Characterization and Antifungal Properties of Wheat Nonspecific Lipid Transfer Proteins

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This study simultaneously considered the phylogeny, fatty acid binding ability, and fungal toxicity of a large number of monocot nonspecific lipid transfer proteins (ns-LTP). Nine novel full-length wheat ns-LTP1 clones, all possessing coding sequences of 348 bp, isolated from abiotic- and biotic-stressed cDNA libraries from aerial tissues, exhibited highly conserved coding regions with 78 to 99 and 71 to 100% identity at the nucleotide and amino acid levels, respectively. Phylogenetic analyses revealed two major ns-LTP families in wheat. Eight wheat ns-LTP genes from different clades were cloned into the expression vector pPICZ α and transformed into *Pichia pastoris*. Sodium dodecyl sulfate polyacrylamide gel electrophoresis, Western blotting, and in vitro lipid binding activity assay confirmed that the eight ns-LTP were all successfully expressed and capable of in vitro binding fatty acid molecules. A comparative in vitro study on the toxicity of eight wheat ns-LTP to mycelium growth or spore germination of eight wheat pathogens and three nonwheat pathogens revealed differential toxicities among different ns-LTP. Values indicating 50% inhibition of fungal growth or spore germination of three selected ns-LTP against six fungi ranged from 1 to 7 μ M. In vitro lipid-binding activity of ns-LTP was not correlated with their antifungal activity. Using the fluorescent probe SYTOX Green as an indicator of fungal membrane integrity, the in vitro toxicity of wheat ns-LTP was associated with alteration in permeability of fungal membranes.

Additional keywords: antimicrobial, fungal pathogen, multigene family, plant defense.

Plant nonspecific lipid transfer proteins (ns-LTP) are abundant and ubiquitous, small, soluble, basic cysteine-rich proteins exhibiting four α -helices which are stabilized by four intramolecular disulphide bonds (Elmorjani et al. 2004). To date, ns-LTP or their cDNAs have been reported in more than 45 species of monocots and dicots, including agriculturally important crops such as rice (Liu et al. 2002; Vignols et al. 1994), wheat (Jang et al. 2004), barley (Monnet et al. 2001), pea (Reddy et al. 1999), cotton (Hsu et al. 2005), and tobacco (Fleming et al. 1992). There are two groups of ns-LTP, ns-

LTP1 and ns-LTP2, with molecular masses of 9 and 7 kDa (Kader 1996).

The biological roles of plant ns-LTP still remain largely speculative, although they have been implicated in plant development and responses to numerous abiotic and biotic stresses. Owing to their in vitro activity of transferring lipids between membranes, ns-LTP originally were postulated to facilitate intracellular lipid transfer in vivo (Kader 1975). Subsequently, it was shown that they all possess an N-terminal signal peptide that directs the ns-LTP to the endoplasmic reticulum for extracellular export via the secretory pathway (Thoma et al. 1993). Therefore, other functions have been suggested, including involvement in biosynthesis or deposition of hydrophobic monomers which form the waxy and polymeric cutin layers of most aerial organs (Hollenbach et al. 1997; Pyee et al. 1994; Sterk et al. 1991), or participation in embryogenesis and transportation of fatty acids and acyl coenzyme A as carrier proteins (Jung et al. 2003). ns-LTP also have been implicated in plant responses to salinity in tomato and wheat (Jang et al. 2004; Torres-Schumann et al. 1992), drought in tomato and wheat (Jang et al. 2004; Trevino and O'Connell, 1998), and low temperatures in barley (Hughes et al. 1992). Cabbage cryoprotectin, an ns-LTP unable to bind any lipids, has been implicated in the stabilization of plant membranes during cold stress (Sror et al. 2003). Several ns-LTP also have been implicated in plant defense responses to diseases. A barley type 1 ns-LTP, acting synergistically with thionins, inhibited the growth of bacterial pathogens in infected plants (Molina and García-Olmedo 1997). Broccoli ns-LTP were expressed at sufficiently high concentrations over the plant surface and in the vascular tissues to inhibit sensitive pathogens (Pyee et al. 1994). ns-LTP expression has been associated in cell differentiation in zinnia (Demura and Fukuda 1993), in developing rice seed (García-Garrido et al. 1998), during nodulation in *Vigna unguiculata* root hairs (Krause et al. 1994), and in plant defense in barley and maize (Buhot et al. 2001; Molina and García-Olmedo 1997; Molina et al. 1993). Overexpression of a barley type 2 ns-LTP in transgenic tobacco and *Arabidopsis* has been shown to enhance resistance to *Pseudomonas syringae* pvs. *tabaci* and *tomato* (Molina and García-Olmedo 1997). *DIR1*, an ns-LTP from *Arabidopsis*, was reported to be involved in long-distance signaling, possibly by binding a lipid molecule during systemic acquired resistance (Maldonado et al. 2002). ns-LTP have been found to be important food allergens (Asero et al. 2000).

Plant ns-LTP are encoded by a multigene family. At least 15 different ns-LTP genes have been found in *Arabidopsis* (Arondel et al. 2000). To date, 28 different full-length wheat ns-LTP genes have been reported in public databases. Although the nucleotide and amino acid sequences exhibit considerable di-

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vergence, several features are highly conserved in the encoded ns-LTP, including eight cysteine residues that form four conservative intramolecular disulphide bonds to stabilize the proteins (Kader 1996). A diversity of expression patterns of ns-LTP in plants subjected to biotic and abiotic stress conditions (Gaudet et al. 2003; Jung et al. 2003) suggests that individual members of the gene family may be involved or specifically expressed under a myriad of different physiological conditions.

Numerous diseases caused by fungi affect the growth and yield of spring and winter wheat worldwide. Previous studies have shown an association between ns-LTP and resistance to low-temperature diseases and bunt in wheat (Gaudet et al. 2003; Lu et al. 2005a). To better understand the role of ns-LTP in plant disease resistance, we isolated 11 full-length wheat ns-LTP cDNA clones, compared their sequences at both the nucleotide and amino acid levels, and conducted a phylogenetic analysis of monocot ns-LTP. Eight ns-LTP were expressed in the methylotrophic yeast *Pichia pastoris*. We demonstrated that expressed wheat ns-LTP are differentially toxic, in vitro, to a wide range of wheat and nonwheat fungal pathogens. The ability of wheat ns-LTP to bind lipids and induce permeability changes in membranes of fungal pathogens also was demonstrated.

RESULTS

Analysis of wheat LTP cDNA clones.

Eleven wheat full-length ns-LTP cDNA clones (Fig. 1A) that were isolated from two wheat suppression subtractive hybridization (SSH) cDNA libraries from tissues exposed to either a low-temperature stress or infection by *Tilletia tritici* using array-based differential hybridization techniques and sequencing information were compared. The 11 different wheat ns-LTP cDNA sequences were designated TaBs108F7, TaBs108G5, TaBs109A5, TaBs112C7, TaBs116G9, TaLt10B6, TaLt10E10, TaLt10F9, TaLt19C10, TaLt709L6, and TaLt710H24. Nine of the ns-LTP cDNA sequences are novel while the other two have been reported previously (TaLt709L6, GenBank accession number CK166650, identical to AF334185; and TaLt710H24, GenBank accession number CK166832, identical to AF302588). The length of the cDNA coding region for all 11 ns-LTP cDNA clones was 348 bp. The coding regions of these 11 novel ns-LTP cDNA clones were highly conserved, with 78 to 99% homology at the nucleotide level and 71 to 100% identity at the amino acid level (Fig. 1B). The deduced precursor proteins coded by the 11 ns-LTP cDNAs have molecular masses of 11.1 to 11.2 kDa with 115 amino acids (aa), which included a typical hydrophobic signal peptide region at the N-terminus that lead to a mature polypeptide of 90 aa (Fig. 1B). The cleavage site was predicted to be between positions Ala25 and Ala26 in all 11 gene products. Based on the predicted structure using the RasMol software, all the ns-LTP contained eight cysteine (cys) residues that form four pairs of conserved disulfide bonds at Cys4-Cys52, Cys14-Cys29, Cys30-Cys72, and Cys50-Cys86. The predicted mature proteins coded by the 11 ns-LTP cDNAs have molecular masses of approximately 9 kDa (8.6 to 8.7 kDa) with 90 aa and a basic isoelectric point (pI) varying from 8.85 to 9.58, characteristic of the ns-LTP1 family.

The hydrophobic amino acid residues alanine (Ala), isoleucine, (Ile), leucine (Leu), and valine (Val) involved in the formation of the hydrophobic cavity were Val7, Leu11, Ala18, Val33, Leu36, Ala40, Leu60, Ala65, Ile68, and Ile80. These were highly conserved among the 11 different sequences (Fig. 1B). Two conserved hydrophobic tyrosine (Tyr) residues, Tyr17 and Tyr78, located toward the N- and C-termini of the polypeptide backbone, respectively, are likely to be involved in the interaction with lipid or fatty acids located in the hydrophobic cavity of ns-LTP (Fig. 1B). The highly conserved resi-

dues reported in all other plant ns-LTP1 sequences (Douliez et al. 2000), including one consensus pentapeptide Pro-Tyr-X-Ile-Ser (Pro77-Ser81) motif, was conserved in the 11 wheat ns-LTP (Fig. 1B). A second pentapeptide, Thr/Ser-X1-X2-Asp-Arg/Lys (Ser42-Thr43-Ala44-Asp45-Lys46), also was observed in all the ns-LTP except for TaBs108G5, where the motif Ser42-Thr43-Ala44-Gly45-Lys46 was identified instead. Taken together, these results demonstrate the very high level of amino acid conservation among these 11 different ns-LTP1.

Among the predicted structures of mature protein of the 11 wheat ns-LTP, there were either 10 or 11 highly conserved, positively charged cationic residues, mainly Arg and Lys, which previously have been identified as key constituents of positively charged membrane patches (Gomar et al. 1996), a common feature of antimicrobial peptides (Zasloff 2002). Based on the predicted structure using RasMol software, each of the 11 ns-LTP forms four α -helices (Fig. 1B) with the potential to adopt an amphipathic tertiary structure.

A phylogenetic tree constructed using the amino acid sequences of 71 ns-LTP from different monocots—wheat (*Triticum aestivum*, $n = 28$), rice (*Oryza sativa*, $n = 33$), barley (*Hordeum vulgare*, $n = 7$), maize (*Zea mays*, $n = 1$), brome grass (*Bromus inermis*, $n = 1$), and millet (*Setaria italica*, $n = 1$)—is shown in Figure 2. Two distinct phylogenetic groups (type I and type II), were evident, suggesting that there are at least two major ns-LTP gene families in wheat (Fig. 2). The amino acid length variability of type I ns-LTP is very low, ranging between 114 and 118 aa residues for the precursor protein, whereas it is much higher in the type II ns-LTP, with either 91 to 110 or 120 to 162 aa residues. Eight ns-LTP (TaBs108F7 [#63], TaLt19C10[#71], TaBs116G9[#67], TaLt10B6[#68], TaLt10F9[#70], TaLt709L6[#45], TaLt710H24[#44], and BQ241565[#47]) were selected for expression in *Pichia pastoris* yeast. The ns-LTP BQ241565 belongs to the ns-LTP2 family while the seven others are members of the ns-LTP1 family.

Expression, purification, and binding activity assay of ns-LTP.

The *Pichia pastoris* expression system was employed for expressing the eight recombinant wheat ns-LTP. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3) and Western blot (Fig. 4) confirmed that the purified fusion peptide bands had the expected molecular masses of 12.9 to 14.5 kDa, which represented 8.6 to 9.7 kDa of protein mass of ns-LTP plus the mass of the vector N-terminal signal peptide and C-terminal *c-myc* epitope and polyhistidine tag; therefore, the native ns-LTP represented 66 to 69% of the total size of expressed protein produced in *Pichia* spp. Overloading gels did not reveal any contaminant proteins (Fig. 3). To ensure that the expressed proteins assumed their native form and exhibited typical binding functionality of ns-LTP, the lipid-binding activity of the purified proteins was analyzed. In vitro lipid-binding activity for all eight ns-LTP was demonstrated in Figure 5. D3821 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diazas-indacene-3-hexadecanoic acid) is a hexadecanoic acid molecule possessing a fluorescent group that has a very weak fluorescence intensity when it is dissolved in aqueous solution, but emits a strong intensity of fluorescence when it enters into the hydrophobic cavity of an ns-LTP molecule. Increasing the molar ratio of D3821/ns-LTP from 0 to 3.0 led to an initial sharp increase in fluorescence intensity due to the fatty acid analog entering into the hydrophobic cavity within the molar ratio of D3821/ns-LTP range of 0.01 to 0.60, whereas further increases led to a decrease in the signal (Douliez et al. 2000; Ge et al. 2002). Between molar ratio of 1.0 and 3.0, the decrease in fluorescence intensity was minimal (Fig. 5). Based on their ability to bind the fluorescent fatty acid analog D3821, wheat

A	TaBs112C7	ATGGCCCGTGTCTGCAGCTGCTCAACTCGTGTGTTTACCCTCGTGGCAGCAATGGTCTCTC	60
	TaLt19C10	ATGGCCCGTGTCTGCAGCTGCTCAACTCGTGTGTTTACCCTCGTGGCAGCAATGGTCTCTC	60
	TaBs108F7	ATGGCTCGCGGTGCAGCTACTCAGCTCGTGGTGGTGGCGCTATGCTCTCTC	60
	TaBs109A5	ATGGCTCGCGGTGCAGCTACTCAGCTCGTGGTGGTGGCGCTATGCTCTCTC	60
	TaBs108G5	ATGGCTCGCGGTGCAGCTACTCAGCTCGTGGTGGTGGCGCTATGCTCTCTC	60
	TaLt10E10	ATGGCCATTCTGCTGTGCTCAGGTCGTGCTCGTGGTGGTGGCGCTATGCTCTCTC	60
	TaLt10F9	ATGGCCATTCTGCTGTGCTCAGGTCGTGCTCGTGGTGGTGGCGCTATGCTCTCTC	60
	TaBs116G9	ATGGCCCGTGTCTGCTGTGCTCAGGTCGTGCTCGTGGTGGTGGCGCTATGCTCTCTC	60
	TaLt10B6	ATGGCTCGCACTGCAGCAACAAAGCTCGTGGTGGTGGCGCAATGCTCTCTG	60
	TaLt710H24	ATGGCTCGCACTGCAGCAACAAAGCTCGTGGTGGTGGCGCAATGCTCTCTG	60
	TaLt709L6	ATGGCTCGCACTGCAGCTACTAAACTCGTGGTGGTGGCGCAATGATCTCTC	60
		***** *	
	TaBs112C7	ACAGCCACAGACGCTGCAATCTCTGTGGCCAGGTGAGCTCTGCCTTGAGCCCCCTGCATC	120
	TaLt19C10	ACAGCCACAGACGCTGCAATCTCTGTGGCCAGGTGAGCTCTGCCTTGAGCCCCCTGCATC	120
	TaBs108F7	GTAGCCTGCGATGCGGCGATATCTCTGCGGTGAGGTGACCTCTGCCTTGAGCCCCCTGCATC	120
	TaBs109A5	GTAGCCTGCGATGCGGCGATATCTCTGCGGTGAGGTGACCTCTGCCTTGAGCCCCCTGCATC	120
	TaBs108G5	GTAGCCTGCGATGCGGCGATATCTCTGCGGTGAGGTGACCTCTGCCTTGAGCCCCCTGCATC	120
	TaLt10E10	GCAGCCACGGAGGCGGCCGTATCGTGGGTGAGGTGAGCTCTGCCTTGAGCCCCCTGCATC	120
	TaLt10F9	GCAGCCACGGAGGCGGCCGTATCGTGGGTGAGGTGAGCTCTGCCTTGAGCCCCCTGCATC	120
	TaBs116G9	GCAGTCACGGAGGCGGCTGTATCGTGGGTGAGGTGAGCTCTGCCTTGAGCCCCCTGCATC	120
	TaLt10B6	GTAGCCTCCGACGCGGCGATCTCTGCGGTGAGTGAACCTGCGCTGGCCCTCTGCGTC	120
	TaLt710H24	GTAGCCTCCGACGCGGCGATCTCTGCGGTGAGTGAACCTGCGCTGGCCCTCTGCGTC	120
	TaLt709L6	GCAGCCTCCGACGCGGCCATCAGCTGCGGCCAGGTGAGCTCTGCCTTGAGCCCCCTGCGTC	120
		* *	
	TaBs112C7	TCCTATGCACGCGGCGAGCGGCTCCAGCCACCTGCGGCCCTGCTGCAGTGGTGTGAGGAGT	180
	TaLt19C10	TCCTATGCACGCGGCGAGCGGCTCCAGCCACCTGCGGCCCTGCTGCAGTGGTGTGAGGAGT	180
	TaBs108F7	TCCTATGCACGCGGCAATGGCGCCAACCCGCTGCGGCCCTGCTGCAGCGGCGTCAAGGAGT	180
	TaBs109A5	TCCTATGCACGCGGCAATGGCGCCAACCCGCTGCGGCCCTGCTGCAGCGGCGTCAAGGAGT	180
	TaBs108G5	TACTATGCACGCGGTAATGGCGCCAACCCGCTGCGGACTGCTGCAGCGGCGTCAAGGAGT	180
	TaLt10E10	TCCTATGCACGCGGCAACGGCGCCAACCCGCTGCGGCCCTGCTGCAGCGGCGTTAGGAGT	180
	TaLt10F9	TCCTATGCACGCGGCAACGGCGCCAACCCGCTGCGGCCCTGCTGCAGCGGCGTTAGGAGT	180
	TaBs116G9	TCCTATGCACGCGGCAACGGCGCCAGCCATCTGCGGCCCTGCTGCAGCGGCGTTAGGAGT	180
	TaLt10B6	TCCTACGCAAAAGGCGAGCGGGGCCAGCCGCTGGGGCTGCTGTTCCGGAGTTAGGAGA	180
	TaLt710H24	TCCTACGCAAAAGGCGAGCGGGGCCAGCCGCTGGGGCTGCTGTTCCGGAGTTAGGAGA	180
	TaLt709L6	GCCTATGCGAAGGCGAGCGGGACAGCCGCTGCGGCCCTGCTGCAGCGGCGTCAAGAAA	180
		* *	
	TaBs112C7	CTGGCCGCGCAGCGCGGAGCACCGCTGACAAGCAAGCGGCGTGAAGTGCATTAAGAGT	240
	TaLt19C10	CTGGCCGCGCAGCGCGGAGCACCGCTGACAAGCAAGCGGCGTGAAGTGCATTAAGAGT	240
	TaBs108F7	CTGGCCGCTGACGCGCGGAGCACCGCTGACAAGCAAGCAGCGTGAAGTGCATCAAGAGC	240
	TaBs109A5	CTGGCCGCTGACGCGCGGAGCACCGCTGACAAGCAAGCAGCGTGAAGTGCATCAAGAGC	240
	TaBs108G5	CTGGCCGCTGACGCGCGGAGCACCGCTGGAAGCAAGCAGCGTGAAGTGCATCAAGAGC	240
	TaLt10E10	CTAGCCAGCTCAGCCCGGAGCACCGCTGACAAGCAAGCGGCGTGAAGTGTATCAAGAGC	240
	TaLt10F9	CTAGCCAGCTCAGCCCGGAGCACCGCTGACAAGCAAGCGGCGTGAAGTGTATCAAGAGC	240
	TaBs116G9	CTAGCCAGCTCAGCCCGGAGCACCGCTGACAAGCAAGCGGCGTGAAGTGCATCAAGAGC	240
	TaLt10B6	TTGGCCGCTTAGCGCGGAGCACCGCGGACAGCAAGCAAGCGGCGTGAAGTGCATCAAGAGT	240
	TaLt710H24	TTGGCCGCTTAGCGCGGAGCACCGCGGACAGCAAGCAAGCGGCGTGAAGTGCATCAAGAGT	240
	TaLt709L6	TTGGCCGCTTAGCGCGGAGCACCGCGGACAGCAAGCTACATGCAGGTGCCTCAAGAGT	240
		* *	
	TaBs112C7	GCTGCTGGTGGGCTCAACGCTGGAAAGGCCGCTGGCATCCCCTCCAAGTGGCGGCTCAGC	300
	TaLt19C10	GCTGCTGGTGGGCTCAACGCTGGAAAGGCCGAGGCATCCCCTCCAAGTGGCGGCTCAGC	300
	TaBs108F7	GCTGCTGGTGGGCTCAACGCTGGCAAGGCCGCGGCATCCCCTTCAAAGTGGCGGCTCAGC	300
	TaBs109A5	GCTGCTGGTGGGCTCAACGCTGGCAAGGCCGCGGCATCCCCTTCAAAGTGGCGGCTCAGC	300
	TaBs108G5	GCTGCTGGTGGGCTCAACGCTGGCAAGGCCGCGGCATCCCCTTCAAAGTGGCGGCTCAGC	300
	TaLt10E10	GCTGCTGCTGGGCTCAACGCTGGCAAGGCCGCGGCATCCCCACAAAGTGGCGGCTTAGC	300
	TaLt10F9	GCTGCTGCTGGGCTCAACGCTGGCAAGGCCGCGGCATCCCCACAAAGTGGCGGCTTAGC	300
	TaBs116G9	GCTGCTGCTGGGCTCAACGCTGGCAAGGCCGCGGCATCCCCACAAAGTGGCGGCTTAGC	300

Continued on following page.

Fig. 1. Comparison of the **A**, nucleotide and **B**, amino acid sequences for the coding regions of 11 nonspecific lipid transfer protein (ns-LTP) cDNA genes from wheat used in this study: TaLt710H24 (GenBank accession number CK166832), TaLt709L6 (GenBank accession number CK166650), TaBs108F7 (GenBank accession number DN551584), TaBs108G5 (GenBank accession number DN551585), TaBs109A5 (GenBank accession number DN551587), TaBs112C7 (GenBank accession number DN551617), TaBs116G9 (GenBank accession number DN551652), TaLt10B6 (GenBank accession number DQ465677), TaLt10E10 (GenBank accession number DQ465678), TaLt10F9 (GenBank accession number DQ465676), and TaLt19C10 (GenBank accession number DQ465679). Amino acids were deduced from their respective cDNA sequences. Symbols: + above the amino acid marks the eight conserved cysteine residues that form the four disulphide bridges and | indicates the position of putative leader cleavage site in the 11 wheat ns-LTP. * indicate the identical nucleotides or amino acids among the 11 distinct sequences, while ':' and '.' represent conserved and semiconserved amino acids, respectively. Colored letters are highly conserved. Red P (Pro) and pink V (Val) indicate involvement in loops and turns. The two red pentapeptide motifs may be involved in loading, binding, and transferring lipid and fatty acids, whereas the red Y (Tyr) would be involved in the interaction with lipid or fatty acids located in the hydrophobic cavity.

the wheat biotrophic pathogens *Puccinia graminis* Pers., *Puccinia triticina* Erikss., and *Tilletia tritici* (Bjerk.) G. Winter; the wheat necrotrophs pathogens *Fusarium graminearum* Schwabe, *Pyrenophora tritici-repentis* (Died.) Drechsler, *Stagonospora nodorum* (Berk.) E. Castell. & Germano, *Typhula incarnata* Lasch, and a low-temperature basidiomycete (LTB); and the nonwheat fungal pathogens *Botrytis cinerea* Pers., *Sclerotinia sclerotiorum* (Lib.) de Bary, and *Verticillium dahliae* Kleb. Growth inhibition was determined on both liquid and solid growth media. Because *Puccinia graminis*, *Puccinia triticina*, and *Tilletia tritici* are obligate biotrophs, and not free of contaminant microbes, their inhibition by the ns-LTP could

not be assessed by radial growth on potato dextrose agar (PDA) or in liquid potato dextrose broth (PDB) culture. Consequently, inhibition of germination and radial extension of hyphae or promycelium was evaluated by placing paper disks containing ns-LTP on petri plates containing 2% water agar and sprayed with a suspension of uredospores or teliospores.

There was a very close association in reactions between the fungi and ns-LTP observed on solid PDA plates and on liquid PDB in the 96-well plates (Fig. 6). Fungal growth in the plate assay was measured as an increase of absorbance at 570 nm while the growth inhibition on solid plate was measured on a semiquantitative, five-step scale ranging from absence of inhibi-

Fig. 2. Phylogenetic tree of 28 wheat (*Triticum aestivum*) (number 44–71 boxed), 33 rice (*Oryza sativa*) (numbers 10 to 35 and 37 to 43), 7 barley (*Hordeum vulgare*) (numbers 1 to 5 and 7 to 8), 1 maize (*Zea mays*) (number 9), 1 broom grass (*Bromus inermis*) (number 6), and 1 millet (*Setaria italica*) (number 36) nonspecific lipid transfer proteins (ns-LTP) based on amino acid sequences indexed in GenBank databases or deduced from our two suppression subtractive hybridization cDNA libraries of wheat. The length of each pair of branches represents the distance between sequence pairs. 1, Z37114; 2, Z37115; 3, X96979; 4, AY662492; 5, AF109195; 6, AY057932; 7, AF039024; 8, HVU88090; 9, ZMU66105; 10, AJ704771; 11, NM_197681; 12, NM_197680; 13, NM_197679; 14, NM_197676; 15, NM_197675; 16, NM_197674; 17, NM_197672; 18, NM_197671; 19, NM_197670; 20, NM_197249; 21, NM_197248; 22, NM_197243; 23, NM_197242; 24, NM_197241; 25, NM_197239; 26, NM_195931; 27, NM_195930; 28, NM_195927; 29, NM_195926; 30, NM_195157; 31, AJ704770; 32, AY466109; 33, AY466108; 34, AY335485; 35, AY327042; 36, AF439446; 37, AF017361; 38, AF017360; 39, AF017359; 40, AF017358; 41, AF051369; 42, U77295; 43, U31766; 44, TaLt710H24*; 45, TaLt709L6*; 46, AF551849; 47, BQ241565*; 48, AJ784895; 49, AJ784896; 50, AJ784897; 51, AJ784898; 52, AJ784899; 53, AJ784900; 54, AJ784901; 55, AJ784902; 56, AY226580; 57, AY566607; 58, AY754742; 59, AY789644; 60, AY796184; 61, BE213412; 62, BE217027; 63, TaBs108F7*; 64, TaBs108G5; 65, TaBs109A5; 66, TaBs112C7; 67, TaBs116G9*; 68, TaLt10B6*; 69, TaLt10E10; 70, TaLt10F9*; and 71, TaLt19C10*. An asterisk (*) marks the ns-LTP expressed in yeast.

tion (–) to a very strong inhibition (+++++) between the ns-LTP-infused disk and the growing fungal colony. The toxicity of the eight ns-LTP toward the 11 fungal pathogens is summarized in Table 1. The wheat pathogens *Puccinia graminis*, *Puccinia triticina*, and *Pyrenophora tritici-repentis* and the nonwheat pathogens *Botrytis cinerea*, *Verticillium dahliae*, and *Sclerotinia sclerotiorum* were inhibited by the majority of ns-LTP while the snow mold fungi LTB and *Typhula incarnata*, *Stagonospora nordorum*, *Tilletia tritici*, and *Fusarium graminearum* were the least inhibited. There appeared to be some commonalities among fungal types in response to the ns-LTP. For example, the closely related rust species *Puccinia graminis* and *Puccinia triticina* (Table 1; Fig. 7) did not differ substantially in their responses. The nonwheat pathogens *Botrytis cinerea*, *Verticillium dahliae*, and *Sclerotinia sclerotiorum* (Table 1) also reacted similarly to the ns-LTP. Among the ns-LTP, TaBs108F7 and TaLt10B6 induced similar inhibition to the majority of fungi, as did TaLt19C10 and TaLt709L6, and both groups appeared to exert the broadest spectrum inhibitory activity (Table 1). TaBs10B6 and TaBs116G9 were the only ns-LTP that obviously inhibited the LTB fungus (Table 1). The least inhibitory ns-LTP against the different pathogens was BQ241565, the only ns-LTP2 evaluated.

There were no obvious groupings related to the level of toxicity of the ns-LTP toward the various types of fungi and the library of origin. For example, *Tilletia tritici* was inhibited by TaLt19C10 (identical to TaBs112C7) but not by TaBs108F7 and TaBs116G9; all three originated from the *Tilletia tritici*-induced resistance library. Of the three ns-LTP originating from this library, two, TaBs108F7 and TaLt19C10, were toxic to a broad range of fungi, including the nonwheat pathogens. Similarly, *Stagonospora nordorum* was inhibited by the low-temperature-induced ns-LTP TaLt10F9 and TaLt10B6 but not by TaLt19C10, TaLt709L6, or TaLt710H24. Of the five ns-LTP originating from the cold-induced library, TaLt10B6, TaLt19C10, and TaLt709L6 were toxic to a broad spectrum of fungi whereas the two others, TaLt10F9 and TaLt710H24, were specifically toxic to fewer wheat and nonwheat pathogens. There also was no obvious correlation between the lipid-binding affinity and the antifungal activity of ns-LTP. Although TaLt19C10 exhibited a very strong antifungal activity toward *Tilletia tritici* and *Sclerotinia sclerotiorum*, its lipid-binding activity was very low. In contrast, TaLt709L6, which exhibited an antifungal activity

spectrum similar to TaLt19C10, was the most efficient in binding lipid among the eight ns-LTP tested. Both TaBs108F7 and TaLt709L6 were able to bind lipid efficiently (Fig. 5) and exerted a very strong antifungal activity on both wheat and nonwheat fungi (Table 1). Controls containing the vector minus ns-LTP were nontoxic to the fungi (data not shown), demonstrating that the expressed products containing N-terminal signal peptide, and the C-terminal *c-myc* epitope and polyhistidine tag fused to the ns-LTP, were nontoxic to the fungi employed in this study. The toxicity of the ns-LTP TaLt709L6 and TaBs116G9 to fungi, after reduction of the disulphide bridges with 20 mM DTT, also was assayed. The DTT-treated ns-LTP exhibited no toxicity to the five fungi compared with the toxicity observed with the ns-LTP controls (data not shown).

Inhibition of fungal growth or spore germination of LTP.

Three ns-LTP were selected for determination of the lethal dose for 50% inhibition of fungal growth or spore germination (LD_{50}) against *Puccinia graminis*, *Tilletia tritici*, *Fusarium graminearum*, the LTB fungus, *Sclerotinia sclerotiorum*, and *Verticillium dahliae*. Both TaBs108F7 and TaLt19C10 exhibited a broader spectrum of antifungal activity than the other ns-LTP whereas TaBs116G9 exhibited the strongest toxicity to the LTB fungus. A series of dilutions were made starting at approximately 15 μ M because this represented the average maximum concentration of the individual ns-LTP obtained following affinity purification. Where toxic effects were recorded, the inhibition of fungi by TaBs108F7, TaLt19C10, and TaBs116G9 was dose dependent (Fig. 8). The LD_{50} concentrations needed for 50% growth inhibition on the fungi ranged from 1.00 to 7.00 μ M. TaBs108F7 exhibited the strongest antifungal effects among the three ns-LTP (Tables 1 and 2). These results also illustrated a strong interaction between strains and sensitivity to the ns-LTP. An obvious example of this interaction was the moderately high toxicity of TaBs116G9 to the LTB fungus and the lack of any activity of TaBs108F7 and TaLt19C10 on the same fungus (Table 2). Similarly, growth of *Tilletia tritici* was moderately reduced by TaLt19C10 but unaffected by the other two ns-LTP. These results were consistent with previous antifungal assays (Table 1).

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the purified fused wheat nonspecific lipid transfer proteins expressed in yeast. Lane A, TaBs108F7; lane B, TaLt19C10; lane C, TaLt709L6; lane D, BQ241565; lane E, TaLt710H24; lane F, TaBs116G9; lane G, TaLt10F9; lane H, TaLt10B6; and lane M, protein standard marker.

Fig. 4. Western blot of the purified fused wheat nonspecific lipid transfer proteins TaLt10B6 and TaLt709L6 expressed in *Pichia* yeast. Lane A, TaLt10B6; lane B, TaLt709L6; and lane M, protein standard marker.

ns-LTP induce fungal membrane permeabilization.

The proposed mechanism of toxicity of the ns-LTP to fungi and bacteria involves the potential for the basic amphipathic ns-LTP molecules to modify and destabilize the plasma membrane of the pathogens (Caaveiro et al. 1997; Dathe and Wieprecht 1999). SYTOX Green (Invitrogen Canada, Inc., Mississauga, ON, Canada) readily penetrates cells that have compromised plasma membranes and binds to nucleic acid, where it induces an intense fluorescence emission when excited under UV-light (Giudici et al. 2004). The effect of TaBs108F7 and TaLt19C10 on SYTOX Green uptake by *Fusarium graminearum*, *Verticillium dahliae*, *Puccinia graminis*, and *Tilletia tritici* mycelia or spores and observed by fluorescence microscopy is illustrated in Figure 9. Among treatments without the ns-LTP (water controls), the SYTOX Green stain was not observed inside fungal cells in mycelia and spores that remained intact (Figures 9A, C, E, and G). Occasionally, a small amount of fluorescence was observed in water-treated mycelia and was attributed to mechanical damage of the hyphae during slide preparation (Fig. 9C). Following addition of ns-LTP, nuclei in *Fusarium graminearum* spores and *Verticillium dahliae* hyphae stained deeply (Fig. 9B and D). In the corresponding treatments involving *Puccinia graminis* and *Tilletia tritici* possessing pigmented spores (Fig. 9F and H), intracellular absorption of the dye appeared more generalized. Among the thick-walled *Tilletia*

tritici spores (Hoffmann 1982), the uptake was variable and fewer spores contained fluorescent dye after 90 min. However, upon germination of spores of both *Puccinia graminis* and *Tilletia tritici* in the presence of ns-LTP, the SYTOX green stain was preferentially observed in the nuclei of the emerging hyphae (data not shown).

For mycelial or conidial stages of the fungi tested, including the conidiospores of *Fusarium graminearum*, the rate of incorporation of the SYTOX Green stain was rapid, frequently approaching 90% after 30 min (Fig. 10A). Similar profiles were observed for *Puccinia graminis*, *Fusarium graminearum*, the LTB fungus, *Sclerotinia sclerotiorum* and *Verticillium dahliae* (not shown). Conversely, intracellular accumulation of the dye within *Tilletia tritici* teliospores exposed to ns-LTP was much less rapid; less than 5% *Tilletia tritici* spores had a fluorescent signal following exposure to ns-LTP TaLt19C10 for 30 min. However, the proportion of cells with a signal reached 42% after 16 h (Fig. 10B). This likely was due to the very thick and resistant nature of the walls of these resting spores that constitutes a barrier to putative targets of ns-LTP on the plasma membrane (Giudici et al. 2004). Collectively, these results demonstrated that ns-LTP were able to permeabilize or perturb fungal membranes of several different species, and strongly suggested that alteration of fungal membrane permeability is the primary mode of action in the toxicity of these eight ns-LTP.

Fig. 5. Binding activity of nonspecific lipid transfer proteins (ns-LTP) with fatty acid D3821. Different concentrations (from 0 to 30 μ M) of the fatty acid analog 4, 4-difluoro-5, 7-dimethyl-4-bora-3a, 4a-diaza-s-indacene-3-hexadecanoic acid (D3821, Invitrogen Canada, Inc.) from a 1-mM stock solution in ethanol were incubated with the purified 10.0 μ M ns-LTP (final concentration), 8 mM 2-mercaptoethanol, 10 mM morpholinepropanesulfonic acid, pH 7.2, and placed in a fluorometer cuvette for fluorescence binding assay according to Zachowski and associates (1998). The relative fluorescence values (RFU) was measured at 520/10 nm (excitation at 490/10 nm). Lysozyme and protease were employed as controls. 1, TaLt709L6; 2, TaLt10F9; 3, TaBs108F7; 4, TaLt710H24; 5, TaBs116G9; 6, BQ241565; 7, TaLt10B6; 8, TaLt19C10; 9, protease; and 10, lysozyme.

Table 1. Inhibitory activity of wheat nonspecific lipid transfer proteins toward wheat and nonwheat fungal pathogens^a

Pathogen	TaBs108F7	TaLt10B6	TaLt19C10	TaLt709L6	TaLt10F9	TaLt710H24	TaBs116G9	BQ241565
<i>Fusarium graminearum</i>	++++	+++	+	+	++	+	+	-
<i>Stagonospora nodorum</i>	++	+++	-	-	++++	-	-	-
<i>Pyrenophora tritici-repentis</i>	+++	++++	+	++++	+	++++	-	+
<i>Typhula incarnata</i>	+	+	++	+++	++	-	+	-
Low-temperature basidiomycete	-	++	-	-	-	-	+++	-
<i>Puccinia triticina</i>	+++	++++	+++	+++	-	++	++	-
<i>P. graminis</i>	++++	++	++	++	+	++	-	+
<i>Tilletia tritici</i>	-	-	++++	-	+++	-	-	+++
<i>Botrytis cinerea</i>	+++	++	++	+	+	++++	++	-
<i>Verticillium dahliae</i>	++++	++	+++	++	+	+	+	+
<i>Sclerotinia sclerotiorum</i>	+++	++	++++	+++	++	+	++	-

^a Symbols: ++++ = very strong antifungal activity, +++ = strong antifungal activity, ++ = antifungal activity, + = weak antifungal activity, and - = no antifungal activity based on radial growth on potato dextrose agar and growth in 96-well plates on potato dextrose broth or microscopic examination of spore germination on potato dextrose agar.

DISCUSSION

The deduced amino acid sequences for the 11 wheat ns-LTP1 cDNA clones considered in this study share a number of characteristics common to all the other known plant ns-LTP, including a basic pI (8.85 to 9.58), four α -helices, eight cysteine residues forming four disulfide bridges, and an N-terminal signal peptide, characteristic for targeting the secretory pathway (Douliez et al. 2000; Kader 1996). The conserved hydrophobic amino acid sequence Ile Pro Ser/Thr Lys Cys Gly Val Ser Val/Ile Pro Tyr (amino acids 68 to 78) is the putative site of the interaction with the acyl chains of lipids (Jung et al. 2003; Tchang et al. 1988; Torres-Schumann et al. 1992), and the highly conserved residues of the consensus pentapeptide Pro Tyr X Ile Ser (amino acids 77 to 81) of unknown function also were

conserved in the 11 wheat ns-LTP studied. The Thr/Ser X1X2 Asp Arg/Lys (amino acids 42 to 46) pentapeptide, ubiquitous among all known plant ns-LTP1 sequences (Douliez et al. 2000), also was conserved in all but one (TaBs108G5) of the same 11 wheat ns-LTP. These two consensus pentapeptides are possibly important for loading, binding, and transferring lipids and fatty acids. Both pentapeptide motifs are absent in the ns-LTP2 family (Douliez et al. 2000; Monnet et al. 2001).

Phylogenetic analyses based on amino acid sequences of 71 ns-LTP demonstrate the existence of two distinct groups of ns-LTP, type I and type II, among cereals and other monocots. Type I ns-LTP are characterized by low protein length variability, whereas type II exhibits considerable length variability. Recently, Boutrot and associates (2005) reported that two maize and one rice ns-LTP clustered together into a type III ns-

Fig. 6. Nonspecific lipid transfer protein (ns-LTP) in vitro inhibition of mycelial growth of *Fusarium graminearum* with **A**, TaBs108F7 and *Stagonospora nodorum* with **B**, TaLt10F9 and **C**, TaLt709L6. Left, ns-LTP treatment; right, control. Graphs represent corresponding time-course inhibition in 96-well plates in potato dextrose broth medium; $\diamond$ = ns-LTP and $\blacklozenge$ = control.

LTP. These sequences were absent from our data set, and the type III class of ns-LTP was not detected in our phylogenetic tree. The presence of multiple members of the ns-LTP1 family in rice has been interpreted as a consequence of gene duplications and subsequent sequence variation, while those residues crucial to function are conserved (Vignols et al. 1997).

The *Pichia pastoris* expression system was employed for expressing eight recombinant wheat ns-LTP because expressed proteins can properly fold to form active protein and, when co-expressed with a signal peptide, proteins are secreted extracellularly for simplified purification (Klein et al. 1998; Zuidmeer et al. 2005). Lipid-binding assays confirmed that the eight wheat ns-LTP expressed in yeast exhibited lipid binding activity and were comparable with those exhibited by a rice ns-LTP when lipid analogs were used as the fluorescent probe to evaluate lipid binding by the expressed proteins (Ge et al.

2002). The eight ns-LTP selected for expression in *Pichia pastoris* yeast basically exemplified the variability of the known 28 wheat ns-LTP (Fig. 2). The ns-LTP BQ241565 belongs to the ns-LTP type II family whereas the seven others belong to the ns-LTP type I family. Competition experiments and X-ray crystallography studies have shown that maize and wheat ns-LTPs, by adapting their volume, can bind two monoacylated lipids (Charvolin et al. 1999; Gomar et al. 1996; Shin et al. 1995; Zachowski et al. 1998) or a diacylated lipid (Sodano et al. 1997). However, a different wheat ns-LTP has been reported to bind only one molecule of fatty acid (Guerbette et al. 1999). It is not clear from our results if the wheat ns-LTP can bind more than one molecule of fatty acid analog.

Some ns-LTP isoforms strongly inhibited germination of fungal spores or the growth of mycelia whereas others exhibited weaker overall toxicity. Commonalities in response to the differ-

Fig. 7. In vitro inhibitory activity of wheat nonspecific lipid transfer proteins to spore germination of **A** and **B**, *Puccinia triticina*, **C** and **D**, *Puccinia graminis*, and **E** and **F**, *Tilletia tritici* in petri dishes with potato dextrose agar medium. **A**, TaLt10B6; **B**, control; **C**, TaBs108F7; **D**, control; **E**, TaLt19C10; and **F**, control. (magnification $\times 500$). Note the very limited growth for *Puccinia graminis* (**C**) and *Tilletia tritici* (**E**). For *Tilletia tritici*, development consisted of extension of the promycelium but did not progress to the formation of sporidia as in the control treatments (**F**).

ent ns-LTP among the different wheat pathogens such as the wheat rusts and nonwheat fungi were evident. The LD₅₀ values for the three different wheat ns-LTP varied between 1 and 7 µM, depending on the ns-LTP and the pathogen involved. Sunflower HA-AP10 and the mung bean ns-LTP exhibited similar toxicities of 0.23 to 1.0 µM (Cammue et al. 1995; Kristensen et al. 2000; Regente et al. 2005). Disruption of the disulphide bridges following reduction of the ns-LTP with DTT resulted in loss of toxicity to fungi and fatty acid binding activity. This demonstrated the requirement that ns-LTP assume their native tertiary structure in order to create the hydrophobic cavity necessary for lipid binding or form amphipathic molecules capable of interacting with the plasma membrane of fungi.

The mechanism of toxicity observed among wheat and other ns-LTP toward fungal and bacterial pathogens still remains to be elucidated. The lack of any correlation between the lipid-binding activity and antifungal activity of the wheat ns-LTP observed in this study suggests that ns-LTP toxicity and lipid-binding activity are likely independent. *Ace*-AMP1, an antifungal protein extracted from onion seed, exhibits structural similarities to plant ns-LTP but it is unable to bind lipids within its hydrophobic core (Tassin et al. 1998), thus suggesting that the lipid-binding property of ns-LTP is not associated with toxicity to microorganisms. *Ace*-AMP1 may have evolved from an ns-LTP1 precursor but has lost its lipid-binding function while retaining antimicrobial activity. Ge and associates (2003) reported that the antifungal activity of a rice ns-LTP1 gene following mutagenesis was affected whereas the lipid-binding ability was not. To date, there have been no reports in the literature of the existence of a correlation between the lipid-binding activity and antifungal activity of ns-LTP. Thus, it is likely that the activity center for binding fatty acids is unrelated to active sites on the ns-LTP that interact with microorganisms and generate the toxic response.

The notion that cationic residues generate positively charged patches over the surface of ns-LTP which, in turn, would interact with specific negatively charged sites on the membranes of fungal pathogens has been proposed (De Samblanx et al. 1997; Gomar et al. 1996). As predicted by its tertiary structure, *Ace*-AMP1 is capable of interacting with lipid bilayers and inducing permeability changes (Tassin et al. 1998). A fundamental principal underlying all classes of antimicrobial peptides is the ability of the molecule to adopt a shape in which clusters of hydrophobic and cationic amino acids are spatially organized in discrete sectors of the molecule (Zasloff 2002). This feature creates a net positively charged surface which then may bind to the negatively charged membrane domains (Crimi et al. 2006) or putative membrane receptors of microorganisms (Buhot et al. 2004), resulting in an alteration and destabilization

of the membrane structure (De Samblanx et al. 1997; Zasloff 2002). Recently, Lin and associates (2005) demonstrated that a mung bean ns-LTP1 contained 11 charged cationic residues, 7 of which were highly conserved among ns-LTP1s, that could contribute to the antimicrobial activity of this ns-LTP1. Among the deduced mature wheat ns-LTP considered in this study, all but one possesses 10 or 11 positively charged cationic residues. BQ241565, the ns-LTP2, possesses only seven residues and, globally, its antifungal activity was lower than that of other ns-LTP. It is possible that the lower activity of the only ns-LTP 2 evaluated in our study resulted from differences in overall structure or in the number of positively charged residues. A two-step mechanism to explain the antimicrobial activity of small defense peptides has been proposed by Dathe and Wieprecht (1999): i) the peptide binds to the cell surface and ii) the bound peptide destabilizes the membrane, thereby inducing membrane permeabilization. The overwhelming majority of antimicrobial peptides improve their affinities for microbial cell membranes by adopting an amphipathic α -helix or β -sheet conformation (Jin et al. 2005). Membrane permeabilization leads to a breakdown of the transmembrane potential causing leakage of cell contents, resulting finally in cell death (Dathe and Wieprecht 1999).

This study demonstrated a differential toxicity of a large number of individual wheat ns-LTP to a large selection of wheat and nonwheat fungal pathogens in vitro; however, the nature of the differential toxicity of wheat ns-LTP is unknown. The differential affinity of a sunflower ns-LTP1 for different phospholipids leading to a toxic response in a pathogen (*Fusarium solani*) but not to host potato cells in suspension (Regente et al. 2005) could serve as a model for the differential toxicity observed in our study. In the present study, single amino acid residue differences between the mature protein sequences for TaLt10F9 and TaBs116G9 and between TaLt10B6 and TaLt710H24 resulted in substantially different antifungal pro-

Table 2. Lethal dose values for 50% inhibition of fungal growth or spore germination (LD₅₀) for selected wheat nonspecific lipid transfer proteins^a

Fungus species	Lipid transfer protein (µM)		
	TaBs108F7	TaLt19C10	TaBs116G9
<i>Sclerotinia sclerotiorum</i>	1.75	1.25	3.00
Low-temperature basidiomycete	–	–	1.00
<i>Fusarium graminearum</i>	1.5	5.00	4.00
<i>Verticillium dahliae</i>	2.75	5.00	7.00
<i>Puccinia graminis</i>	1.00	3.50	–
<i>Tilletia tritici</i>	–	3.00	–

^a LD₅₀ was determined from dose-response curves representing percentage of growth inhibition versus protein concentration; – = no inhibition.

Fig. 8. Dose-response curves depicting the antifungal activity of nonspecific lipid transfer protein **A**, TaBs108F7 and **B**, TaLt19C10 to *Verticillium dahliae* and *Sclerotinia sclerotiorum*, respectively, after the fungal mycelia were incubated for 3 days at 25°C (*Sclerotinia sclerotiorum*) or 6 days at 15°C (*Verticillium dahliae*). Bars represent standard error values.

files in vitro. A single amino acid change could alter the net charge within and among cationic patches, and this could affect the nature of the interaction at the surface of the fungal membrane. Possibly, these single amino acid residue replacements were sufficient to alter the tertiary structure of ns-LTP and result in a different distribution of cationic patches that

ultimately affect their antifungal properties. In the particular case of TaLt10F9 and TaBs116G9, the difference occurs at amino acid 24, which are asparagine ($\text{NH}_2\text{-CO-CH}_2\text{-CHNH}_3^+\text{-COO}^-$) and serine ($\text{HO-CH}_2\text{OH-CHNH}_3^+\text{-COO}^-$), respectively. This amino acid is not located in one of the conserved domains but rather occurs on the surface of the ns-LTP based on three-

Fig. 9. Uptake of SYTOX green by fungal mycelium and spores 90 min following treatment with nonspecific lipid transfer protein (ns-LTP) TaBs108F7 and TaLt19C10. **A** and **B**, *Fusarium graminearum*; **C** and **D**, *Verticillium dahliae*; **E** and **F**, *Puccinia graminis*; **G** and **H**, *Tilletia tritici*. **A**, **C**, **E**, and **G**, water controls; **B**, **D**, and **F**, ns-LTP TaBs108F7; **H**, ns-LTP TaLt19C10. (magnification $\times 500$). Note that in fungi treated with SYSTOX green only (**A**, **C**, **E**, and **G**), there is no fluorescence inside spores or mycelium. Following addition of nsLTP, nuclei in *Fusarium graminearum* spores and *Verticillium dahliae* hyphae stain deeply (**B** and **D**). In the corresponding treatments involving *Puccinia graminis* and *Tilletia tritici* possessing pigmented spores (**F** and **H**), intracellular absorption of the dye appears more generalized. Among the thick-walled *Tilletia tritici* spores, the uptake is variable and fewer spores contain fluorescent dye after 90 min.

dimensional display using RasMol (J.-Y. Sun, D. A. Gaudet, M. Frick, and A. Laroche, *unpublished results*). The additional NH₂-CO group from asparagine clearly would have the potential to affect the ns-LTP–fungal pathogen interaction due to different spatial organization, possibly altering the net cationic charge of the domain. Development of a series of ns-LTP mutational variants possessing altered cationic amino acid residues, expressed in *Pichia* spp., would be a useful strategy in resolving the mechanism of differential toxicity of ns-LTP to different fungal pathogens.

Some elicitors such as cryptogin, known as elicitors of plant defense mechanisms, share some structural and functional properties with ns-LTP; both are able to load and transfer lipids (Buhot et al. 2001). One ns-LTP purified from wheat seed has been shown to interact with plasma membrane receptors that previously have been identified as elicitor receptors (Blein et al. 2002; Buhot et al. 2001). These receptors could be associated to a general mechanism involving ns-LTP in the effecting plant defense responses. The structure of the receptors and the ligands for ns-LTPs remains to be elucidated (Buhot et al. 2001). Interestingly, when the defense signaling compound jasmonic acid formed a complex with a tobacco LTP1, this complex bound more strongly to plasma membrane receptors to induce a protective response to *Phytophthora parasitica* compared with an LTP1-linolenic acid complex or with LTP1 and jasmonic acid alone. Thus, it is possible that jasmonic acid directly participates in the defense reaction by binding to ns-LTP, and this complex directs an induced protective response against an invading pathogen (Buhot et al. 2004). It is not known yet whether wheat LTP also can bind jasmonic acid.

This comprehensive study has simultaneously considered the phylogeny, fatty acid binding ability, and in vitro toxicity of a large number of members of the ns-LTP family within a species. We demonstrated a differential in vitro toxicity of the wheat ns-LTP to a broad range of host and nonhost fungal pathogens and this toxicity was related to induced permeability changes in the fungal membranes. The ns-LTP TaLt19C10 was expressed during both low-temperature (abiotic) stress (Gaudet et al. 2003) and the incompatible wheat-bunt interaction (Lu et al. 2005a), suggesting that the same ns-LTP can be induced under both abiotic and biotic stresses. Analysis of the promoter regions for several of the wheat ns-LTP demonstrates the presence of *cis*-acting elements homologous to known elicitor-, abscisic acid-, jasmonic acid-, salicylic acid-, drought-, and cold-responsive elements (J.-Y. Sun, D. A. Gaudet, M. Frick, and A. Laroche, *unpublished results*). Thus, collectively, the different inducible and constitutively expressed ns-LTP may play a role in an enhanced generalized nonspecific defense re-

sponse induced during abiotic and biotic stresses that provide interim protection against a wide variety of potential pathogens. Because ns-LTP also are constitutively expressed (Gaudet et al. 2003), it is possible that ns-LTP contribute to nonhost resistance. The ns-LTP antifungal properties and their possible mode of action in the present study were based on in vitro experiments. Overexpression and silencing studies of specific ns-LTP in wheat will be central to establishing the role of wheat ns-LTP in plant defense responses and the amount required for effective protection. Those with broad inhibitory activity may be useful in developing wheat cultivars exhibiting resistance to several wheat pathogens.

MATERIALS AND METHODS

Sequence analysis of wheat ns-LTP cDNA genes.

Eleven different full-length ns-LTP genes were obtained from two SSH cDNA libraries involving wheat infected with *Tilletia tritici* (Lu et al. 2005a) or subjected to low-temperature stress (Gaudet et al. 2003). The cDNA clones were sequenced on an ABI Prism 377 DNA sequencer using the ABI Prism BigDye Terminator sequencing kit (Applied Biosystems, Inc., Foster City, CA, U.S.A.) with M13 forward or reverse primers, according to the manufacturer's instructions. DNA sequence analysis was performed using BLAST at the National Center for Biotechnology Information (NCBI) website services, European Molecular Biology Laboratory European Bioinformatics Institute (EMBL-EBI) website network services, and DNATools software (dnaTools, Inc., Ft. Collins, CO, U.S.A.). Phylogenetic analysis of those sequences subsequently was performed through comparison with sequences at EMBL-EBI website network services and the TreeView software program. Protein tertiary structure analysis was performed by using the RasMol 2.7.3 software program (Molsoft L.L.C., Inc., La Jolla, CA, U.S.A.).

Vector construction.

The coding regions of eight wheat ns-LTP TaBs108F7 (GenBank accession number DN551584), TaLt19C10 (GenBank accession number DQ465679), TaLt709L6 (GenBank accession number CK166650), TaLt710H24 (GenBank accession number CK166832), TaBs116G9 (GenBank accession number DN551652), TaLt10F9 (GenBank accession number DQ465676), and TaLt10B6 (GenBank accession number DQ465677) from our laboratory, and an ns-LTP2 (GenBank accession number BQ241565, supplied by S. Cloutier, Cereal Research Centre, Winnipeg, Canada) were generated by polymerase chain reaction (PCR) and cloned into the yeast expres-

Fig. 10. Timecourse uptake of SYTOX Green by **A**, *Fusarium graminearum* and **B**, *Tilletia tritici* spores following treatment with nonspecific lipid transfer proteins (ns-LTP) TaBs108F7 and TaLt19C10, respectively. Spores were treated with 10 μ M ns-LTP for the indicated times. After the addition of SYTOX Green, fluorescent spores were visualized and counted under a fluorescence microscope. Results were expressed as percentage of fluorescent spores in relation to total spores. Error bars represented the standard deviations. A range of 50 to 138 spores was examined for each repetition. Values were the means of three independent experiments.

sion vector pPICZ α A or pPICZ α B (Invitrogen Canada Inc.). Ns-LTP cDNA sequences were inserted into the alcohol oxygenase locus of pPICZ α vector of the methanotrophic yeast *Pichia pastoris*, whereby the expression of ns-LTP was induced by the addition of methanol to the growth medium buffered methanol-complex medium (BMMY). All ns-LTP-pPICZ α constructs were verified for accuracy by DNA sequencing. These constructed vectors included an N-terminal signal peptide and C-terminal *c-myc* epitope and polyhistidine tag to facilitate purification and identification of ns-LTP. A control vector pPICZ α A, containing the N-terminal signal peptide, and the C-terminal *c-myc* epitope and polyhistidine tag without the ns-LTP sequence, also were transformed into *Pichia pastoris* to determine whether the resulting peptide was toxic to fungi.

Expression and purification of ns-LTP.

Linearized ns-LTP-pPICZ α s and pPICZ α A digested by *Bst*X I (New England Biolabs, Ipswich, MA, U.S.A.) were electroporated into *Pichia pastoris* strain GS115 competent cells according to the manufacturer's instruction (Invitrogen Canada, Inc.). Electroporated yeast cells were spread on yeast extract peptone dextrose sorbitol agar plates containing different amounts of zeocin (0.5 to 2 mg/ml) for selection as reported by Lu and associates (2005b). Plates were incubated at 30°C for 2 to 4 days. Resistant clones that grew on elevated concentrations of zeocin were screened and used to express the fused ns-LTP and the *c-myc*-histidine tag peptide. Resistant clones were further screened for high levels of expression of wheat ns-LTP and *c-myc*-histidine tag peptide by Western blot using polyclonal anti-*c-myc*-antibodies (Invitrogen Canada, Inc.). The clones expressing the highest levels of each ns-LTP and *c-myc*-histidine tag peptide were chosen for subsequent protein expression studies. The supernatant of expressed fused ns-LTP was collected by centrifugation and the culture medium BMMY was substituted with 20 mM imidazole, 0.5 M NaCl, and 20 mM phosphate, pH 7.4 (binding buffer) by centrifugation using Centricon Plus-70 centrifugal filters (Millipore, Billerica, MA, U.S.A.). The supernatant was loaded on an affinity chromatography Ni-NTA resin column (GE Healthcare, Baie d'Urfé, QC, Canada) and the purified protein was eluted with 8 ml of 500 mM imidazole, 0.5 M NaCl, and 20 mM phosphate, pH 7.4 (elution buffer) according the manufacturer's instructions. Due to its small size, it was not possible to use a centrifugal filter to concentrate the empty vector used as the control. Consequently, an ammonium sulfate precipitation step was employed, in which 460 g of ammonium sulfate was added gradually into 600 ml of supernatant containing *c-myc*-histidine tag peptide by gently stirring at 25°C until the ammonium sulfate was completely dissolved. After all the ammonium sulfate dissolved, it was stirred for another 2 h at 4°C. The saturated ammonium sulfate supernatant was stored at 4°C overnight, then centrifuged at 4,400 $\times$ g for 2 h. The supernatant was decanted and the precipitate dissolved in 20 ml of the above binding buffer, loaded on an affinity chromatography Ni-NTA resin column, and eluted as above. The final protein concentration was determined using the Protein Assay Kit (Bio-Rad Laboratories [Canada] Ltd., Mississauga, ON, Canada).

SDS-PAGE and Western blotting of ns-LTP.

Wheat ns-LTP were dissolved in 2 $\times$ loading buffer containing 50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue (wt/vol), and 0.1 M DTT, and separated by SDS-PAGE (Mini-Protein II; Bio-Rad Laboratories [Canada] Ltd.) using 15% separating gel at 200 V for 1 h. Proteins then were transferred to 0.1- μ m-pore-sized Protran nitrocellulose transfer membrane (Schleicher & Schuell BioScience Inc.,

Keene, NH, U.S.A.) using a Bio-Rad mini Trans-Blot Cell at 200 mA for 35 min. The filters were incubated with a 1:3000 dilution of anti-myc-antibodies (Invitrogen Canada, Inc.) and the bands were visualized with goat anti-rabbit immunoglobulin-G-AP according to Brodzik and associates (2000).

Lipid-binding assay.

The purified ns-LTP proteins in their elution buffer were substituted with 8 mM 2-mercaptoethanol and 10 mM morpholinepropanesulfonic acid, pH 7.2, using a Centricon YM-10 centrifugal filter device (Millipore). Different concentrations (from 0 to 30 μ M) of the fatty acid analog D3821 (Invitrogen Canada, Inc.) from a 1-mM stock solution in ethanol were incubated with the purified 10.0 μ M ns-LTP (final concentration), 8 mM 2-mercaptoethanol, and 10 mM MOPS, pH 7.2, and placed in a fluorometer cuvette for fluorescence-binding assay according to Zachowski and associates (1998). To determine whether reduced ns-LTP retained their lipid-binding activity, the in vitro binding activity of the fused ns-LTP (TaLt709L6 and TaBs116G9) in 8 mM 2-mercaptoethanol, 10 mM MOPS, and 20 mM of dithiothreitol (DTT), pH 7.2, also were tested. The solutions of protease, lysozyme, and *c-myc*-histidine tag peptide in 8 mM 2-mercaptoethanol and 10 mM MOPS, pH 7.2, were used as control. The excitation wavelength employed was 490/10 nm and the emission spectrum was recorded at 520/10 nm on a Perkin-Elmer LS-5 spectrofluorometer to measure the relative fluorescence value. The relative fluorescence value of 200 μ l of 10 mM MOPS, pH 7.2, and 8 mM 2-mercaptoethanol, and 0.05 μ l of 1 mM D3821 containing different concentrations of ethanol (0 to 50%) was determined as above to evaluate fluorescence quenching by alcohol.

Inhibition tests.

Inhibition tests of ns-LTP were evaluated on 11 wheat and nonwheat pathogens: the wheat biotrophic pathogens *Puccinia graminis* (WPG-03), *Puccinia triticina* (WPG LR01), and *Tilletia tritici* (race T1); the wheat necrotrophic pathogens *Fusarium graminearum* (N2), *Pyrenophora tritici-repentis*, *Stagonospora nodorum*, *Typhula incarnata* (LRC-034), and an unnamed LTB (LRC-013); and the nonwheat fungal pathogens *Botrytis cinerea* (LRC-2421), *Sclerotinia sclerotiorum* (LRC-2148), and *Verticillium dahliae* (LRC-2165).

Growth inhibition was determined on both liquid and solid growth media. On solid media, a standard quantity of 100 μ l of 10 μ M ns-LTP was added to 0.5-cm Whatman no. 1 filter paper disks and placed on 10 ml of PDA in 100-by-15-mm petri plates adjacent to a growing colony or among germinating spores, and directly rating the inhibition of growth of spores or mycelia compared with that of the water controls. The rating scale was developed to evaluate overall growth inhibition ranged from – = no inhibition to ++++ = very strong inhibition. Because *Puccinia graminis*, *Puccinia triticina*, and *Tilletia tritici* are obligate biotrophs, their inhibition by the ns-LTP could not be assessed by radial growth on PDA or in liquid PDB culture. Consequently, paper disks containing ns-LTP were placed in petri plates containing 2% water agar and sprayed with a suspension of uredospores or teliospores. Inhibition of germination and radial extension of hyphae or promycelium was evaluated after 5 days of incubation at 15°C for *Tilletia tritici* and after 2 days at room temperature for *Puccinia graminis* and *Puccinia triticina*. Growth inhibition of mycelium and spores also was evaluated in 30 μ l of 10 μ M ns-LTP added into 150 μ l of PDB in 96-well plates; growth at 22°C was measured as the daily increase in absorbance at 570 nm over 5 to 6 days, except for the snow mold fungi LTB and *Typhula incarnata* grown at 15°C for which absorbance was recorded daily for 8 days. The toxicity of the reduced ns-LTP

(TaL709L6 and TaBs116G9) solutions with 20 mM DTT to fungi *Fusarium graminearum* (N2), LTB (LRC-013), *B. cinerea* (LRC-2421), *Sclerotinia sclerotiorum* (LRC-2148), and *Verticillium dahliae* (LRC-2165) also was assayed on PDA medium. The elution buffer from the affinity chromatography purification step and the *c-myc*-histidine tag peptide solution were used as control for radial growth and liquid growth studies.

For determination of LD₅₀ of fungal growth on solid or spore germination on liquid media, a series of dilutions for the ns-LTP TaBs108F7, TaL719C10, and TaBs116G9 were made starting at approximately 15 µM, the average maximum concentration of the individual LTP obtained following purification. Growth inhibition tests for determination of LD₅₀ values were as described above for individual fungi that included *Puccinia graminis*, *Tilletia tritici*, *Fusarium graminearum*, the LTB fungus, *Sclerotinia sclerotiorum*, and *Verticillium dahliae*. All the tests were repeated at least three times.

Assay for fungal membrane permeabilization.

Mycelia or spores of the fungal strains *Puccinia graminis*, *Tilletia tritici*, *Fusarium graminearum*, the LTB fungus, *Sclerotinia sclerotiorum*, and *Verticillium dahliae* were treated with 10 µM (final concentration) ns-LTP TaBs108F7, TaL719C10, and TaBs116G9 or water as a control. ns-LTP were mixed with SYTOX Green (Invitrogen Canada, Inc.) to a final concentration of 1 mM according to Giudici and associates (2004) and immediately viewed at ×500 magnification with an Olympus BX80 microscope using a HBO103W/2 light source and the U-MWU filter combinations consisting of a 330-385 excitation filter, a 400-nm chromatic beam splitter, and a 420 barrier filter. For *Puccinia graminis*, *Fusarium graminearum*, the LTB fungus, *Sclerotinia sclerotiorum*, and *Verticillium dahliae*, the percentage of fungal cells in mycelium or spores possessing fluorescent nuclei in the LTP treatments and water controls were determined at 5-min intervals for 30 min. Because of the thick-walled, impermeable nature of *Tilletia tritici* teliospores (Hoffmann 1982), the assay for percent cells possessing fluorescent nuclei was determined at daily intervals for 6 days.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

NCBI website: www.ncbi.nlm.nih.gov/BLAST/
 dnaTOOLS software: www.dnatools.com
 EMBL-EBI website: www.ebi.ac.uk/clustalw/
 Glasgow's Treeview software:
taxonomy.zoology.gla.ac.uk/rod/treeview.html
 Swiss Federal Institute of Technology Zurich Computer Science
 Department website: www.isg.inf.ethz.ch/bin/view/Main