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Isolation and Expression of a Host Response Gene Family Encoding Thaumatin-Like Proteins in Incompatible Oat-Stem Rust Fungus Interactions

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Four cDNA clones (corresponding to *tlp-1*, *-2*, *-3*, and *-4* genes) encoding thaumatin-like (TL), pathogenesis-related proteins were isolated from oat (*Avena sativa*) infected by an incompatible isolate *Pga*-1H of the oat stem rust fungus (*Puccinia graminis* f. sp. *avenae*). All four cDNA clones contained an open reading frame predicted to encode a 169-amino acid polypeptide with a signal peptide of 21 amino acids at the N-terminus, suggesting that these proteins are transported through a secretory pathway. The amino acid sequences revealed high homology among the four cDNA clones, 80 to 99% identity and 86 to 100% similarity. The *tlp* genes and several TL protein genes of certain cereals are clustered into a small group that is phylogenetically separate from the major group of TL protein genes of several plant species. In plants infected with the incompatible isolate *Pga*-1H, or an inappropriate isolate *Pgt*-8D of *P. graminis* f. sp. *tritici*, high levels of *tlp* gene transcripts accumulated at 42 to 48 h AI and thereafter when hypersensitive host cell death occurred and hyphal growth was inhibited, whereas in plants infected with a compatible isolate *Pga*-6A, relatively lower amounts of transcripts were detected. Overall, transcript levels were higher with *tlp-1* than with the three other genes. Spray with a light mineral oil used as a spore carrier induced transient expression of *tlp-1*, *-2*, and *-3* genes at 16 to 30 h AI which obscured the initial induction of the *tlp* genes in response to infection by the pathogens. In contrast, *tlp-4* was induced very little by oil spray, so that induction was clearly observed in response to either compatible, incompatible, or inappropriate isolates at 24 to 30 h AI. Wounding leaves by either slicing or puncturing them strongly induced *tlp-1* and *tlp-3*, moderately induced *tlp-2*, but had no effect on *tlp-4*. Taken together, the results showed that *tlp* genes displayed differential responses to oil spray, mechanical wounding, and pathogen infection and that the expression of *tlp* genes, especially *tlp-1*, in oat is associated with resistance reactions in response to infection by incompatible and inappropriate isolates of the stem rust fungi.

Additional keywords: gene-specific probe, phylogenetic analysis.

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Resistance genes are postulated to trigger the induction of host response genes whose products participate in disease resistance (Lamb 1994; Freialdenhoven et al. 1994; Staskawicz et al. 1995). A wide variety of host response genes are induced in plants in response to pathogen attack (Bowles 1990; Bol and Linthorst 1990). The products of many of these host response genes have direct antifungal activities, such as chitinase and β-1,3-glucanase, or are involved in biosynthesis of compounds with antifungal activity, including phytoalexins, or in cell wall thickening and lignin synthesis. One group of these induced proteins, known as pathogenesis-related (PR) proteins, has been induced in several plant species in response to pathogen infection, stress, or developmental signals (Van Loon 1985 and 1987; Bol and Linthorst 1990; Cutt and Klessig 1992). The PR proteins in tobacco infected by tobacco mosaic virus (TMV) have been classified into five families (Cutt and Klessig 1992): PR-1, function unknown; PR-2 and PR-3, β-1,3-glucanase and chitinase activity, respectively; PR-4, function unknown; and PR-5, having amino acid sequences similar to thaumatin, the sweet-tasting proteins isolated from fruit of the West African shrub, *Thaumatococcus daniellii*, designated also as thaumatin-like (TL) proteins (Cornelissen et al. 1986).

Whereas some TL proteins are constitutively produced in seeds (Roberts and Selitrennikoff 1990; Hejgaard et al. 1991; Vigers et al. 1991; Malehorn et al. 1994), TL proteins have been reported to be induced in several plant species by pathogens, e.g., TMV (Cornelissen et al. 1986; Pierpoint et al. 1987), *Rhynchosporium secalis* (Hahn et al. 1993), *Erysiphe graminis* (Schweizer et al. 1989; Bryngelsson and Green 1989), *Pseudomonas syringae* (Uknes et al. 1992; Reimmann and Dudler 1993), and by other stimuli, e.g., salicylic acid (Uknes et al. 1992; Jung et al. 1993), 2,6-dichloroisonicotinic

acid (Uknes et al. 1992), salt (Singh et al. 1987), abscissic acid (Singh et al. 1989), ethylene and methyl jasmonate combination (Xu et al. 1994), wounding (Brederode et al. 1991; Neale et al. 1990), and UV light (Brederode et al. 1991). In most cases, the induction of TL protein genes is correlated either with reduced pathogenesis or associated with systemic acquired resistance.

Although the exact role of TL proteins in disease resistance is unclear, TL proteins with antifungal activity have been purified from seeds of several plant species, including zeamatin from maize (Roberts and Selitrennikoff 1990), zeamatin-like proteins from maize and other cereals (Vigers et al. 1991; Malehorn et al. 1994), AP24 from tobacco (Woloshuk et al. 1991), and PR-R and PR-S from barley (Hejgaard et al. 1991). These TL proteins can inhibit hyphal growth of *Candida albicans*, *Neurospora crassa*, *Trichoderma reesei*, *T. viride*, or *Phytophthora infestans*. Overexpression of a gene for osmotin, a TL protein which accumulates in salt-adapted tobacco cells, delays the development of disease symptoms caused by *P. infestans* in transgenic potato, but not the devel-

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pCRL101  .CCATA.... ACAAGCTCGG C.CACAGCA. ....AGCAAC AGTAGC.AAA GCTTGTAGCA 48
pCRL102  C-----.... G--------- -A-------A CACT-----A GC-T--T-G- ---------G 56
pCRL103  .......... .........- -.-------A CAGT-----A GC-T--T--- ---------G 41
pCRL104  C-----CCTA G-------.- AA--A---GA AACT-A-C-. GC----T..- -----C---- 56

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pCRL101  ATGGCGACCT CCTCCGCGGT GCTGTTTCTG CTGCTCGCCG TTTTCGCCGC CGGTGCCAGC 108
pCRL102  ---------- ---------- -------T-C --C------- -C-------- ---------- 116
pCRL103  ---------- ---------- ---C-----C --C------- -C-------- ---------- 101
pCRL104  ---------- -----A---- ---T-----C --C--T---- ---------- -A-------- 116

pCRL101  GCGGCCACCT TCCGCATCAC CAACAACTGC GGCTTCACGG TGTGGCCGGC GGGCATCCCG 168
pCRL102  ---------- ---------- ---------- ---------- ---------- ---------- 176
pCRL103  ---------- ---------- ---------- ---------- ---------- ---------- 161
pCRL104  ---------- --AC------ ---------- ----A----- ---------- --C------A 176

pCRL101  GTGGGCGGAG GCTTCCAGCT CAACTCGAAG CAGTCGTCCA ACATCAACGT GCCCGCGGGC 228
pCRL102  ---------- ---------- ---------- ---------- ---------- ---------- 236
pCRL103  --------G- ---------- ---------- ---------- ---------- ---------- 221
pCRL104  --------C- --CAG----- TG--CA-GGC ---A---GG- C-C------- ---T--C--- 236

pCRL101  ACCAGCGCCG GCAGGATATG GGGCCGCACC GGCTGCTCCT TCAACAACGG GAGAGGGAGC 288
pCRL102  ---------- ---------- ---------- ---------- ---------- ---------- 296
pCRL104  ---------- -T-------- ---------- --G------- ---------- ---------- 281
pCRL104  ----A-T--- ---------- ------T--- --G------- -----GG--- A--C------ 296

pCRL101  TGCGCGACCG GCGACTGCGC CGGCGCGCTG TCCTGCACCC TCTCCGGGCA GCCGGCGACG 348
pCRL102  ---------- -A-------- ---------- ---------- ---------- ---------- 356
pCRL103  ---------- ---------- ---------- ---------- ---------- ---------- 341
pCRL104  ---CA----- ---------- ---------- ---------T -A-------- ---------- 356

pCRL101  CTGGCCGAGT ACACCATCGG CGGCTCCCAG GACTTCTACG ACATTTCGGT GATCGACGGC 408
pCRL102  ---------- ---------- ---------- ---------- ----C----- ---------- 416
pCRL103  ---------- ---------- ---------- ---------- ----C----- ---------- 401
pCRL104  ---------- T--G------ ----GAG--C ----A----- ---------- --------T- 416

pCRL101  TTCAACCTCG CCATGGACTT CTCCTGCAGC ACCGGCGTCG CGCTCAAGTG CAGGGATGCC 468
pCRL102  -A-------- ---------- ---------- ---------- ---------- ---------- 476
pCRL103  -A-------- ---------- ---------- --------G- ----T----- -------T-- 461
pCRL104  -A-------- ---------- ---------- -------AT- -----C---- -------T-- 476

pCRL101  AACTGCCCCG ATGCCTATCA CCACCCCAAC GATGTCGCCA CGCACGCCTG CAACGGCAAC 528
pCRL102  ---------- -C-------- ---------- --C------- -------T-- ---------- 536
pCRL103  GG-------- ---------- ---------- --C------- ---------- ---------- 521
pCRL104  -G-------- ---------- ---A---G-- --CCCAAAA- -C---T-A-- ----AC---- 536

                                    ***
pCRL101  AGCAACTACC AGATCACCTT CTGCCCATGA AG.......G CCAC.CCGCC AATAAACGGC 580
pCRL102  ---------- ---------- ---------- --ACCCTAT- --G-G----- -----C---- 596
pCRL103  ---------- ---------- ---------- --ACCTTAC- --G-G--A-- ---------. 580
pCRL104  ---------- ---------- ---------- -.ACCCTAC- T-G-G-A--- ------.... 591

pCRL101  GCGCGTATAT ACGACCGTAT AAATAGTGTA AACTGTGTAA TGCTTACATC GCGGTATCGT 640
pCRL102  -TA-..---- --------.. ---------- ---------- ---------- --------A- 654
pCRL103  .......... .......... .......... ..-------- ---------- ---------- 608
pCRL104  .......... T--------- ---------- ....--A-G- -A-A-.---A -T-C----.. 629

pCRL101  ATATCTGTAT TCCAGCCGTT .......... ..GT....AG T.......... ....TG..AC 669
pCRL102  ---------- ---------- GT........ .A--....-- -.......... ....--.--- 686
pCRL103  ---------- ---------- .......... ..-.....-- -.......... ....--.--- 637
pCRL104  ..-------- --T------- GTGCATGTGC AT--GCTG-- -GCTATCATC ATAG--CT-T 687

pCRL101  AAGCGG...C C.A.AATAAA GTTCAATAAA GACGGTGCAC ACATGTGTGC ATGTCGACGT 724
pCRL102  --A---...- -.-.------ ---------- ---------- ---------- ---------- 741
pCRL103  ------...T -.-.------ ---------- --G-A----- -----C--C- ------.... 688
pCRL104  C-T-T-TATT -T-TC----- ---A------ T-.....-A T-GA-G---- ----.-CT-A 740

pCRL101  TATCTATTTA .... 734
pCRL102  ---------- AAA. 754
pCRL103  .......... .... 688
pCRL104  GTG-A-AAA- AAAA 754
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Fig 1. Alignment of nucleotide sequences of four cDNA clones encoding thaumatin-like proteins. The nucleotide sequences which were identical to pCRL101 are shown as dashes. Dots represent gaps introduced for optimal alignment. The asterisks denote the start and stop codons for translation. Three potential polyadenylation signals are indicated as rectangles. Nucleotide regions corresponding to oligonucleotides used for gene-specific probes are underlined. The nucleotide sequences were aligned using the "pileup" program from the GCG Package (Genetics Computer Group, Inc. Wisconsin). The nucleotide sequences have been submitted to the Genbank library as accession numbers L39774, L39775, L39776, and L39777 for pCRL101, 102, 103, and 104, respectively.

opment of symptoms caused by *P. parasitica* var. *nicotianae* in transgenic tobacco (Liu et al. 1994). Linthorst et al. (1989) found that constitutive expression of PR-5 in transgenic tobacco does not alter the susceptibility of transgenic plants to infection by TMV and suggested that TL proteins need to work cooperatively with other host response gene products in order to contribute to disease resistance.

Puccinia graminis Pers. f. sp. *avenae* Eriks. and E. Henn. is the causal agent of stem rust on oat (*Avena sativa* L.). Resistance to the oat stem rust fungi is race-specific and follows the gene-for-gene hypothesis (Stakman et al. 1923; Flor 1947; Martens et al. 1969). Limited information is available on physiological, biochemical, and molecular factors which mediate the resistance of oat against the oat stem rust fungi. In a survey of mRNA accumulation patterns of heterologous host response genes in oat in response to infection by the stem rust fungi, transcripts with homology to WIR2 (Schweizer et al. 1989; Rebmann et al. 1991), a TL protein gene from wheat, accumulated to high levels in incompatible host-parasite combinations (K.-C. Lin and W. R. Bushnell, unpublished). The temporal expression patterns coincided well with the onset of resistance reactions determined by both inhibition of fungal growth and the hypersensitive response. Because of the possible involvement of TL protein genes in disease resistance, we undertook to clone and characterize such genes from infected, resistant oat. Here we report the isolation of four cDNA clones which encode proteins with amino acid sequences similar to those of the TL proteins and demonstrate that the induction of the genes encoding these cDNA clones is associated not only with resistance reactions in oat against stem rust fungi but also with reactions in response to oil spray and physical wounding. A preliminary report of this research has been presented in abstract form (Lin et al. 1995).

RESULTS

Isolation and characterization of cDNA clones homologous to WIR2.

Total RNA was extracted from Rodney (*Pg-2*) at 36, 42, 48, 54, and 60 h after inoculation (AI) by an incompatible isolate, *Pga*-1H, of the oat stem rust fungus. Total RNA was pooled and used for poly(A)$^+$RNA isolation. A cDNA library was constructed from the isolated poly(A)$^+$RNA. Approximately 5,000 plaque-forming units of the cDNA library were screened by using WIR2 (Schweizer et al. 1989; Rebmann et al. 1991) as a heterologous probe. After three cycles of screening, 11 cDNA clones with strong hybridization signals were purified for further analysis. Based on partial DNA sequences of both the 5′ and 3′ ends, these clones were grouped into 4 classes. The cDNA clones with the longest insert from each class were selected for further DNA sequence analysis and designated as pCRL101, pCRL102, pCRL103, and pCRL104. The genes corresponding to the four cDNA clones were designated as *tlp-1*, *-2*, *-3*, and *-4* (thaumatin-like proteins).

The entire nucleotide sequences of the four cDNA clones are shown in Figure 1, whereas Table 1 shows the principal characteristics of the four cDNA clones. The sizes of the four cDNA clones range from 688 to 754 bp, somewhat smaller than the corresponding mRNAs (approximately 900 nucleotides). These cDNA clones contain complete coding regions with translational initiation and stop codons as well as multiple copies of the polyadenylation signal, AATAAA. Nucleotide sequences for the coding regions among pCRL101, pCRL102, and pCRL103 had identity of 97% or higher, and approximately 87% identity was found between pCRL104 and the other three cDNA clones. The high percentages of identity indicate that all four cDNAs are very similar. All four cDNA clones contain a single open reading frame of 507 bp which encodes a putative polypeptide of 169 amino acids with a molecular weight range from 17.3 to 17.6 kDa. These polypeptides were designated as TLP-1, -2, -3, and -4. The amino acid sequences among TLPs are highly homologous to one another with 80 to 99% identity and 86 to 100% similarity. The first 21 amino acids in the N-terminus of the four polypeptides have the typical features of a signal peptide, including length, a hydrophobic core, and a potential cleavage site (Von Heijne 1983), suggesting that these polypeptides are synthesized as proproteins and that the signal peptides are processed during translocation. The molecular weights of the mature proteins range from 15.3 to 15.6 kDa with predicted pI values of 6.7 for TLP-1, -2, and -3, and 4.2 for TLP-4. Based on comparison of DNA sequences, protein sequences, and pI values, *tlp-1*, *-2*, and *-3* are more similar to each other than to *tlp-4* (Table 1).

A search for proteins with amino acid sequences similar to TLP-1 indicated significant homology (44 to 84% identity and 61 to 89% similarity) to published sequences of thaumatins and other thaumatin-like (TL) proteins as represented by thaumatin II and five TL proteins in Figure 2. Among the amino acid sequences compared, the highest degrees of similarity were found between TLP-1 and PWIR2 from wheat (84% identity and 89% similarity) and PRHv-1 from barley (82% identity and 88% similarity). Less similarity was shown between TLP-1 and thaumatin II or other TL proteins (44 to 53% identity and 61 to 70% similarity). The molecular weights of the mature TLPs (15.3 to 15.6 kDa) were more like those of PWIR2 (15.6 kDa) and PRHv-1 (15.6 kDa) than those of thaumatin II or other TL proteins (20 to 26 kDa),

Table 1. Characteristics of cDNA clones encoding TL proteins

Clone	pCRL101	pCRL102	pCRL103	pCRL104
Corresponding gene	*tlp-1*	*tlp-2*	*tlp-3*	*tlp-4*
mRNA (nt)[a]	900	900	900	900
cDNA (bp)	734	754	688	754
Protein (amino acids)[b]	169	169	169	169
Signal peptide (amino acids)[c]	21	21	21	21
M.W. of proprotein (kDa)[b]	17.4	17.4	17.3	17.6
M.W. of mature protein (kDa)[b]	15.4	15.4	15.3	15.6
Isoelectric point (pI)[b]	6.2	6.2	6.2	4.1
% identity to DNA[d] sequence of *tlp-1*	100	98.0	97.1	86.5
% similarity to protein[b] sequence of TLP-1	100	100	98.8	85.8

[a] The size of mRNA was estimated by interpolation from RNA ladders.
[b] Data were predicted from the amino acid sequences using GCG Package (Genetics Computer Group, Inc. Wisconsin).
[c] Data were predicted by using GCG Package and by comparing to other TL proteins.
[d] Data were predicted from the DNA sequences of coding regions of each cDNA clone using GCG Package.

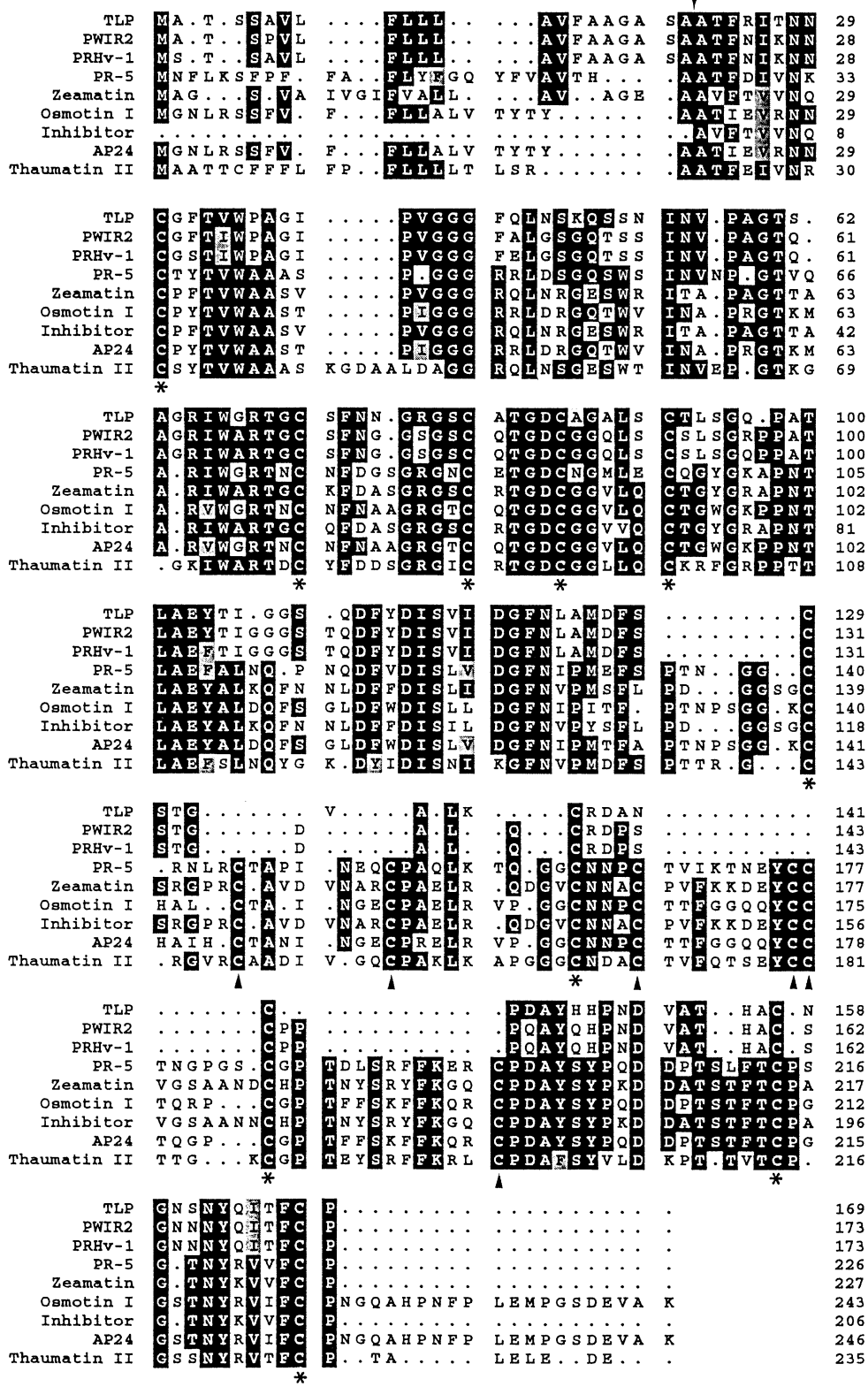

Fig. 2. Alignment of the deduced TLP-1 amino acid sequence with other thaumatin-like (TL) proteins, shown in single letter code, from different plant species in order of decreasing similarity. Identical or similar amino acids were shaded. Dots were introduced for optimal alignment. The arrow denotes the putative cleavage site of N-terminal signal peptide. The positions of cysteine residues conserved in all the proteins are shown with asterisks. Arrowheads indicate the cysteine residues that were present in six TL proteins but not in TLP-1, PWIR2, and PRHv-1. PWIR2, a thaumatin-like, pathogenesis-related protein from wheat infected by barley powdery mildew fungus (Rebmann et al. 1991); PRHv-1, an elicitor-induced thaumatin-like, pathogenesis-related protein from barley (Hahn et al. 1993); PR-5, a TMV-induced, pathogenesis-related protein from tobacco (Cornelissen et al. 1986; Payne et al. 1988); Zeamatin, an antifungal protein from maize seeds (Vigers et al. 1991); Osmotin I, a salt-induced protein from tobacco (Singh et al. 1989); Inhibitor, a bifunctional trypsin/α-amylase inhibitor from maize (Richardson et al. 1987); AP24, an antifungal protein from tobacco (Woloshuk et al. 1991); Thaumatin II, a sweet-tasting protein from *Thaumatococcus daniellii* (Edens et al. 1982). The amino acid sequences were aligned using the "pileup" program from the GCG Package (Genetics Computer Group, Inc. Wisconsin).

mainly due to the lack of 58 amino acids in the internal region near the C-terminus (from position 137 to 199 in thaumatin II; Fig. 2). Thaumatin II and other thaumatins contain 16 cysteine residues which participate in 8 disulfide bonds in the tertiary structure of thaumatin molecules (Ogata et al. 1992; Fig. 2). These 16 cysteine residues were found in the conserved positions of a tobacco PR-5 protein, zeamatin, osmotin I, bifunctional trypsin/α-amylase inhibitor and AP24. In contrast, only 10 of the 16 cysteine residues were found in TLPs, PWIR2 and PRHv-1. The 6 missing cysteines are located in the internal region near the C-terminus of the thaumatin protein which is lacking in the TLPs, PWIR2 and PRHv-1.

Phylogenetic analysis of 24 aligned amino acid sequences was used to compare further the relationship between TLPs and other TL proteins. Parsimony analysis showed that TLPs grouped with PRHv-1 and PWIR2 and that this group was distinct from the other TL proteins (Fig. 3). The separation of these clusters was well supported by bootstrap analysis, with 100% of trees in 1,000 bootstrap replicas showing this separation. To determine whether the distinction between these two groups is due solely to the presence or absence of this 58 amino acid block, the parsimony analysis was repeated with this region deleted from all the sequences. Similar results were obtained, indicating that the distinction between these two groups is not dependent on the presence or absence of this amino acid block.

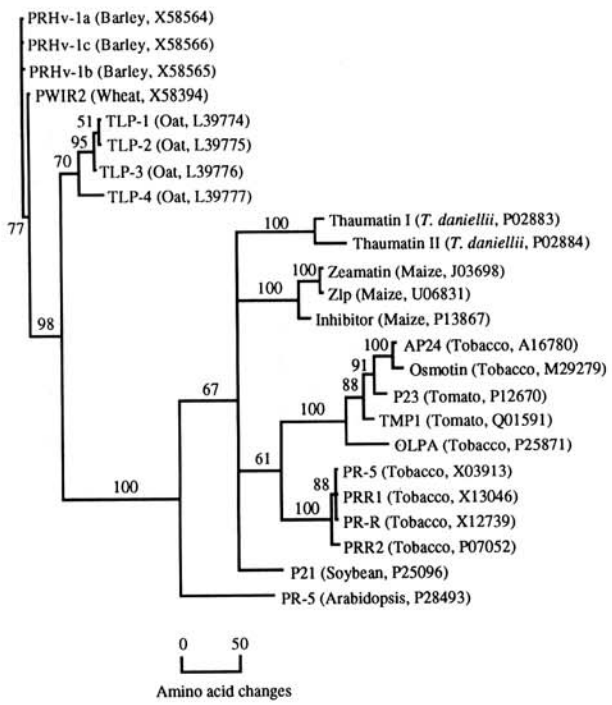

Fig. 3. Parsimony analysis of TLPs and 21 other thaumatin-like proteins based on amino acid sequence data. The amino acid sequences were aligned using the "pileup" program from the GCG package (Genetics Computer Group, Inc., Wisconsin) and manually adjusted to obtain optimal alignment. Parsimony analysis was performed using the heuristic search option of PAUP (Swofford 1993). For each protein, the plant source and accession number are listed in parentheses. The number on each branch represents the percentage of 1,000 bootstrap replicas supporting the branch. The scale bar represents the numbers of amino acid substitutions to obtain branch lengths.

DNA blot analysis of oat.

To estimate the copy number of *tlp* genes in the oat genome, DNA blot analysis was carried out with genomic DNA isolated from uninoculated Rodney (*Pg-2*) using the pCRL101 cDNA insert as a probe. This probe hybridized to 8 to 14 bands of genomic DNA depending on the restriction enzyme used (Fig. 4). No change in hybridization pattern or intensity was detected using high versus low stringency wash conditions (data not shown). DNA blot analysis using pCRL104 cDNA insert as a probe revealed identical hybridization patterns to those detected by the pCRL101 cDNA insert but with differences in intensity of some bands (data not shown).

Gene expression in response to infection by *P. graminis*.

To determine whether *tlp* genes were induced in response to infection by *P. graminis*, 8-day-old seedling leaves of Rodney (*Pg-2*) were mock-inoculated with mineral oil, or inoculated with a compatible isolate *Pga*-6A, an incompatible isolate *Pga*-1H of *P. graminis* f. sp. *avenae*, and an inappropriate isolate *Pgt*-8D of *P. graminis*, and sampled from 16 to 72 h AI at 6-h intervals. Accumulation of *tlp* transcripts in the samples was determined by RNA blot analysis using pCRL101 as a probe as shown in Figure 5; all values were corrected for loading based on hybridization signals for 28S rRNA. Two phases of transcript accumulation were observed. The first

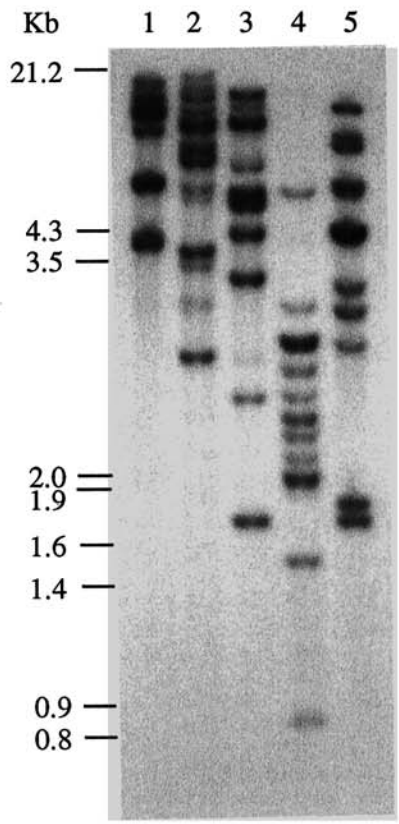

Fig. 4. DNA blot hybridization of oat genomic DNA showing homology with *tlp* genes. Rodney (*Pg-2*) genomic DNA (20 µg/lane) was digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Eco*RV (lane 3), *Ssp*I (lane 4), and *Nsi*I (lane 5), and electrophoresed on a 0.8% agarose gel. The DNA gel was blotted onto a nylon filter and probed with ^{32}P-labeled pCRL101 cDNA insert. Molecular weight markers are shown and denoted in kilobase pairs.

phase (16 to 36 h AI) occurred in plants inoculated with each of the isolates and also in plants mock-inoculated with oil. In this phase, the *tlp* mRNAs started to accumulate at the first sampling time (16 h AI), reached a peak at 24 h, and declined until 36 h. The induction of *tlp* genes during this period was mainly due to oil spray as shown in later experiments (Fig. 7). In the second phase (42 to 72 h AI), relatively small amounts of *tlp* mRNAs were detected in plants either mock-inoculated with oil spray or inoculated with the compatible isolate *Pga*-6A, whereas large amounts of *tlp* gene transcripts accumulated in plants infected by the incompatible isolate *Pga*-1H or inappropriate isolate *Pgt*-8D (Fig. 5B). The amount of *tlp* gene transcripts in plants infected by incompatible *Pga*-1H or inappropriate *Pgt*-8D was two- to threefold the amount of transcripts in plants infected by compatible *Pga*-6A at 42 h and sixfold at 72 h. In this experiment the exact time when *tlp* genes were first induced in the early stage of infection was obscured by *tlp* gene transcripts induced by oil spray. Similar

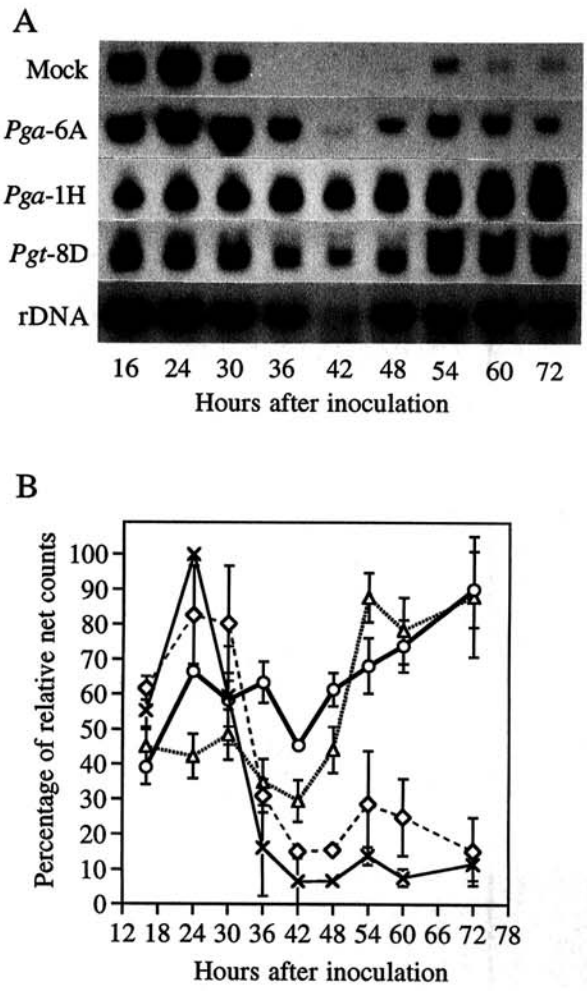

Fig. 5. Accumulation of *tlp* gene transcripts in Rodney (*Pg-2*) 16 to 72 h after inoculation with *Puccinia graminis*. **A,** RNA blot analysis of total RNA isolated from 8-day-old seedling leaves of Rodney (*Pg-2*) inoculated with oil (Mock), a compatible isolate of *P. graminis* f. sp. *avenae* (*Pga*-6A), an incompatible isolate (*Pga*-1H), or an isolate of *P. graminis* f. sp. *tritici* (*Pgt*-8D). Total RNAs (30 µg per lane) isolated from the four treatments were electrophoresed on a single 1.2% agarose formaldehyde gel, blotted onto a nylon filter, and hybridized to ^{32}P-labeled pCRL101 cDNA insert. Autoradiography was carried out for 18 h. Verification of the amount of RNA loaded per lane was obtained by hybridizing the same filter with 28S subunit ribosomal DNA from maize. Only the result from mock-inoculation leaves is shown (rDNA). **B,** Radioanalytic image analysis of RNA filters. Mock (inoculation) (×), *Pga*-6A (diamond), *Pga*-1H (circle), and *Pgt*-8D (triangle). Data plotted as the percentage of net count values for mock-inoculation at 24 h AI. Each value is given as the mean ± standard deviation of three replicates.

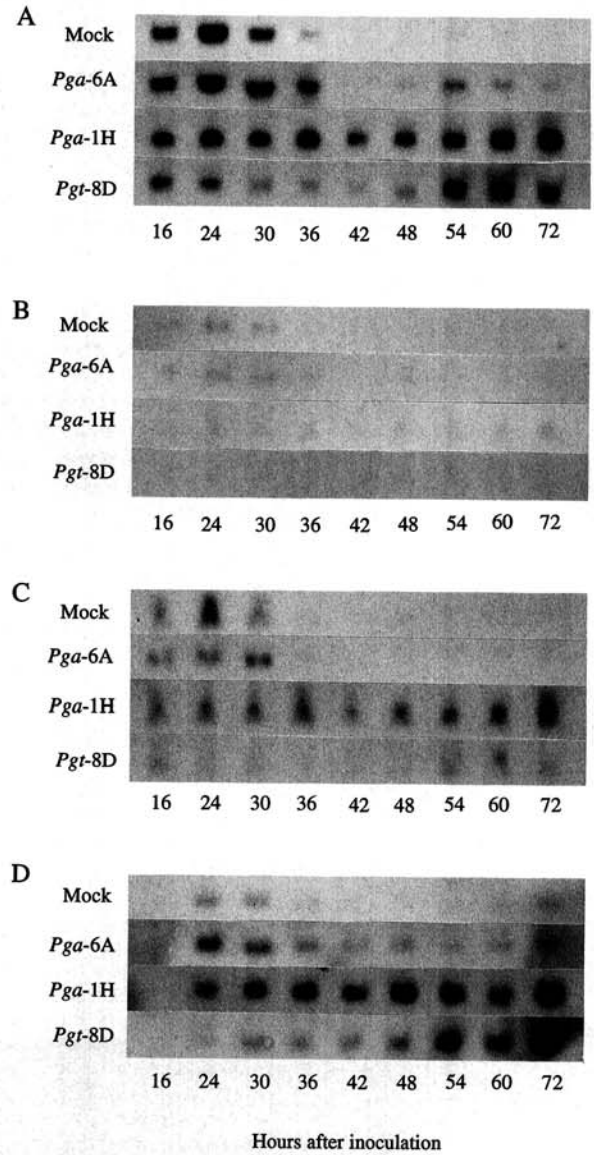

Fig. 6. Accumulation of *tlp-1*, *-2*, *-3*, and *-4* gene transcripts in response to infection by stem rust fungi as determined by RNA blot analyses. Total RNAs were extracted from Rodney (*Pg-2*) inoculated with oil (Mock), *Pga*-6A, *Pga*-1H, and *Pgt*-8D from 16 to 72 h after inoculation with 6-h intervals. Total RNAs (30 µg per lane) isolated from the four treatments were electrophoresed and blotted onto a single nylon filter, and four duplicated filters were made. The four filters were hybridized to the gene-specific, end-labeled oligonucleotides (Fig. 1) corresponding to *tlp-1* (**A**), *tlp-2* (**B**), *tlp-3* (**C**), *and tlp-4* (**D**) at the same time. Autoradiography was carried out at the same time but the X-ray films were exposed for 12 days for *tlp-1*, 28 days for *tlp-2 and -3*, and 21 days for *tlp-4*. Although the signal for *tlp-2* was too weak to be visible in the photograph, temporal expression patterns similar to those of *tlp-3* (**C**) were observed on the X-ray film. The specificity of each probe was confirmed by its strong differential hybridization to the respective cDNAs inserts as shown in Figure 8B.

expression patterns were obtained from a separate experiment which was sampled from 0 to 120 h AI (data not shown).

To determine which *tlp* genes were activated in response to infection by incompatible isolate *Pga*-1H and inappropriate isolate *Pgt*-8D, four gene-specific oligonucleotides corresponding to sequences at either the 5′ or 3′ end of pCRL101, pCRL102, pCRL103, and pCRL104 (Fig. 1) were synthesized and used as probes to hybridize to total RNA from inoculated leaves in RNA blot analyses (Fig. 6). The specificity of hybridization for each probe was confirmed by its differential hybridization to the respective cDNA insert as shown in Figure 8B. Since the gene-specific probes were labeled with radioisotopes only at the 5′ termini, long exposure times were needed for each RNA blot analysis. A preliminary trial with 4-day exposures showed more radioactivity from *tlp-1* than from the other probes which gave little or no signal. To partially compensate for the observed differences in radioactivity, we used exposure time of 12 to 28 days for the analyses of Figure 6. Thus, radioactivity was strongest for *tlp-1* (12 days exposure) and weakest for *tlp-2* (28 days exposure). The weak signal for *tlp-2* was due primarily to low hybridization efficiency since the *tlp-2* oligonucleotide probe carried as much radioactivity as did the other *tlp* probes but gave relatively weak signal when used to probe for *tlp-2* in a control experiment (Fig. 8B).

The temporal patterns of transcript accumulation were generally the same for *tlp-1* (Fig. 6A), *tlp-2* (Fig. 6B), and *tlp-3* (Fig. 6C), except that *tlp-1*, but not *tlp-2* or *tlp-3*, was induced at 36 h after inoculation by compatible isolate *Pga*-6A. On the other hand, the expression patterns for *tlp-4* (Fig. 6D) clearly differed from those obtained for *tlp-1*, *-2*, and *-3* in that no hybridization occurred in any of the samples at 16 h AI and only traces of transcript accumulation were induced by mock inoculation at 24 to 30 h AI. Consequently, the time when transcripts first accumulated in response to fungal attack was not obscured as it had been for *tlp-1*, *-2*, and *-3*. By 24 h AI, *tlp-4* transcripts began to accumulate in response to compatible *Pga*-6A and incompatible *Pga*-1H and by 30 h in response to inappropriate *Pgt*-8D. Thereafter, the patterns resembled those obtained for *tlp-1*, *-2*, and *-3* as expression remained at high levels with incompatible isolate *Pga*-1H and inappropriate isolate *Pgt*-8D but dropped to low levels with the compatible isolate *Pga*-6A.

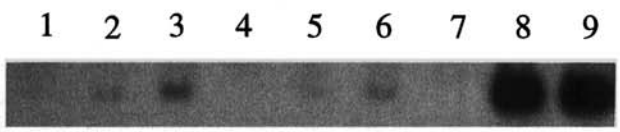

Fig. 7. Accumulation of *tlp* gene transcripts in response to oil spray as shown by RNA blot analysis. Eight-day-old seedling leaves of Rodney (*Pg-2*) were sprayed with oil or air. One group of sprayed leaves was incubated in a dew chamber for 16 h and then transferred to a growth chamber for an additional incubation of 8 h; the remaining sprayed leaves were kept in a growth chamber for 24 h. Unsprayed plants were incubated in the same condition as sprayed plants and used as a control. The leaves were sampled at 0 and 24 h after spray and used for total RNA isolation. Total RNA of 30 µg per lane was separated in a 1.2% agarose formaldehyde gel, blotted onto a nylon filter, and hybridized to ^{32}P-labeled pCRL101 cDNA insert. Lanes 1-3, unsprayed leaves at 0 h, after 24 hr incubation in a growth chamber, and after 24 hr incubation in a dew chamber, respectively; lanes 4-6, air sprayed leaves at 0 hr, after 24 h of incubation in a growth chamber, and after 24 h of incubation in a dew chamber, respectively; lanes 7 to 9, oil sprayed leaves at 0 h, after 24 h of incubation in a growth chamber, and after 24 h of incubation in a dew chamber, respectively. Autoradiography was carried out for 18 h.

Expression of *tlp* genes in response to oil spray and wounding.

The accumulation of *tlp* mRNA in the mock-inoculated plants during the period from 16 to 36 h AI (Figs. 5 and 6) suggested that the induction was due to some factors associated with the inoculation or incubation process. To determine if the responses in mock-inoculated plants at 16 to 36 h were caused by the oil spray or some other aspect of the inoculation or incubation procedure, we compared responses to oil and air spray with and without incubation in the dew chamber after treatment (Fig. 7). As indicated by RNA blots probed with pCRL101 insert, the *tlp* gene transcripts were strongly induced by the oil spray, slightly induced by incubation in the dew chamber, but were not induced by the air spray. Transcripts were more strongly induced by incubation in the dew chamber than in the growth chamber.

To test whether *tlp* genes were activated in response to wounding, 8-day-old seedling leaves of Rodney (*Pg-2*) were mechanically injured by slicing or puncturing. RNA blot analysis, using pCRL101 as a probe, indicated that *tlp* genes were induced by both kinds of wounding but to a higher level in the sliced leaves than in the punctured leaves (data not shown). Transcript accumulation for each *tlp* gene in response to wounding was studied by using the four gene-specific oligonucleotides as probes (Fig. 8). The *tlp-1*, *-2*, and *-3* genes responded to both slicing and puncturing wounds at 24 h after wounding with the highest expression level for *tlp-1* and *tlp-3*. The *tlp-4* gene, however, was not induced by either wounding

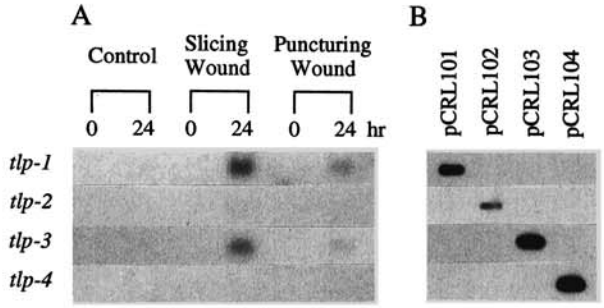

Fig. 8. Accumulation of *tlp-1*, *-2*, *-3*, and *-4* gene transcripts in response to wounding. **A**, RNA blot analysis of total RNA isolated from wounded oat leaves. Eight-day-old seedling leaves were wounded by either slicing into pieces (2 to 3 mm width) with a razor blade or puncturing with a brush (0.5 to 1 mm holes 2 to 3 mm apart). Samples were collected at 0 and 24 h after treatment. Detached leaves without any treatment were incubated with the wounded leaves in the dew chamber for 24 h and used as a control. Total RNA of 30 µg per lane was electrophoresed and blotted onto a nylon filter, and four duplicate filters were made. Each duplicate filter was hybridized to a gene-specific, end-labeled oligonucleotide corresponding to a *tlp* gene (Fig. 1). Autoradiography was carried out for 28 days for each filter. **B**, Specificity of hybridization using gene-specific oligonucleotide probes. The four pCRL cDNA inserts (1 ng each), each corresponding to a *tlp* gene, were loaded on a 1% agarose gel. After electrophoresis, the pCRL cDNA inserts were blotted onto a nylon filter, and four duplicate filters were made. Each duplicate filter was hybridized to a gene-specific probe in the same hybridization bag with a corresponding RNA filter from panel A. Both the DNA and RNA filters were washed together under the same conditions. Autoradiography for DNA filters was carried out for 4 days.

method. With the three gene-specific probes, a higher level of transcripts was detected in the sliced than in the punctured leaves.

DISCUSSION

In this study, four classes of cDNA clones that encode thaumatin-like (TL) proteins were isolated from oat infected by an incompatible isolate, *Pga*-1H, of the oat stem rust fungus by using a heterologous probe, WIR2, a cDNA clone of a gene encoding a TL protein in wheat. The nucleotide sequences of the four cDNA clones revealed high homology to TL protein genes in other plant species. The four genes corresponding to the cDNA clones, *tlp-1*, *-2*, *-3*, and *-4*, were all induced by both incompatible isolate *Pga*-1H and inappropriate isolate *Pgt*-8D from 42 to 48 h after inoculation (AI) and thereafter, with the highest expression level for *tlp-1* as determined using gene-specific probes.

With *tlp-1*, *-2*, and *-3*, the exact timing for the initial induction in response to incompatible *Pga*-1H and inappropriate *Pgt*-8D infection was partially obscured by the response to oil spray. Notably, *tlp* genes were neither expressed constitutively nor induced by oil spray at the time of inoculation (0 h AI) as shown in Figure 7. By 16 to 30 h AI, oil-induced high levels of these *tlp* transcripts in both inoculated and mock-inoculated leaves (Fig. 6). By 36 h AI, as the response to oil declined, levels of *tlp-1*, *-2*, and *-3* were higher in inoculated than mock-inoculated leaves, especially with the incompatible isolate *Pga*-1H (Fig. 6). However, in contrast to these three genes, *tlp-4* was induced very little by oil spray. In this case, a response to infection at 24 to 30 h AI could be seen clearly with plants inoculated with all three isolates; but decreasing at 36 h AI with the compatible isolate, and increasing with the incompatible and inappropriate isolates. We suspect, therefore, that transcript levels of *tlp-1*, *-2*, and *-3* increase at 24 to 30 h AI as did levels of *tlp-4*, declining at 36 h AI and thereafter with the compatible isolate but, as is clear from Figure 6, remaining high and increasing with incompatible isolate *Pga*-1H and inappropriate isolate *Pgt*-8D at 42 to 48 h AI and later. The phenomenon of decreasing transcript level in compatible host-parasite interactions while maintaining high transcript level in incompatible interactions has been observed in barley-powdery mildew combinations with certain host response genes, such as β-1,3-glucanase, chitinase, and TL protein genes (Clark et al. 1993; Boyd et al. 1994).

The temporal patterns of *tlp* gene induction did not relate to the alternating 16 h light and 8 h dark periods in which plants were incubated. Lights were on at 16 to 28 h AI, a period of high transcript levels, and on again at 36 to 52 h AI, a period of relatively low transcript levels. This contrasts with results for a 3-phosphoglycerate kinase gene (a gene involved in glycolysis induced preferentially by incompatible or inappropriate isolates) in parallel experiments in which transcript levels increased in each light period (K.-C. Lin and W. R. Bushnell, unpublished).

The increasing expression levels of *tlp* genes at 42 to 48 h AI (Fig. 6) occurred at the time when resistance was expressed by hypersensitive cell death and inhibition of fungal growth (K.-C. Lin and W. R. Bushnell, unpublished). Mesophyll cell death was visible at 4 to 6% of infection sites at 36 h AI and increased to 10 to 16% at 42 to 48 h in Rodney (*Pg-2*) in response to infection by incompatible isolate *Pga*-1H or inappropriate isolate *Pgt*-8D. Likewise, significant reduction in hyphal growth as a consequence of resistance was first detected at 42 to 48 h AI. The period 42 to 48 h AI was when transcript levels of *tlp* genes increased with incompatible and inappropriate isolates compared to declining levels with the compatible isolate (Figs. 5 and 6). Thus, *tlp* genes were induced in Rodney (*Pg-2*) in response to infection by incompatible or inappropriate isolates when resistance was expressed.

The induction of TL protein gene expression in response to infection by incompatible isolates has been reported in both barley-*Erysiphe graminis* f. sp. *hordei* (Bryngelsson and Green 1989; Boyd et al. 1994) and barley-*Rhynchosporium secalis* (Hahn et al. 1993) interactions and was assumed to be involved in race-specific resistance. In addition, the TL protein genes are also induced in wheat infected by the inappropriate barley powdery mildew fungus (Schweizer et al. 1989; Rebmann et al. 1991) and in rice attacked by *Pseudomonas syringae* pv. *syringae*, the inappropriate bean bacterial pathogen (Reimmann and Dudler 1993), much as with the inappropriate wheat stem rust fungus in our experiment with oat. Clearly, TL protein gene induction occurs in both race-specific resistance and resistance to inappropriate pathogens. Although induction of TL protein genes accompanies physiological expression of resistance, the correlation does not necessarily indicate a direct role for TL proteins in resistance.

In our experiments, only a small proportion of the total number of leaf cells were in contact with fungal hyphae by 42 to 72 h AI, especially in the incompatible interactions. For example, only one or two out of 10 stomata were penetrated by the rust pathogens (data not shown). For each penetrated stoma at 42 h AI, only a few host cells were in contact with haustorial mother cells (which, in turn, may have produced haustoria within host cells); e.g., 2 to 5 host cells with the incompatible isolate; 5 to 10 host cells with the compatible isolate (data not shown). Furthermore, the infection rates (see Materials and Methods) of incompatible isolate *Pga*-1H and inappropriate isolate *Pgt*-8D were 39 and 69%, respectively, of the rate obtained with compatible isolate *Pga*-6A. Nevertheless, activation of *tlp* genes by incompatible and inappropriate isolates was clearly evident from the results of RNA blot analyses (Figs. 5 and 6), suggesting that the *tlp* genes were either strongly induced at each infection site or that tissues away from infection sites were induced. On the other hand, differences in transcript levels between incompatible and compatible interactions was only two- to sixfold (e.g., at 42 to 72 h AI, Fig. 5B), less than has been reported in some other diseases (Cornelissen et al. 1986; Singh et al. 1989; Uknes et al. 1992), but comparable to those obtained for host response genes in barley-powdery mildew (Clark et al. 1993; Boyd et al. 1994).

The oat *tlp* genes investigated here displayed differential responses to pathogen attack, oil spray, and wounding (Figs 6 and 8). The *tlp-1*, *-2*, and *-3* genes were induced by all three treatments but to differing degrees, while *tlp-4* was induced by pathogen attack and oil spray but not by wounding. To investigate the mechanisms for differential induction of *tlp* genes, further analyses are required of copy number, mRNA lifetime, and the *cis*-acting elements required for induction of each *tlp* gene by pathogen infection, oil spray, and wounding. The in-

duction of *tlp* genes by slicing or puncturing oat leaves is consistent with reports of induction of TL protein genes in other plants by wounding (Neale et al. 1990; Brederode et al. 1991). However, induction by the light mineral oil used as spore carrier was unexpected. The level of oil used did not cause any visible sign of injury to oat leaf tissues. Higher levels of light mineral oil, however, are phytotoxic to oats (data not shown). We did not investigate what constituent or property of the oil acted as the inducer. Repeated attempts to inoculate oat plants without using oil as a carrier, such as applying dry spores in settling towers or using other liquid carriers, have failed to give uniform or high infection density as compared to that of oil. This failure is largely due to clumping of spores by dew droplets during incubation resulting in self-inhibition of spore germination (Rowell 1985).

The deduced amino acid sequences of four *tlp* gene products indicate that all TLPs are acidic proteins, with TLP-1, -2, and -3 having identical pI values of 6.2, and TLP-4 with pI value of 4.1 (Table 1). The presence of isoforms with diverging pI values has been reported among other PR proteins, including TL protein of PR-5 (Bol and Linthorst 1990; Jung et al. 1993). According to the available evidence, all the PR proteins are synthesized as precursors with an N-terminal signal peptide which mediates the transport of PR proteins through the secretory pathway (Bol and Linthorst 1990). Whereas the acidic PR proteins are exclusively transported to the intercellular spaces, the basic PR proteins are targeted to vacuoles as well as intercellular spaces depending on the presence of a C-terminal extension (Bol and Linthorst 1990; Chrispeels and Raikhel 1992). The presence of a putative signal peptide at the N-terminus and the absence of C-terminal extension signal or sorting signal in the mature TLP proteins needed for targeting proteins to vacuoles (Chrispeels and Raikhel 1992) suggest that TLPs, like other acidic PR proteins, are transported to the intercellular spaces through the secretory pathway rather than being retained in the vacuoles. Fink et al. (1990) found that the acidic β-1,3-glucanase and chitinase, but not the basic isoforms, were induced and transported into the intercellular spaces in oat leaves in response to infection by wheat stem rust and leaf rust fungi. The probable destination of TLPs in intercellular spaces is consistent with the possible involvement of TLPs in inhibiting growth of intercellular hyphae of incompatible *Pga*-1H and inappropriate *Pgt*-8D (K.-C. Lin and W. R. Bushnell, unpublished). The exact localization of TLPs needs to be shown by immunolocalization. Furthermore, activity against rust fungi needs to be demonstrated by infiltrating TLPs into infected leaves or by other means.

Osmotin (Singh et al. 1987) and thaumatin itself (Edens et al. 1982) are both basic proteins and have C-terminal extensions which are assumed to target both to either vacuoles or vesicle-like organelles in the cytosol. Zeamatin from maize (Roberts and Selitrennikoff 1990; Vigers et al. 1991), zeamatin-like proteins from maize and some other cereals (Vigers et al. 1991; Malehorn et al. 1994), and bifunctional trypsin/α-amylase inhibitor from maize (Richardson et al. 1987) are basic proteins, but do not contain a C-terminal extension. Although it is unclear whether these proteins are secreted to intercellular spaces in their original plants, zeamatin-like proteins were transported into intercellular spaces in transgenic *Arabidopsis thaliana* and tomato (Malehorn et al. 1994). The intercellular localization of basic PR-5 proteins has also been reported in sunflower (Jung et al. 1993).

Comparison of the amino acid sequences of TLPs to other TL proteins revealed that TLPs have more similarity to PRHv-1 from barley and PWIR2 from wheat than to thaumatin and the other TL proteins (Fig. 2). In addition, the molecular weights of TLPs, PRHv-1, and PWIR2 are similar to each other and are smaller than the other TL proteins, mainly because 58 amino acid residues that are present near the C-terminus of thaumatin are absent in TLPs, PRHv-1, and PWIR2. Whereas 16 cysteines are present in PR-5 of tobacco, zeamatin, osmotin, trypsin/α-amylase inhibitor, and thaumatin, only 10 cysteines are present at almost the identical positions in TLPs, PRHv-1, and PWIR2 (Fig. 2). These structural differences suggest that TLPs, PRHv-1, and PWIR2 are more closely related to each other than to other TL proteins. This suggestion was supported by phylogenetic analysis of TLPs and 21 other TL proteins from various plant species (Fig. 3), which shows that TLPs, PRHv-1, and PWIR2 were clustered into a group that is distinct from other TL proteins. A similar phylogenetic tree was obtained using the protein sequences without the extra 58 amino acid residues, suggesting that the primary structures of TL proteins are adequate to differentiate the phylogenetic relationships between TL proteins regardless of the extra 58 amino acid residues.

Seeds of oat and wheat contain zeamatin-like proteins (avematin and trimatin, respectively) which have antifungal characteristics (Vigers et al. 1991). These constitutive seed TL proteins are definitely different from the pathogen-induced leaf TL proteins of oat (TLPs) and wheat (PWIR2). Complete amino acid sequence data are not available for these seed TL proteins, but they are similar in molecular weight to zeamatin and thaumatin and larger than those of TLPs and PWIR2. Based on partial protein sequence data available at the N-termini, the amino acid sequences of avematin and trimatin (Vigers et al. 1991) are different from those of TLPs and PWIR2. The coexistence of the pathogen-induced leaf TL protein genes with the constitutively expressed seed TL protein genes in cereals suggests that these two groups may confer resistance to attack by different pathogens or stresses.

The isolation of four *tlp* genes and the detection of 8 to 14 fragments from genomic DNA by DNA blot analysis (Fig. 4) suggested the presence of a *tlp* gene family in oat. This supports previous reports that TL proteins are encoded by a gene family in several plant species (Ledeboer et al. 1984; Singh et al. 1989; Hahn et al. 1993). However, since Rodney (*Pg-2*) is hexaploid (6N = 42), some of the *tlp* genes may be from the corresponding (orthologous) loci of different genomes, especially *tlp-1*, *-2*, and *-3* which have similar expression kinetics in response to a given stimulus (Figs. 6 and 8). Mapping the location of each *tlp* gene on the genetic map of oat would shed light on this possibility.

MATERIALS AND METHODS

Plant and fungal materials.

All experiments were done with an oat (*Avena sativa* L.) line, Rodney (*Pg-2*) derived from the cultivar Rodney containing the oat stem rust resistance gene, *Pg-2*. The stem rust fungi used to inoculate Rodney (*Pg-2*) were isolate *Pga*-6A of race 6A (CRL culture number 59NE06), and isolate *Pga*-1H

of race 1H (CRL culture number PGR6812) of *Puccinia graminis* Pers. f. sp. *avenae* Eriks. & Henn., and isolate *Pgt*-8D of race *Pgt*-SCM (CRL culture number 74-36-924-A) of *P. graminis* f. sp. *tritici*, an inappropriate *formae specialis* for oat. Rodney (*Pg-2*) was compatible with isolate *Pga*-6A giving infection type 4, incompatible with *Pga*-1H giving infection type 1, and inappropriate with *Pgt*-8D giving infection type 0 (Roelfs 1984).

Urediniospore stock cultures of the isolates were derived from three cycles of single pustule isolation, and maintained in a desiccator with 30% relative humidity at 4°C. Large numbers of urediniospores were produced by inoculating 8-day-old seedlings of the susceptible cultivars Rodney (*Pg-0*) for *Pga*-6A and *Pga*-1H or McNair 701 for *Pgt*-8D. Urediniospores were harvested 12 days after inoculation and used immediately for inoculating Rodney (*Pg-2*).

Rodney (*Pg-2*) seedlings used for RNA blot analysis were grown in vermiculite (Strong-Lite Products Corp., Seneca, IL) in pots (7 × 7 cm) in a greenhouse at 25 ± 3°C under supplemental fluorescent light for 16 h per day. Eight-day-old seedlings (about 20 plants/pot) were inoculated with about 5 mg/pot of freshly harvested spores of each isolate. The spores were suspended in 0.25 ml of light weight mineral oil (SOLTROL 170; Phillips Chemical Company, Bartlesville, OK) and sprayed onto each pot with an atomizer designed by Browder (1965) using air pressure of 2 to 3 × 10^3 kg/m^2. After inoculation, the plants were allowed to dry for 30 to 60 min and were then incubated in a dew chamber (79 × 58 × 41 cm) located in a growth chamber (Conviron CMP3244, GIBBCO Scientific, Inc., MN). Dew was made by an ultrasonic humidifier (HOLMES AIR, Holmes Products Corp., Holliston, MA) at lowest mist volume for 15 min. The inoculated plants were incubated for 16 h in the dark at 20°C, and then for 3 h in fluorescent light (250 to 350 μE/m^2/s, 25°C) according to Kochman and Brown (1976). The plants were then moved from the dew chamber to the same growth chamber, kept for another 9 h in fluorescent light (250 to 350 μE/m^2/s, 25°C) and were then incubated under controlled conditions of 8 h of darkness (20°C) alternating with 16 h of light (250 to 350 μE/m^2/s, 21°C).

To determine the infection rate of each isolate, seven leaves of each host-pathogen combination were collected 24 h after inoculation. Inoculated leaf surfaces were stained with an acid fuchsin solution according to Rowell (1984), and observed using a Zeiss standard microscope. A successful infection site was defined as the penetration of a stoma beneath an appressorium as indicated by an empty appressorium. Empty appressoria were counted using 100× magnification in the microscopic field (2.4 mm^2/field). Each leaf was counted at positions 1, 3, and 5 cm away from the leaf tip on both the adaxial and abaxial surfaces. For each position, 3 fields were counted with a total of 18 fields per leaf. Therefore, a total of 126 fields were measured for each host-pathogen combination. The infection rate was determined as number of infection sites per centimeter square. Mean infection rates for *Pga*-6A, *Pga*-1H, and *Pgt*-8D were 680, 266, and 468 infection sites/cm^2, respectively.

RNA extraction and RNA gel blot analysis.

Approximately 60 inoculated plants (30 g fresh weight) from three pots were harvested at 6-h intervals from 16 to 72 h after inoculation. Extraction of total RNA was done according to Chirgwin et al. (1979). Approximately 30 μg of total RNA per lane was denatured and electrophoresed in 1.2% (w/v) agarose gels containing formaldehyde, transferred to a nylon filter (Nytran, Schleicher & Schuell, Inc., Keene, NH), and hybridized to ^{32}P-labeled probes according to manufacturer's instructions. Probes were labeled using a Random Primer DNA Labeling Kit (GIBCO-BRL, Life Technologies, Inc. Gaithersburg, MD). After hybridization, the filter was rinsed with 1× SSPE/0.1% SDS and then washed in 1× SSPE/0.1% SDS at 42°C for 15 min, twice in 1× SSPE/0.1% SDS at 60°C for 30 min each, and once in 0.1× SSPE/0.1% SDS at 60°C for 30 min. Levels of radioactivity on filters were quantified by using an AMBIS Radioanalytic Image Analyzer (AMBIS 4000, AMBIS, Inc., San Diego, CA). Background hybridization, as measured in membrane areas outside the hybridizing bands, was subtracted automatically by the Analyzer. Radioactivity of hybridization signals after subtraction of background was normalized with respect to the amount of total RNAs loaded in each lane. Normalization was based on hybridization with an internal control, ^{32}P-labeled 28S subunit rDNA from maize. Resultant data were plotted as the percentage of maximum net counts.

Oligonucleotides AS2-1 (5′-TTTGCTACTGTTGCTTGCTGTGG-3′), AS2-2 (5′-CGGTCGTATATGTACGCCGG-3′), AS2-3 (5′-GCATTACACAGCCGTTTATTGGT-3′), and AS2-4 (5′-GCACTCAGCACATGCACATGC-3′) corresponding to sequences of pCRL101, pCRL102, pCRL103, and pCRL104 (Fig. 1), respectively, were used as gene-specific probes. Hybridization of RNA gel filters with gene-specific probes was carried out according to the manufacturer's recommendation (Schleicher & Schuell, Inc., Keene, NH). Probes were end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Promega Corporation, Madison, WI). The probes all had specific activity of approximately 1 × 10^9 cpm/μg DNA. After hybridization, filters were washed in 6× SSPE/0.1% SDS three times at room temperature for 15 min each, and then washed in the same solution at the calculated melting temperature (Tm) for each of the probes, [Tm = 2 (A-T) + 4 (C-G) – 5°C], for 1 to 3 min.

Construction and screening of a cDNA library.

Poly(A)$^+$RNA was isolated by using an oligo(dT)-cellulose column (GIBCO-BRL, Life Technologies Inc., Gaithersburg, MD) according to the manufacturer's instructions. The quality of isolated poly(A)$^+$RNA was checked by RNA blot analysis using a peroxidase cDNA clone (WIR3) as a probe (Schweizer et al. 1989). A cDNA library was constructed from the isolated poly(A)$^+$RNA using a ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA). The titer of the library was approximately 10^7 plaque-forming units (PFU)/100 ng of insert DNA with approximately 1% nonrecombinant plaques. After amplification of the library, about 10^9 PFU/ml was obtained. The cDNA library was screened with a WIR2 cDNA probe (Schweizer et al. 1989) according to the manufacturer's protocol (Schleicher & Schuell, Inc., Keene, NH). Three cycles of plaque purification were carried out to obtain single independent plaques. After isolation of single plaques, DNA was excised in vivo as pBluescript II SK(–) phagemids (Stratagene, La Jolla, CA) and isolated according to Ausubel et al. (1987).

DNA sequencing and phylogenic analysis.

cDNA clones were sequenced by the double-stranded dideoxy chain termination method using a DNA sequencing kit (Sequenase Version 2.0, USB, Cleveland, OH). Both strands of the cDNA inserts were sequenced. Regions of compression were resolved according to McCrea et al. (1993) by using terminal deoxynucleotidyl transferase (Promega Corporation, Madison, WI). DNA sequence data were analyzed with the GCG Package (Version 7.2, Genetics Computer Group, Inc. Wisconsin). Phylogenic analysis was determined using the PAUP package (Swofford 1993).

Genomic DNA isolation and DNA blot analysis.

Genomic DNA was isolated from uninfected leaves of Rodney (*Pg-2*) as described by Ausubel et al. (1987). DNA samples of about 20 µg were digested with different restriction enzymes, electrophoresed on a 0.8% (w/v) agarose gel, transferred to a nylon filter (Nytran, Schleicher & Schuell, Inc., NH), and hybridized to a ^{32}P-labeled probe according to the manufacturer's instructions. The pCRL101 cDNA insert was used as a probe and was labeled by using the Random Primer DNA Labeling Kit. After hybridization, filters were rinsed with 1× SSPE/0.1% SDS, washed once in 1× SSPE/0.1% SDS at 42°C for 15 min, twice in 1× SSPE/0.1% SDS at 60°C for 15 min each, and subjected to autoradiography. For high stringency of wash, filters were washed in 0.1× SSPE/0.1% SDS at 60°C for 1 h.

Wounding of leaves.

Eight-day old seedling leaves were detached and sliced into pieces (2 to 3 mm wide) with a razor blade or punched with a brush leaving 0.5 to 1 mm holes spaced 2 to 3 mm apart. A sample of wounded leaves was immediately frozen in liquid nitrogen and kept as a sample for 0 h after wounding. The remaining wounded leaves were incubated for 24 h over moist filter paper in partially covered petri dishes in a dew chamber, following the light and temperature regime used for incubating inoculated plants. Detached leaves without wounding were incubated in the dew chamber for 24 h and used as a control.

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LITERATURE CITED

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl. K. 1987. Current Protocols in Molecular Biology. Vols. I and II. Greene Publishing Associate and Wiley-Interscience, New York.

Bol, J. F., and Linthorst, H. J. M. 1990. Plant pathogenesis-related proteins induced by virus infection. Annu. Rev. Phytopathol. 28:113-138.

Bowles, D. J. 1990. Defense-related proteins in higher plants. Annu. Rev. Biochem. 59:873-907.

Boyd, L. A., Smith, P. H., Green R. M., and Brown, J. K. 1994. The relationship between the expression of defense-related genes and mildew development in barley. Mol. Plant-Microbe Interact. 7:401-410.

Brederode, F. T., Linthorst, H. J. M., and Bol, J. F. 1991. Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. Plant Mol. Biol. 17:1117-1125.

Browder, L. E. 1965. An atomizer for inoculating plants with spore-oil suspension. Plant Dis. Rptr. 49:455.

Bryngelsson, T., and Green, B. 1989. Characterization of pathogenesis-related, thaumatin-like protein isolated from barley challenged with an incompatible race of mildew. Physiol. Mol. Plant Pathol. 35:45-52.

Chirgwin, J. M., Przybyia, A. E., MacDonald, R. J., and Rutter, W. J. 1979. Isolation of biological active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294-5299.

Chrispeels, M. J., and Raikhel, N. V. 1992. Short peptide domains target proteins to plant vacuoles. Cell 68:613-616.

Clark, T. A., Zeyen, R. J., Smith, A. G., Bushnell, W. R., Szabo, L. J., and Vance, C. P. 1993. Host response gene transcript accumulation in relation to visible cytological events during *Erysiphe graminis* attack in isogenic barley lines differing at the Ml-a locus. Physiol. Mol. Plant Pathol. 43:283-298.

Cornelissen, B. J. C., Hooft van Huijsduijnen, R. A. M., and Bol, J. F. 1986. A tobacco mosaic virus-induced tobacco protein is homologous to the sweet-tasting protein thaumatin. Nature 321:531-532.

Cutt, J. R., and Klessig, D. F. 1992. Pathogenesis-related proteins. Pages 209-243 in: Genes Involved in Plant Defense. T. Boller and F. Meins, eds. Springer-Verlag Wien, New York.

Edens, L., Heslinga, L., Klok, R., Ledeboer, A. M., Maat, J., Toonen, M. Y., Visser, C., and Verrips, C. T. 1982. Cloning of cDNA encoding the sweet-tasting plant protein thaumatin and its expression in *Escherichia coli*. Gene 18:1-12.

Fink, W., Liefland, M., and Mendgen, K. 1990. Comparison of various stress responses in oat in compatible and nonhost resistant interactions with rust fungi. Physiol. Mol. Plant Pathol. 37:309-321.

Flor, H. H. 1947. Inheritance of reaction to flax. J. Agric. Res. 74:241-262.

Freialdenhoven, A., Scherag, B., Hollricher, K., Collinge, D. B., Thordal-Christensen, H., and Schulze-Lefert, P. 1994. *Nar-1* and *Nar-2*, two loci required for Mla_{12}-specified race-specific resistance to powdery mildew in barley. Plant Cell 6:983-994.

Hahn, M., Jungling, S., and Knogge, W. 1993. Cultivar-specific elicitation of barley defense reactions by the phytotoxic peptide NIP1 from *Rhynchosporium secalis*. Mol. Plant-Microbe Interact. 6:745-754.

Hejgaard, J., Jacobsen, S., and Svendsen, I. 1991. Two antifungal thaumatin-like proteins from barley grain. FEBS. 291:127-131.

Jung, J.-L., Fritig, B., and Hahne, G. 1993. Sunflower (*Helianthus annuus* L.) pathogenesis-related proteins: induction by aspirin (acetylsalicylic acid) and characterization. Plant Physiol. 101:873-880.

Kochman, J. K., and Brown, J. F. 1976. Host and environmental effects on the penetration of oats by *Puccinia graminis avenae* and *Puccinia coronata avenae*. Ann. Appl. Biol. 82:251-258.

Lamb, C. J. 1994. Plant disease resistance genes in signal perception and transduction. Cell 76:419-422.

Ledeboer, A. M., Verrips, C. T., and Dekker, B. M. M. 1984. Cloning of the natural gene for the sweet-tasting plant protein thaumatin. Gene 30:23-32.

Lin, K.-C., Bushnell, W. R., Szabo, L. J., and Smith, A. G. 1995. Expression of genes encoding thaumatin-like proteins is associated with resistance in oat against *Puccinia graminis*. (Abstr.) Phytopathology 85:1203.

Linthorst, H. J. M., Meuwissen, R. L. J., Kauffmann, S., and Bol, J. F. 1989. Constitutive expression of pathogenesis-related proteins PR-1, GRP, and PR-S in tobacco has no effect on virus infection. Plant Cell 1:285-291.

Liu, D., Raghothama, K. G., Hasegawa, P. M., and Bressan, R. A. 1994. Osmotin overexpression in potato delays development of disease symptoms. Proc. Natl. Acad. Sci. USA 91:1888-1892.

Malehorn, D. E. Borgmeyer, J. R., Smith, C. E., and Shah, D. M. 1994. Characterization and expression of an antifungal zeamatin-like protein (*Zlp*) gene from *Zea mays*. Plant Physiol. 106:1471-1481.

Martens, J. W., Mckenzie, R. I. H., and Green, G. J. 1969. Gene-for-gene relationships in the *Avena:Puccinia graminis* host-parasite system in Canada. Can. J. Bot. 48:969-975.

McCrea, K. W., Marrs, C. F., and Gilsdorf, J. R. 1993. Gel compressions and artifact banding can be solved in the same DNA sequence reaction. BioTechniques 15:843-844.

Neale, A. D., Wahleithner, J. A., Lund, M., Bonnett, H. T., Kelly, A., Meeks-Wagner, D. R., Peacock, W. J., and Dennis, E. S. 1990. Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell 2:673-684.

Ogata, C. M., Gordon, P. F., de Vos, A. M., and Kim, S-H. 1992. Crystal structure of a sweet tasting protein thaumatin I, at 1.65 Å resolution. J. Mol. Biol. 228:893-908.

Pierpoint, W. S., Tatham, A. S., and Pappin, D. J. C. 1987. Identification of the virus-induced protein of tobacco leaves that resembles the sweet-protein thaumatin. Physiol. Mol. Plant Pathol. 31:291-298.

Rebmann, G., Mauch, F., and Dudler, R. 1991. Sequence of a wheat cDNA encoding a pathogen-induced thaumatin-like protein. Plant Mol. Biol. 17:283-285.

Reimmann, C., and Dudler, R. 1993. cDNA cloning and sequence analysis of a pathogen-induced thaumatin-like protein from rice (*Oryza sativa*). Plant Physiol. 101:1113-1114.

Richardson, M., Valdes-Rodriguez, S., and Blanco-Labra, A. 1987. A possible function for thaumatin and a TMV-induced protein suggested by homology to a maize inhibitor. Nature 327:432-434.

Roberts, W. K., and Selitrennikoff, C. P. 1990. Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity. J. Gen. Microbiol. 136:1771-1778.

Roelfs, A. P. 1984. Race specificity and methods of study. Page 131-164 in: The Cereal Rusts. Vol. 1. A. P. Roelfs and W. R. Bushnell, eds. Academic Press, Inc. Orlando, FL.

Rowell, J. B. 1984. Controlled infection by *Puccinia graminis* f. sp. *tritici* under artificial conditions. Page 291-332 in : The Cereal Rusts. Vol. 1. A. P. Roelfs and W. R. Bushnell, eds. Academic Press, Inc. Orlando, FL.

Schweizer, P., Hunziker. W., and Mosinger, E. 1989. cDNA cloning, in vitro transcription and partial sequence analysis of mRNAs from winter wheat (*Triticum aestivum* L.) with induced resistance to *Erysiphe graminis* f. sp. *tritici*. Plant Mol. Biol. 12:643-654.

Singh, N. K., Bracker, C. A., Hasegawa, P. M., Handa, A. K., Buckel, S., Hermodson, M. A., Pfankoch, E., Regnier, F. E., and Bressan, R. A. 1987. Characterization of osmotin. A thaumatin-like protein associated with osmotic adaptation in plant cells. Plant Physiol. 85:529-536.

Singh, N. K., Nelson, D. E., Kuhn, D., Hasegawa, P. M., and Bressan, R. A. 1989. Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. Plant Physiol. 90:1096-1101.

Stakman, E. C., Levine, M. N., and Bailey, D. L. 1923. Biological forms of *Puccinia graminis* on varieties of *Avena spp.* J. Agric. Res. 24:1013-1018.

Staskawicz, B. J., Ausubel, F. M., Baker, B. J., Ellis, J. G., and Jones, J. D. G. 1995. Molecular genetics of plant disease resistance. Science 268:661-667.

Swofford, D. L. 1993. PAUP: Phylogenetic analysis using parsimony, version 3.1.1. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. 1992. Acquired resistance in Arabidopsis. Plant Cell 4:645-656.

Van Loon, L. C. 1985. Pathogenesis-related proteins. Plant Mol. Biol. 4:111-116.

Van Loon, L. C., Gerritsen, Y. A. M., and Ritter, C. E. 1987. Identification, purification and characterization of pathogenesis-related proteins from virus-infected Samsun NN tobacco leaves. Plant Mol. Biol. 9:593-609.

Vigers, A. J., Roberts, W. K., and Selitrennikoff, C. P. 1991. A new family of plant antifungal proteins. Mol. Plant-Microbe Interact. 4:315-323.

Von Heijne, G. 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. 133:17-21.

Woloshuk, C. P., Meulenhoff, J. S., Sela-Buurlage, M., van der Elzen, P. J. M., and Cornelissen, B. J. C. 1991. Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell 3:619-628.

Xu, Y., Chang, P.-F. L., Liu, D., Narasimhan, M. L., Raghothama, K. G., Hasegawa, P. M., and Bressan, R. A. 1994. Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell 6:1077-1085.