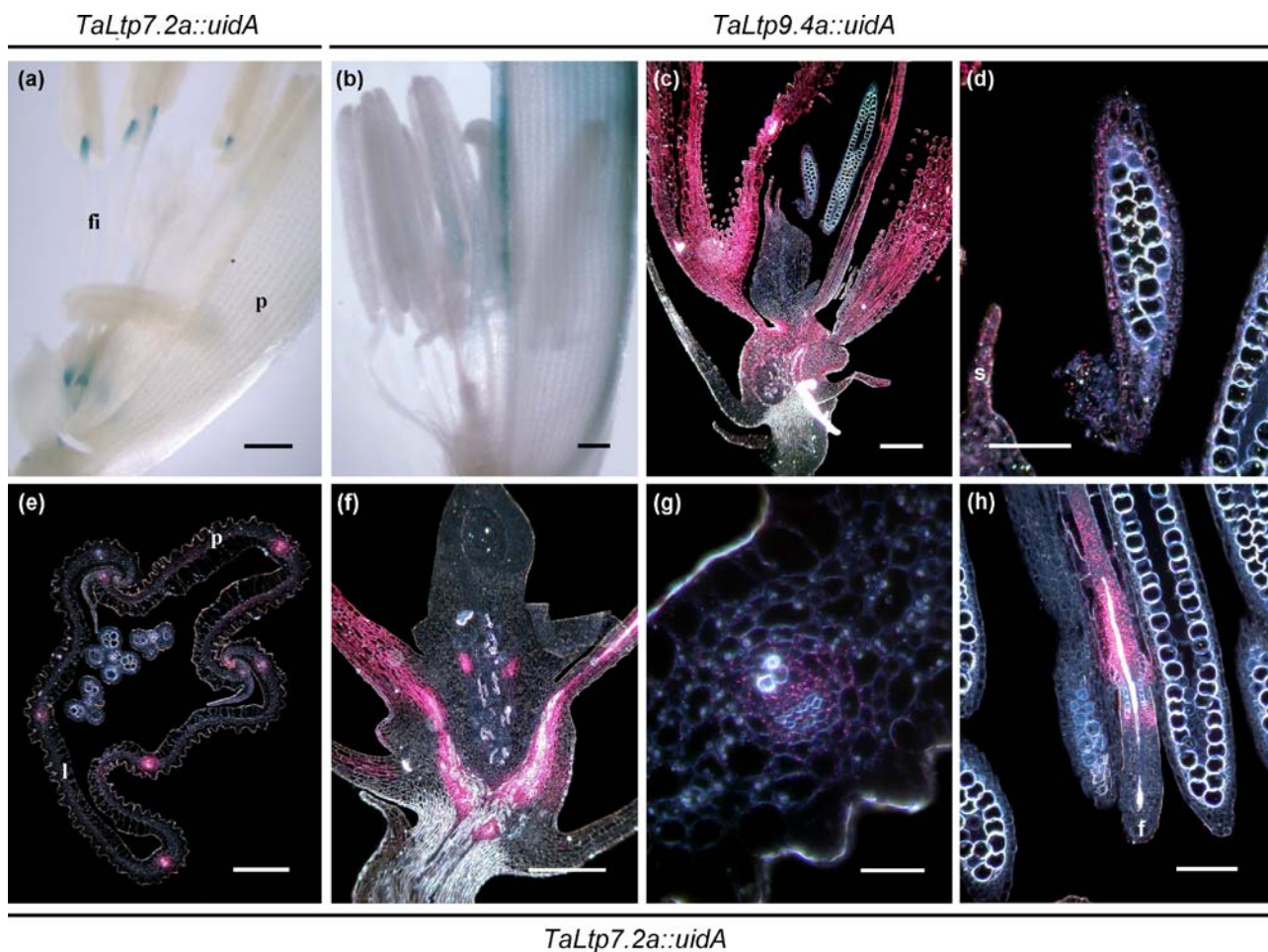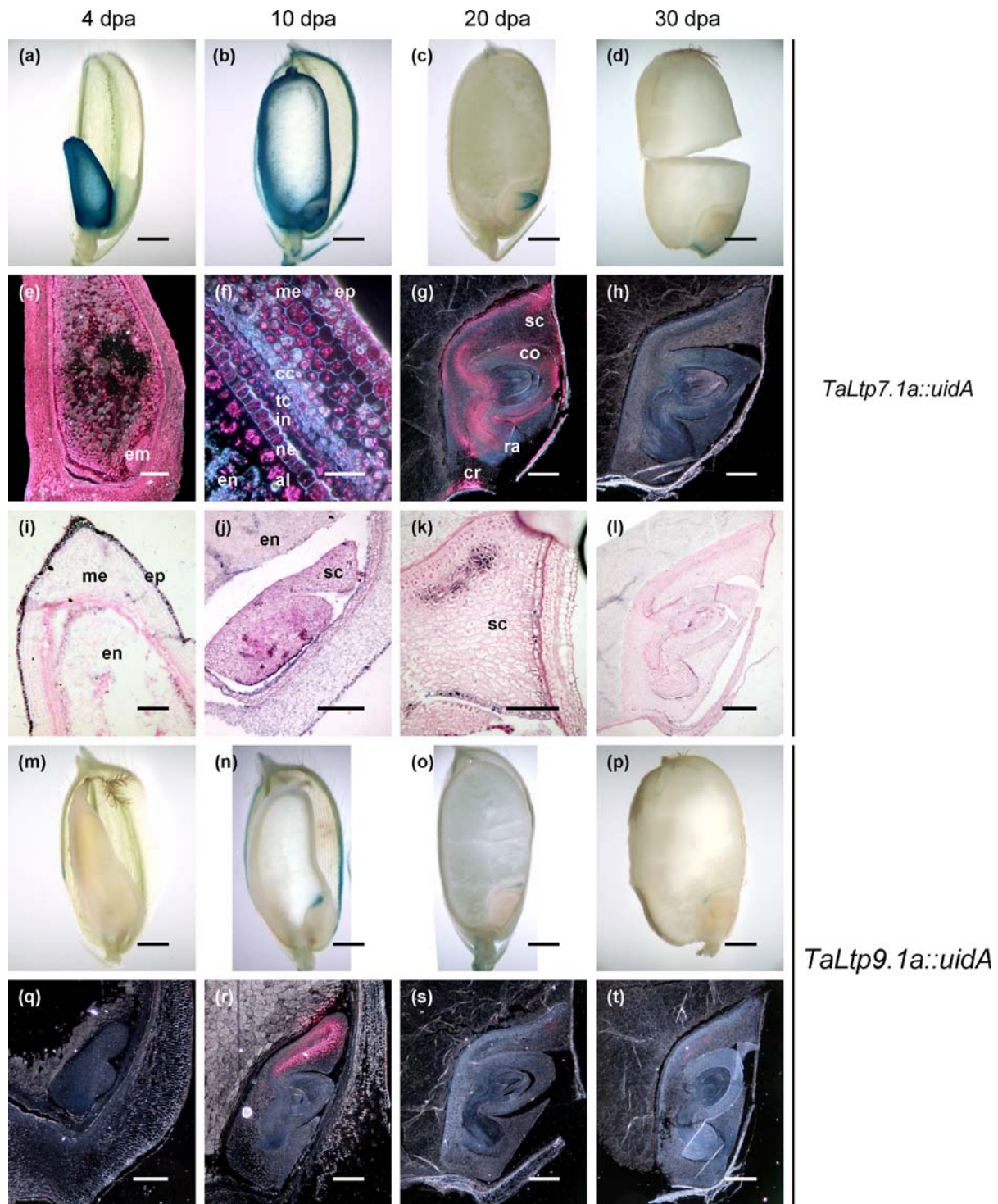
Fig. 6 Histochemical localization of GUS activity in florets of T$_0$ transgenic rice plants harboring *TaLtp::uidA* fusions. Florets (*O. sativa* 'Zhongzuo321') were collected prior to anthesis in emerged panicles. **a, e–h** GUS activity in plants carrying the *uidA* reporter gene under the control of the *TaLtp7.2a* promoter. **a** Binocular microscope observation of a floret with the lemma removed. **e** Transverse section of floret and **g** detailed observation of a vessel element of a lemma. Under dark-field illumination GUS crystals appear pink and xylem vessels have autofluorescent walls. **f** Longitudinal section of floret. **h** Longitudinal section of anthers. **b–d** *TaLtp9.4a::uidA* florets. **b** Binocular microscope observation of open floret. **c** Longitudinal section of floret and **d** detailed observation of anthers. *fi* Filament, *l* Lemma; *p* Palea, *s* Style. *Bars*, 1 mm (**a, b**), 250 μm (**c, e, f**), 100 μm (**d, h**), 25 μm (**g**)

important in understanding nsLTP functions. In the present study, we isolated different members of the wheat *nsLtp* gene family, and showed that six of them have overlapping but distinct expression patterns in transgenic rice plants. Because we are interested in wheat seed development, we first focused our work on the identification of *nsLtp* genes expressed in seed. Nineteen of 22 *TaLtp* genes cloned in this work were isolated by chromosome walking demonstrating that this procedure was particularly productive to clone genes from multigene families and complex genomes as wheat. However, a prerequisite for this technique is to dispose of sequence data from cDNAs or genes to design primers for the LM-PCR. Another important requirement with multigene families composed of closely related members is to avoid the creation of chimera genes by carefully checking the contiguity of fragments amplified from the successive walks.

The 23 *TaLtp* genes reported here are 420–3,448 bp long and each contains a complete ORF encoding a putative nsLTP. Our phylogenetic analysis indicates that the deduced mature proteins are distributed within distinct clades. Based on this analysis, the proposed wheat *nsLtp* gene nomenclature (Boutrot et al. 2005) was refined and led to the distribution of 35 identified wheat nsLTPs in two groups representing the types 1 and 2 nsLTPs usually reported, and ten subfamilies characterized by 1–7 members. The nomenclature criteria settled on the basis of amino acid homology allows to discriminate the three nsLTPs types, which display distinct eight-cysteine motifs (data not shown). TaLTP7.1, TaLTP9.3, and TaLTP9.7 subfamilies are

Fig. 7 Histochemical localization of GUS activity and GUS immunolocalization in T$_1$ grains of transgenic rice carrying different *TaLtp::uidA* constructs. **a–h** GUS activity in grains of *TaLtp7.1a::uidA* plants. **a–d** Longitudinal half-sections of grains at 4 dpa (**a**), 10 dpa (**b**), 20 dpa (**c**), and 30 dpa (**d**). **e–h** Histological sections of grains under dark-field illumination at 4 dpa (**e**), 10 dpa (**f**), 20 dpa (**g**), and 30 dpa (**h**). **i–l** GUS immunolocalization in longitudinal sections of *TaLtp7.1a::uidA* grains at 4 dpa (**i**), 10 dpa (**j**), 20 dpa (**k**), and 30 dpa (**l**). **m–t** GUS activity in grains of *TaLtp9.1a::uidA*. (**m–p**) Longitudinal half-sections of rice grains at 4 dpa (**m**), 10 dpa (**n**), 20 dpa (**o**), and 30 dpa (**p**). **q–t** Histological sections of rice grains visualized under dark-field illumination at 4 dpa (**q**), 10 dpa (**r**), 20 dpa (**s**), and 30 dpa (**t**). *al* Aleurone, *cc* Cross cells, *co* Coleoptile, *cr* Coleorhiza, *ep* Epicarp, *em* Embryo, *en* Endosperm, *in* Integument, *me* Mesocarp, *ne* Nucellar epidermis, *ra* Radicle, *sc* Scutellum, *tc* Tube cells. *Bars*, 1 mm (**a–d**, **m–p**), 300 μm (**s**, **t**), 250 μm (**e**, **g**, **h**, **l**), 200 μm (**j**, **q**, **r**), 100 μm (**i**, **k**), 40 μm (**f**)

represented by more than three homoeologous copies, which suggests that several genes are paralogs resulting from recent gene duplication events. Evolution of wheat *nsLtp* genes was also revealed by the study of their structure. Type 2 *nsLtp* genes are intronless whereas the majority of type 1 *nsLtp* genes examined to date are interrupted by a single intron (Kader 1996). Clearly, within wheat type 1 *nsLtp* genes only those belonging to the two closely related *TaLtp9.2* and *TaLtp9.5* subfamilies failed to show an intron that could have been independently lost in the ancestor gene. Intron diversity was also highlighted by the *TaLtp9.3d* gene that carries a *Stowaway* miniature inverted-repeat transposable element. Sequence alignments indicated that the five other members of the *TaLtp9.3* gene subfamily harbor a footprint that could have resulted from the excision of the *Stowaway* element. As variable footprints could be generated following excision of the *Stowaway* element (Petersen and Seberg 2000), no consensus sequence is indicative that a *Stowaway* foldback element was effectively excised from the five other members of the *TaLtp9.3* gene subfamily. Absence of footprint could be evidence of the insertion/excision mechanism but to date no member of the *TaLtp9.3* gene subfamily has been shown to harbor this original sequence. With more than 22,000 *Stowaway* MITEs identified in the rice genome (Feschotte et al. 2003) and a population size which remains to be evaluated in the wheat genome, these repetitive elements present an important disruptive mutation capability. However, the *Stowaway* element inserted in the *TaLtp9.3d* intron did not prevent gene transcription since a corresponding cDNA (pTaD2-2) was identified. Because most wheat *nsLtp* genes were characterized on the basis of sequence information deduced from cDNA clones isolated from developing seed cDNA libraries, it is obvious that we did not identify the full set of wheat *nsLtp* genes. In higher plants, nsLTPs are encoded by multigene families and 53 *nsLtp* genes were identified in the rice diploid genome (F. Boutrot et al. unpublished data). Taking into account the synteny between the rice and wheat genomes and the fact that several wheat *nsLtp* gene subfamilies (*TaLtp7.1*, *TaLtp9.3*, and *TaLtp9.7*) are represented by more than three homoeologous copies, we estimate that the hexaploid wheat *T. aestivum* contains a minimum of 100–150 *nsLtp* genes. The LM-PCR procedure that we adapted for walking in the wheat genome proved to be a powerful tool to identify *nsLtp* genes. An EST data mining strategy has been initiated in order to identify a more complete set of wheat *nsLtp* genes and appreciate the complexity of this family.

Expression of the wheat *nsLtp* genes

The complexity of plant *nsLtp* gene families and their large spatio-temporal expression patterns raised the question whether the expression of each member is distinctly regulated, and if the corresponding proteins support distinct physiological functions. In wheat, in a preliminary study we showed that *nsLtp* genes displayed a complex pattern of expression in developing seeds (Boutrot et al. 2005). However, the identification of new wheat *nsLtp* genes that share high-sequence similarities means that RT-PCR analysis is not really suitable for the evaluation of gene expression. We therefore used a reporter gene strategy and the rice lifecycle to monitor GUS activity driven by the promoter of six *TaLtp* genes belonging to different subfamilies.

Plant nsLTPs were first isolated from spinach leaves (Kader et al. 1984) and then purified from leaves of different species as broccoli (Pyee et al. 1994). There are also many reports of *nsLtp* gene expression in leaves of monocots and dicots (Molina and García-Olmedo 1993; Clark and Bohnert 1999). All the six wheat *nsLtp* gene promoters we studied were active in leaves. However, statistically significant differences in GUS activity were observed in leaves of transgenic lines where the *uidA* gene is under the control of the *TaLtp7.1a*, *TaLtp7.2a*, *TaLtp9.1a*, *TaLtp9.3e*, and *TaLtp9.2d* gene promoters. Our results indicate that the highest GUS activity is observed in the youngest leaf and declines as the leaf ages. Likewise, it was previously reported that the transcript levels of the tobacco *Ltp1* gene (Fleming et al. 1992) and broccoli *wax9* gene (Pyee et al. 1994) were higher in the youngest leaves. This pattern was correlated with a decrease in nsLTP found in the wax surface (Pyee et al. 1994) and is consistent with an involvement of nsLTP in the secretion of extracellular lipophillic material, including cutin monomers (Sterk et al. 1991). The variability of promoter activities between leaves was also reported for the *Arabidopsis AtLtp1* (van Leeuwen et al. 2001) and rice *Ltp1* (Guiderdoni et al. 2002) genes. Finally, as described by Guiderdoni et al. (2002), we observed that the decline in GUS staining was progressively limited to the vascular bundles and, then disappeared in the oldest leaves. In immature leaf sheaths of 4-week-old wheat seedlings, *TaLtp3* (*TaLtp9.4c* in our nomenclature) transcripts were only detected in phloem vessels (Jang et al. 2005). This gene is likely a homoeologous copy of the *TaLtp9.4a* gene considered in this study. Nevertheless, the two genes present differential transcriptional regulation, since the GUS activity driven by the *TaLtp9.4a* gene promoter was

not restricted to immature leaf sheaths. Many studies demonstrated that the expression of *nsLtp* genes in leaves was mainly associated with epidermal cells and suggested that nsLTPs were involved in the formation of the cuticle (Fleming et al. 1992; Sohal et al. 1999). Nevertheless, we report wheat *nsLtp* gene promoter activity mainly associated with leaf vascular tissues, suggesting that the physiological function of these wheat nsLTPs in leaves is not be related to cuticle formation, and that their function remains to be elucidated.

All the six wheat *nsLtp* gene promoters we studied were active in roots while expression of *nsLtp* genes in root tissues was more rarely reported than in leaves. For example the *Arabidopsis Ltp1* gene (Thoma et al. 1994), the tobacco *Ltp1* gene (Canevascini et al. 1996), the rape *BnLTP* gene (Sohal et al. 1999), and the rice *Ltp1* gene (Guiderdoni et al. 2002) present a broad pattern of expression that includes roots. Whereas, the tobacco *Ltp1* gene promoter drove *uidA* expression in root hair epidermal cells, the three other gene promoters resulted in GUS staining localized at the emission sites of lateral roots similar to that observed in this study. Since T_1 seeds were germinated on a moist paper, the GUS activity in rice seedlings was not related to wound induction. The presence of nsLTPs was also identified in xylem sap (Buhtz et al. 2004), however the GUS activity reported in this study would not support efficient nsLTP translocation in the xylem sap since reporter gene expression under the control of wheat *nsLtp* promoters was not associated with the seminal root vascular system. On the contrary, the localization of the GUS activity in regions of developing vascular tissues supports the hypothesis of wheat nsLTP involvement in the process of vascular differentiation as reported for a Zinnia nsLTP (Ye and Varner 1994). During this physiological process, nsLTPs might thus be discharged into the xylem sap and travel upward with the flow of sap.

In many plant species, the presence of nsLTPs and *nsLtp* gene expression were reported in stem tissues, mainly associated with xylem (Eklund and Edqvist 2003) and phloem vascular tissues (Pyee et al. 1994; Horvath et al. 2002) or localized either in epidermal cells (Sossountzov et al. 1991) or subepidermal cells (Clark and Bohnert 1999). In contrast no GUS activity was detected in stems of seedlings or mature transgenic rice plants carrying the *TaLtp::uidA* constructs. All these findings suggest that if wheat nsLTPs do have a biological function in vascular tissues of leaves and roots as hypothesized above, this function cannot be generalized to the vascular tissues of all vegetative organs.

In rice flowers, three different expression profiles were observed depending on the wheat *nsLtp* promoter concerned. Under the control of the *TaLtp7.1a* promoter no *uidA* gene expression was observed in immature and mature flowers. The GUS activity driven by the *TaLtp7.2a*, *TaLtp9.1a*, *TaLtp9.2d*, and *TaLtp9.3e* gene promoters was associated with vascular tissues in glumellas and the extremities of anther filaments. As we observed in seedling roots, *nsLtp* gene expression was not present in all vascular elements since anther filaments and some receptacle autofluorescent xylem vessels were not GUS stained. This supports our hypothesis that in flowers, these wheat nsLTPs may have a physiological function related to particular vascular elements. Unlike in roots, these tissues are not subject to vascular differentiation, so TaLTP7.2a, TaLTP9.1a, TaLTP9.2d, and TaLTP9.3e could be involved either in different physiological processes in roots and flowers or in only one process which remains to be determined. Since GUS staining also appeared in phloem sieve tubes of glumellas, these four nsLTPs could have a function related to these vascular elements. The GUS activity in phloem sieve tubes and companion cells is consistent with reports of the immunodetection of broccoli WAX9 proteins in cell walls of vascular parenchyma cells and sieve elements (Pyee et al. 1994), the highly specific expression of the potato *StnsLTP.2* gene promoter in phloem vessels (Horvath et al. 2002) and the RT-PCR detection of *Arabidopsis dir1* gene transcripts in phloem companion cells (Ivashikina et al. 2003). However, the predicted apoplastic subcellular localization of plant nsLTPs is inconsistent with their presence in the phloem sieve; thus, like the DIR1 protein, nsLTPs may release a systemic signal responsible for SAR in the vascular system (Maldonado et al. 2002).

Finally, the *TaLtp9.4a* gene promoter drove a unique GUS activity profile in flowers and is the first *nsLtp* gene whose broad spatial expression includes the anther epidermal cells. While this profile does not appear to be consistent with a role played by TaLTP9.4a in vascular tissues, GUS activity was not observed in the remaining tapetal cell layer indicating that the protein does not promote pollen development. With GUS staining also present in all glumella cells, the TaLTP9.4a can be assumed to be present in all the flower tissues that protect the pollen. In this way, like several defensins found in flowers displaying antifungal activity (Lay et al. 2003), TaLTP9.4a may support a physiological function by protecting reproductive tissues against potential attack by pathogens.

The six *nsLtp* gene promoters studied were active in transgenic rice grains and drove two distinct expression

profiles. The first profile was observed under the control of the *TaLtp7.1a*, *TaLtp9.2d*, and *TaLtp9.4a* gene promoters and was characterized by GUS activity in all immature grain tissues. Several studies reported induction of *nsLtp* gene expression following wounding (Guiderdoni et al. 2002; Cameron et al. 2006a). As the transgenic rice grains were half-sectioned prior to GUS histochemical analysis, we performed concomitant immunodetection of GUS proteins which was carried out with plant materials fixed immediately after sampling to determine if the *uidA* gene expression was or was not wound-induced following longitudinal sectioning of the grain. These combined analyses revealed that at 4 dpa the *TaLtp9.4a::uidA* construct was expressed in the rice grain. In contrast *TaLtp7.1a* and *TaLtp9.2d* genes were restricted to the grain epicarp and the cell layers surrounding the testa while expression was wound-inducible in all other grain tissues. At 10 dpa, the three promoters displayed a similar expression pattern to the GUS protein detected in embryo peripheral cells and vascular tissues, and *uidA* expression was found to be wound-inducible in grain tissues including embryo, but no longer in endosperm. In subsequent developmental stages, GUS activity and GUS immunolocalization were restricted to the embryo and displayed similar patterns.

Several *nsLtp* genes were preferentially expressed in the outer epidermal layer of fleshy fruits and the corresponding nsLTPs were thought to be involved in fruit cuticle synthesis (Botton et al. 2002; Liu et al. 2006). Our study reports for the first time *nsLtp* gene expression in the epicarp of Poaceae caryopsis, and supports the implication of the corresponding TaLTP7.1a, TaLTP9.2d, and TaLTP9.4a proteins in cuticle synthesis. At 2 and 10 dpa, GUS proteins were also detected in the nucellar epidermis and inner integuments. During grain maturation these cells layers will be compressed to form the testa (seed coat). Cutin layers are found in both sides of the testa, which mechanically protect the developing endosperm from water penetration and from microbial attack (for review see Moise et al. 2005). The presence of wheat nsLTPs coincides yet again with the formation of a cuticular layer and strongly supports the hypothesis of nsLTP involvement in cuticle formation. Nevertheless, since induction of *TaLtp* gene expression following wounding was observed in inner grain tissues, it is tempting to speculate that the three wheat nsLTPs could also be involved in plant defense mechanisms. This presumed physiological function would be limited to immature grains since GUS activity subsequently disappeared from pericarp cell layers and *uidA* gene expression was no longer wound-inducible in the grain after 10 dpa. Many

nsLTPs display intrinsic antimicrobial activity and their involvement in plant defense mechanisms has been widely illustrated (Cammue et al. 1995). In the wheat grain epicarp, TaLTP7.1a, TaLTP9.2d, and TaLTP9.4a are presumed to be constitutively accumulated to become part of antimicrobial and entomotoxic proteins. Such proteins were identified in the epicarp cell layer (Li et al. 2005) and in the testa of different plants (for review see Moise et al. 2005). In the other grain tissues, expression of the corresponding *TaLtp7.1a*, *TaLtp9.2d*, and *TaLtp9.4a* genes is thought to be induced following pathogen development to confer better resistance to biotic stresses.

During grain maturation, GUS activity became restricted to the embryo epidermal cells and embryo vascular tissues, and, in the end, almost no GUS activity was detected in mature transgenic rice grains. Like the maize *LTP2* gene (Sossountzov et al. 1991) and the carrot *EP2* gene (Sterk et al. 1991), transcripts from several *nsLtp* genes displayed a similar developmental time course in epidermal cells of developing embryos. These genes are expressed at the early proembryostage and confined to the outer cell layer of the proembryo (Sterk et al. 1991; Bommert and Werr 2001). As LTP2 and EP2 were thought to be involved in the secretion or in the deposition of extracellular lipophilic molecules on the protoderm layer, TaLTP7.1a, TaLTP9.2d, and TaLTP9.4a could be involved in transport of lipids to the outer surface. Since the *TaLtp7.1a*, *TaLtp9.2d*, and *TaLtp9.4a* gene promoters drove GUS activity in all rice grain tissues covered by a cuticular layer, our results strongly support the hypothesis of nsLTP involvement in cuticle synthesis. This profile was also illustrated by GUS activity and immunolocalization in embryo vascular tissues. In the scutellum vascular system, the developmental regulation of the *uidA* reporter gene expression was similar whatever the *TaLtp* gene promoter. Since the embryo provascular tissue develops from 5 dpa and the vascular bundle system is completed around 10 dpa (Hoshikawa 1993), the appearance of GUS activity between 4 and 10 dpa coincides with the formation of vessel elements. In this way, our results suggest that in grain these six wheat nsLTPs could support a common physiological function related to vessel formation. TaLTP7.2a, TaLTP9.1a, and TaLTP9.3e could be specialized in this function, whereas TaLTP7.1a, TaLTP9.2d, and TaLTP9.4a could be also involved in cuticle formation.

In conclusion, the six wheat *nsLtp* gene promoters studied directed GUS activity mainly in vascular tissues of leaf, root, flower, and embryo, as well as in grain tissues protected by a cuticle layer. Our study clearly shows that there is no specific expression

pattern of type 1 *nsLtp* genes versus type 2 *nsLtp* genes. Our observations also revealed that the activity of these promoters presented both overlapping and distinct patterns of transcriptional regulation. The functional significance of the multiple patterns of expression was not elucidated; however, our results suggest that the six wheat nsLTPs could play a role in vascular tissues. Several GUS profiles suggested that TaLTPs could have multiple physiological functions. In this way, TaLTP7.1a, TaLTP9.2d, and TaLTP9.4a could also be involved in cuticle synthesis in grain tissues, while TaLTP9.4a could play a role in anthers. Strong correlative evidence supports the involvement of wheat nsLTPs in plant defense mechanisms. Further experiments are pending to determine the activity of the six wheat *nsLtp* gene promoters after wounding and microbial infection.

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