Journal of Biochemistry and Molecular Biology, Vol. 38, No. 4, July 2005, pp. 420-431

JOURNAL OF Biochemistry and Molecular Biology

Identification and Characterization of Genes Differentially Expressed in the Resistance Reaction in Wheat Infected with *Tilletia tritici*, the Common Bunt Pathogen

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Received 21 January 2005, Accepted 17 March 2005

The differentially virulent race T1 of common bunt (*Tilletia tritici*) was used to inoculate the wheat lines Neepawa (compatible) and its sib BW553 (incompatible) that are nearly isogenic for the *Bt-10* resistance gene. Inoculated crown tissues were used to construct a suppression subtractive hybridization (SSH) cDNA library. Of the 1920 clones arrayed from the SSH cDNA library, approximately 10% were differentially regulated. A total of 168 differentially up-regulated and 25 down-regulated genes were identified and sequenced; 71% sequences had significant homology to genes of known function, of which 59% appeared to have roles in cellular metabolism and development, 24% in abiotic/biotic stress responses, 3% involved in transcription and signal transduction responses. Two putative resistance genes and a transcription factor were identified among the up-regulated sequences. The expression of several candidate genes including a lipase, two non-specific lipid transfer proteins (ns-LTPs), and several wheat pathogenesis-related (PR)-proteins, was evaluated following 4 to 32 days post-inoculation in compatible and incompatible interactions. Results confirmed the higher overall expression of these genes in resistant BW553 compared to susceptible Neepawa, and the differential up-regulation of wheat lipase, chitinase and PR-1 proteins in the expression of the incompatible interaction.

Keywords: Defense responses, Disease resistance, Gene expression, PR-proteins, Resistance gene

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Introduction

Resistance responses to plant pathogens are the focus of intensive research (Veronese *et al*., 2003a) because current technologies offer the possibility of genetically engineering plants for broad-based, effective resistance in crops species. Resistance responses can be divided into a series of inter-related stages (Keen, 2000; for reviews, see Dangl and Jones, 2001; Glazebrook, 2001; Veronese *et al*., 2003a). There is an initial recognition of the pathogen by the host plant that frequently involves the interaction between host resistance (R) genes and pathogen avirulence (Avr) genes which code for specific elicitors. An incompatible interaction results in a triggering of the defense responses through signaling pathways and includes reactive oxygen intermediates, nitric oxide, salicylic acid (SA), jasmonic acid (JA) and ethylene. Signaling pathways activate a broad series of defense responses that curb or eliminate the pathogen. These responses include the hypersensitive response (HR), up-regulation of phenylalanine ammonium lyase, a key enzyme in plant defense, deposition of cell wall reinforcing materials, and the synthesis of a wide range of antimicrobial compounds including pathogenesis-related (PR)-proteins and phytoalexins (Veronese *et al*., 2003a).

PR-proteins, of which there at least 14 currently known classes, occur widely in the plant kingdom, and constitute a major component of plant defense responses (van Loon and van Strien, 1999). These include the original five classes that consisted of PR-1, β-1,3-glucanase (PR-2), chitinases (PR-3, PR-4), thaumatin-like (PR-5), proteinase inhibitors (PR-6), proteinase (PR-7), additional chitinases (PR-8, PR-11), peroxidase (PR-9), ribonuclease-like (PR-10), and more recently defensins (PR-12), thionins (PR-13) and non-specific lipid transfer proteins (ns-LTPs) (PR-14) (van Loon and van Strien, 1999). The induction of genes PR-1, PR-2, and PR-5 in dicotyledonous species correlates with the onset of systemic acquired resistance (SAR) (Uknes *et al*., 1992; Ryals *et al*., 1996), which can also be triggered by exogenous treatments

of SA and other chemical inducers (Vernooij *et al*., 1995; Friedrich *et al*., 1996; Morris *et al*., 1998).

Based on genomic sequencing in *Arabadopsis,* as many as 2000 out of a total of approximately 25,500 genes are implicated in plant defense (The Arabidopsis Genome Initiative, 2000). Recent findings suggest that individual pathogens induce different defense responses in the host and this may reflect a differential response to individual virulence mechanisms employed by pathogens (Veronese *et al*., 2003a). Temporal expression of defense response genes varies considerably through the course of the host's defense response and frequently, the only apparent differences between incompatible and compatible plant-pathogen interactions is the more rapid and stronger induction of defense reactions in incompatible interactions (Maleck *et al*., 2000; Martinez de Ilarduya *et al*., 2003). Additionally, commonalities between responses to pathogens and other abiotic stresses such as cold, salinity, drought and nutritional stresses have been established (Tronsmo *et al*., 1993; Yun *et al*., 1997; García-Olmedo *et al*., 1998). Defense reactions can also change in function of the host's age and developmental stage (Neale *et al*., 1990; Gaudet *et al*., 2003b; Veronese *et al*., 2003b). Based on this premise, plants can potentially generate a myriad of 'individualized' defense responses (Hahlbrock *et al*., 2003). Several PR-proteins including chitinase, β-1,3-glucanase, and PR-1 (Thordal-Christensen *et al*., 1993; Bryngelsson *et al*., 1994; Molina *et al*., 1999), and the small defensin proteins, γ-thionin, γ-purothionin, and nonspecific lipid transfer proteins (ns-LTPs) (Hughes *et al*., 1992; Gaudet *et al*., 2000, 2003a, 2003b) have been isolated and characterized from monocots but relatively little is known about the defense mechanisms in this group that includes the most important crop species. Identifying the common and most efficacious plant defense response(s) effective against individual pathogens or groups of pathogens will provide a framework for genetically engineering new disease resistant varieties.

Common bunt, incited by the fungi *Tilletia tritici* (Bjerk.) Wint and *Tilletia laevis* Kuhn, can be a serious disease, causing yield and quality losses in spring and winter wheat (*Triticum aestivum L*.) (Hoffmann, 1982). A gene-for-gene interaction exists between Avr genes of the bunt pathogen and the many known wheat R genes (Hoffmann, 1982; Goates, 1996). Germination of the bunt fungus teliospore coincides with germination of the wheat seedling shortly after seeding; spore germination generally occurs four to ten days after seeding (Lowther, 1950; Russell and Mills, 1994) and penetration of emerging wheat coleoptiles occurs seven to ten days post-inoculation (Woolman, 1930; Swinburne, 1963). A successful infection results when the fungus is able to establish itself in the region directly below the apical growing point of the developing seedling following passage through the coleoptile and several embryonic leaves, a process which takes three to five weeks after seeding (Swinburne, 1963; Fernandez *et al*., 1978) although macroscopic symptoms (i.e. presence of the infected heads) are only visible at plant maturity (Hoffmann, 1982). No HR evidence has been observed with any race-specific forms of resistance.

The bunt resistance *Bt-10* gene, effective against the majority of common bunt races (Hoffmann and Metzger, 1976; Gaudet and Puchalski, 1995), is an important resistance source for new wheat varieties. However, the molecular pathways and mechanisms involved in inducing any of the common bunt race-specific resistance genes (such as *Bt-10*), or any of the downstream defense pathways in wheat are still unknown. In this study, we established a suppression subtractive hybridization (SSH) cDNA library and employed differential screening techniques (Diatchenko *et al*., 1996; von Stein *et al*., 1997) to compare mRNA populations in a susceptible and resistant line that are nearly isogenic for the *Bt-10* gene, and enriched for sequences that are more prevalent in the *Bt-10* line. Such differential analysis has been successfully used in monocots to identify downstream-activated genes including defense-related genes and medium-rare transcripts, important to disease resistance (Kim *et al*., 2000; Huckelhoven *et al*., 2001; Xiong *et al*., 2001). Here, we report the first results toward the identification and characterization of wheat genes involved in plant defense pathways following infection by the common bunt pathogen.

Materials and Methods

Biological materials Two spring wheat (*Triticum aestivum* L. em. Thell) lines, cv Neepawa and its near-isogenic sib cv BW553 (Neepawa*6 //Red Bobs/PI 178383), were used as the susceptible, and *Bt-10* resistant cultivars, respectively. The common bunt race employed was T1, virulent on Neepawa but avirulent on BW553 containing *Bt-10* (Gaudet *et al*., 1993). Spores were thoroughly dusted on the wheat seeds just before seeding. Seeds were planted into rootrainers (Spencer-Lemaire Industries, Edmonton, Canada) containing a soil-less potting mixture. Inoculated seeds were germinated and grown in growth cabinets set at 12°C. Fluorescent and incandescent lights in growth cabinets provided an 18 h day-length. Plants were thoroughly washed and crown tissues consisting of a 1 cm section of stem tissues directly above the roots were sampled, flash frozen in liquid nitrogen, and stored at −80°C until used. For the purpose of this study, the term 'days post-inoculation' refers to 'days post-seeding' since neither the fungus nor the plant undergo any development until seeding.

RNA isolation, transcript profiling and library construction To study expression of selected genes and wheat PR-proteins in bunt non-inoculated and inoculated plants, crown tissues were collected after 4-, 8-, 16-, 32-days after sowing (Zadoks stages 9, 11, 13, and 22, respectively) (Tottman and Makepeace, 1979). For library construction, crown tissues from treated seedlings were sampled 16 days after seeding (Zadoks stage 13). Total RNA was extracted from all crown tissues using Trizol (Invitrogen, San Diego, USA) at a ratio of 0.8 g fresh tissue per 10 ml of Trizol and stored at −80°C until used. Messenger RNA was further purified from total RNA using PolyA Pure (Ambion). The SSH approach, based on the

Table 1. DNA primers used to measure expression of different genes using real-time PCR

Gene	GenBank acces. No.	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplicon size (bp)	Tm value (°C)	Gene description
18S	X03205	cggctaccacatccaaggaa	gctggaattaccgcggct	187	63; 64	Ribosomal RNA 18S
Actin	DN551593	ggaaaagtgcagagagacacg	tacagtgtctggatcggtggt	150	60; 60	Actin (TaBs109G1 clone)
LTP-1	DN551584	acgtaggtactcctctcgctgt	gttgatcgaccacttcttctca	148	59; 59	Lipid transfer protein (TaBs108F7 clone)
LTP-2	DN551617	ggtcacacacacacacacaca	cgggagagaagtaacaaccaa	145	59; 59	Lipid transfer protein (TaBs112C7 clone)
Lipase	DN551653	cacaaaatatcgacccaccac	actgggtattcgtctgtcagc	149	60; 59	Lipase (TaBs117A2 clone)
ChiA	X95000	ccctacacatggggctactg	cctgcccgtagttgtagttgt	145	60; 59	ChiA 0.1 basic chitinase
PR-1.1	AJ007348	actacgactacgggtccaaca	tcgtagttgcaggtgatgaag	145	60; 59	Basic PR-1
PR-1.2	AJ007349	cgtcttcatcacctgcaacta	caaacataaacacacgcacgta	144	59; 60	Neutral PR-1
Glu 2	Z22874	agcagaactggggactcttct	cacatacgtaccgcatacacg	150	60; 60	β-1,4-glucanase
Glu 3	AY091512	ccttgcctctttgtatgctga	tcatcttttgtgggttcttgc	146	60; 60	β-1,4-glucanase

Clontech PCR-Select cDNA Subtraction Kit (K1804-1), was used for construction of the cDNA library. However, restriction of the cDNA was omitted in order to obtain full and near-full length sequences. In the library construction, inoculated BW553 mRNA was used as a tester and inoculated Neepawa mRNA was used as a driver. The SSH subtraction was also performed using inoculated Neepawa as tester and inoculated BW553 as driver to prepare reverse subtracted cDNA for differential screening. cDNA fragments smaller than 400 bp were removed by exclusion chromatography before ligation of cDNA to the vector pGEM-T (Promega, Madison, USA). Clones were picked and arrayed in 96-well plates, duplicated and kept at −80°C for long-term storage. A total of 1920 clones were arrayed in duplicate at low density on 10 nylon membranes. Differential screening was applied to isolate up- and down-regulated clones. Both cDNA and forward and reverse subtracted PCR product probes were used to screen the membranes.

Up- and down-regulated clones were identified, sampled, and cultured in new 96-well plates. Plasmids were extracted (QIAprep Miniprep, QIAGEN, Chatsworth, USA) for each differentially regulated clone and DNA sequencing was carried out using ABI PRISM 377 DNA Sequencer with the ABI Prism Big Dye Terminator (Sequencing Analysis System V3.7, PE Applied Biosystems, Foster City, USA). All sequences were deposited to our central DNA database server and the National Center for Biotechnology Information (NCBI) GenBank database (USA). BLASTN and BLASTX from NCBI and other bioinformatic tools were applied to analyze all sequence data. Sequences with signal peptides were identified using SignalP (http://www.cbs.dtu.dk/services/SignalP). Contigs were generated using CAP3, a fragment assembly program (X. Huang, xqhuang@iastate.edu). A contig is defined as a group of overlapping sequences of a gene or gene family that exhibit a minimum of 150 overlapping nucleotides. A singleton is any sequence that does not overlap with other sequences in the library data set.

Real-time quantitative PCR An iCycler iQ (BIO-RAD, Richmond, USA) was used for detection of all amplicons. Reverse transcription of total RNA was carried out using SuperScript II Reverse Transcriptase (Invitrogen). Real-time PCR analysis of individual sequences was carried out using QuantiTect SYBR Green PCR Kit (QIAGEN) according to the manufacturer's instructions. Primer pairs for real-time PCR analyses were designed using Primer3 software (Rozen and Skaletsky, 2000; http://cbr-rbc.nrc-cnrc.gc.ca/). Sequences and general guidelines for primer design were based on the recommendations provided by QIAGEN. The gene specific primers employed, the melting temperature (T_m) of the primers and the expected size of amplified fragments in base pair (bp), are listed in Table 1. Threshold (Ct) values and gene transcript numbers were used to determine the gene expression profiles from different cDNA samples. Real-time PCR data were analyzed with the REST software (Pfaffl *et al*., 2002), and wheat ribosomal RNA 18S and actin genes were used as reference controls (Table 1). Raw data of gene transcript numbers was analyzed using a General Linear Model (Proc GLM) (SAS Software, North Carolina, USA) whereby all main effects and interactions were considered. Within group means were separated using Fisher's Least Significant Difference (LSD) test.

The time-course expression profiles for three defense-related genes isolated from the SSH cDNA library, LTP-1 (TaBs108F7), LTP-2 (TaBs112C7, Contig 10), lipase (TaBs117A2, Contig 24) were determined by real-time PCR analyses (Table 1), 4-, 8-, 16-, and 32-days following T-1 inoculation of Neepawa (compatible) and BW553 (incompatible). Additionally, the time-course expression of wheat PR-proteins PR-1.1, PR-1.2, ChiA basic chitinase (ChiA), β-1,4-glucanase (Glu 2), and β-1,4-glucanase (Glu 3) (Table 1) was also established as described above in order to study the expression generalized defense responses in both compatible and incompatible interactions (Table 1). These primers were designed from wheat PR-protein sequences deposited in NCBI GenBank. Real-time PCR was repeated twice using newly synthesized cDNA samples each time, and representative results are presented.

Results

Construction and characterization of the SSH cDNA library Infection levels with the T1 race of *T. tritici* in the inoculated BW553 (incompatible) and Neepawa (compatible) reared to maturity, were 0/30 plants (0%) and 24/31 plants

Table 2. Major contigs of differently regulated sequences from the crown tissues SSH cDNA library

Contig	Clone No. in contig	GenBank accession No. and TaBs clone identifier**	BLAST matching accession No.	Gene description	E-value
TaBs1-Contig 2*	4↑; 2↓	DN551527 (TaBs101E6***); DN551596 (TaBs109H2); DN551609 (TaBs111D1); DN551631 (TaBs114A12); DN551634 (TaBs114B7***); DN551676 (TaBs119A7)	AAA50846	*Triticum aestivum* metallothionein-like protein 1 (MT-1);	1e-46
TaBs1-Contig 3	2↑	DN551556 (TaBs105F5); DN551629 (TaBs113H2)	NP_922860	*Oryza sativa* NADH-ubiquinone oxidoreductase 12 kDa subunit, mitochondrial precursor	4e-43
TaBs1-Contig 7	4↑	DN551546 (TaBs105C11); DN551549 (TaBs105C7); DN551580 (TaBs118C5); DN551669 (TaBs118F4)	NP_565539	*Arabidopsis thaliana* isochorismatase hydrolase	6e-42
TaBs1-Contig 8	2↑	DN551547 (TaBs105C2); DN551693 (TaBs120H5)	AAM92706	*T. aestivum* putative cytochrome C oxidase subunit	1e-49
TaBs1-Contig 10	7↑	DN551550 (TaBs105D10); DN551561 (TaBs106D4); DN551585 (TaBs108G5); DN551587 (TaBs109A5); DN551617 (TaBs112C7); DN551652 (TaBs116G9); DN551692 (TaBs120G10)	X68654	*H. vulgare* nonspecific lipid transfer protein *Cw-21* precursor	6e-36
Tabs1-Contig 18	4↑	DN551580 (TaBs108C5); DN551606 (TaBs111B2); DN551645 (TaBs115A1); DN551680 (TaBs119D8)	P13564	*H. vulgare* glutamine synthetase leaf isozyme chloroplast precursor	1e-118
TaBs1-Contig 19	11↑	DN551610 (TaBs111E3); DN551618 (TaBs112D5); DN551625 (TaBs113B8); DN551628 (TaBs113G1); DN551648 (TaBs116D2); DN551650(TaBs116F9); DN551662 (TaBs118B12); DN551667 (TaBs118D11); DN551677 (TaBs119B7); DN551684 (TaBs119G7; DN551689 (TaBs120C1)	BAB89205	*O. sativa* lipase-like protein	2e-36
TaBs1-Contig 21*	2↑; 1↓	DN551627 (TaBs113F9***); DN551641 (TaBs114G6); DN551661 (TaBs118A4)	A26014	*T. aestivum* histone H3	9e-70
TaBs1-Contig 22	4↑	DN551632 (TaBs114A9); DN551657 (TaBs117G1); DN551659 (TaBs117G8); DN551666 (TaBs118C9)	NP_850218	*A. thaliana* ubiquitin-conjugating enzyme E2	4e-50
TaBs1-Contig 23*	1↑; 1↓	DN551613 (TaBs111G2***); DN551644 (TaBs114H6)	AJ784900	*T. aestivum* lipid transfer protein precursor	5e-43
TaBs1-Contig 24	3↑	DN551653 (TaBs117A2); DN551668 (TaBs118F10); DN551686 (TaBs120A1)	BAB89205	*O. sativa* lipase-like protein	5e-31
TaBs1-Contig 25	2↑	DN551658 (TaBs117G11); DN551672 (TaBs118H8)	P13564	*H. vulgare* glutamine synthetase	4e-56
TaBs1-Contig 26	2↑	DN551664 (TaBs118B4); DN551675 (TaBs119A6)	CAB40943	*A. thaliana* resistance protein	1e-30

*The contig comprises both up-regulated (↑) and down-regulated (↓) clones from the SSH cDNA SSH library.

**The NCBI GenBank accession number was designed to each cDNA sequence, and the corresponding TaBs clone identifier was listed in parentheses.

***The clone was identified as down-regulated in the SSH cDNA library.

Table 3. Major up-regulated or down-regulated singletons from the crown tissues SSH cDNA library.

Singleton*	BLAST matching accession No.	Gene description	E-value
DN551523 (TaBs101A8)	Z34917	*H. vulgare* mRNA for *bas1* protein (thioredoxin-dependent peroxide reductase)	5e-51
DN551524 (TaBs101B3)	AAK82454	*O. sativa* putative transcription factor	4e-48
DN551533 (TaBs102H1)	L27349	*H. vulgare* calreticulin (CRH2) mRNA	1e-61
DN551554 (TaBs105E9)	AAM92707	*T. aestivum* glycine decarboxylase	4e-91
DN551562 (TaBs106D8)	S14959	*T. aestivum* proline rich protein	2e-33
DN551567 (TaBs106F7)	BAC45141	*Z. mays* thiamine biosynthetic enzyme (*thi1-2*) mRNA	3e-22
DN551566 (TaBs106F12)	AAM615568	*A. thaliana* putative ABC transporter ATPase	2e-26
DN551575 (TaBs108A5)	AY009938	H. vulgare Mla1-2	1e-31
DN551582 (TaBs108D2)	T05924	*H. vulgare* mRNA for putative cysteine thiol protease *RG42*	3e-43
DN551584 (TaBs108F7)	2115353A	*H. vulgare* lipid transfer protein	7e-34
DN551593 (TaBs109G1)	P30172	*Solanum tuberosum* actin	2e-38
DN551598 (TaBs110A8)	NP_910009	*O. sativa* putative RNA helicase	1e-23
DN551608 (TaBs111B6)	NP_920667	*O. sativa* profilin A	2e-07
DN551620** (TaBs112F6)	T05721	*H. vulgare* germin-like protein 1	4e-45
DN551639 (TaBs114F5)	AAM88439	*T. aestivum* Rieske Fe-S precursor protein	1e-89
DN551651 (TaBs116G1)	NP_187911	*A. thaliana* putative transporter	1e-52

*The NCBI GenBank accession number was designed to each cDNA sequence, and the corresponding TaBs clone identifier was listed in parentheses.
**The clone was identified as down-regulated in the SSH cDNA library.

(77%), respectively. This demonstrated that there was a sufficient level of disease in the compatible interaction and an effective resistance response had occurred in the incompatible interaction to use the biological material.

Of the1920 independent clones arrayed from the SSH cDNA library from the *Bt-10* BW553 line inoculated with the T1 pathogen, approximately 10% of the total arrayed clones were differentially regulated. Sixty seven up-regulated and 12 down-regulated clones were identified using cDNA probes while 115 up-regulated and 13 down-regulated clones were identified using the subtracted PCR product probes. Only 14 up-regulated clones were identified by both probes. Thus, the subtracted PCR probe was more efficient in identifying low abundant transcripts than the cDNA probe. A total of 168 up-regulated and 25 down-regulated clones were identified and sequenced (Tables 2, 3) and sequences have been deposited in the NCBI dbEST database (GenBank accession number DN551523 to DN551693).

BLAST results for all up-regulated and down-regulated clones indicated that 71% of the clones had significant homology to genes of known function in available public databases. The homology search and function classification indicated that 59% of up-regulated sequences appeared to function in cellular metabolism and development, 24% in abiotic/biotic stress responses, and 3% involved in transcription and signal transduction responses. The remaining clones (14%) exhibited significant homology to rice or *Arabidopsis* sequences for which any functional roles remain to be established. Of the differentially expressed clones, 60.1% were grouped under 26 contigs, 31.6% were singletons while the remaining 8.3% could not be assigned due to failed sequencing reactions.

Among the most abundant up-regulated sequences from the SSH cDNA library were genes encoding a lipase, two ns-LTPs and a sequence homologous to a hypothetical protein from *Oenothera elata* (Tables 2, 3). For the lipase gene, 14 clones were identified which comprised 8% of the differentially up-regulated sequences isolated, 11 of which belonged to Contig 19 while the remaining three clones formed Contig 24 (Table 2). Both contigs represented the same lipase gene since they showed the highest identity to the same *O. sativa* lipase-like protein (GenBank accession no. BAB89205) at the 5' and 3' ends of full-length of gene sequence, but without any overlap as 103 amino acids (aa) are missing between the two contigs.

Non-specific LTPs comprised about 5% of the differentially up-regulated clones (Table 2). There were two distinct contigs for the ns-LTP genes, Contig 10 (7 clones) and Contig 23 (2 clones). Contig 10 corresponded to a full-length gene sequence that was nearly identical (95%) to *Cw21, a* LTP gene isolated from a drought stressed barley library (GenBank accession no. X68654) (Ozturk *et al*., 2002) and *blt4.9* (GenBank accesssion no. U63993) (White *et al*., 1994), a low temperature regulated LTP gene. Contig 23 represented a different full-length ns-LTP gene *ltp9.4* (GenBank accession no. AJ784900), which exhibited 84% identity to the barley *blt4.2* gene and 82% identity to the *Cw21* gene. Contig 23 of the ns-LTP gene was also identical to an up-regulated clone

Table 4. Real-time PCR profiles for the expression of defense-related genes in the crown tissues of wheat cultivars Neepawa and BW553, 16 days post inoculation with the common bunt pathogen

Target gene*	Ct Value**				PCR efficiency	REST outputs			
	Neepawa		BW553			Neepawa		BW553	
	Control	Inoculated	Control	Inoculated		Exp. ration	P value	Exp. ratio	P value
Actin	19.3	19.4	19.3	19.2	1.96	---	---	---	---
LTP-1	23.2	22.7	19.2	18.7	1.87	1.463	0.170	1.278	0.132
LTP-2	23.6	23.3	20.9	20.5	1.92	1.344	0.251	1.162	0.449
Lipase	22.9	22.3	21.1	18.9	1.96	1.602	0.085	4.018***	0.001
ChiA	28.9	28.5	28.3	27.1	1.98	1.358	0.189	2.122***	0.001
PR-1.1	23.3	22.7	21.2	20.0	1.90	1.623	0.120	1.977***	0.033
PR-1.2	22.4	23.1	22.1	21.2	1.93	0.653	0.332	1.690	0.122
Glu 2	25.7	25.2	22.7	22.4	1.82	1.443	0.085	1.097	0.634
Glu 3	23.5	23.2	19.8	19.3	1.87	1.291	0.328	1.226	0.158

*The expression of defense-related genes was normalized by expression of the reference control (wheat actin gene) and the pair wise fixed reallocation randomization test was applied in REST analyses (Pfaffl *et al.*, 2002).
**The Ct value was the mean of two independent readings with three replications, and the threshold baseline 25.0 was selected to calculate the Ct value for each sample.
***The target gene treatment is significantly different from the target gene control (P value < 0.05).

(TaLt19C10) from another SSH cDNA library induced by cold stress generated in our laboratory. LTP genes from both contigs possessed a signal peptide sequence.

Two putative R genes and a transcription factor were identified among the up-regulated sequences. The 509 bp TaBs108A5 clone represented a partial R gene sequence that shared 66% identity with the NB-ARC domain contig encoding the barley powdery mildew resistance gene *Mla1* (GenBank accession no. AY009938) (Zhou *et al.*, 2001) (Table 3). Contig 26, made up of clones TaBs118B4 and TaBs119A6, represented a second putative R gene, which exhibited homology to the *A. thaliana* putative disease resistance protein RPP5 (GenBank accession no. CAB40943) (Bevan *et al.*, 1998). The putative full-length transcription factor, TaBs101B3 clone (Table 3) exhibited a high degree of identity (90%) with an *O. sativa* sequence (GenBank accession no. AAK82454). Up-regulated and defense-related genes homologous to a cysteine thiol protease (TaBs108D2) and a thioredoxin-dependent peroxide reductase (TaBs101A8), implicated in the oxidative burst, were also identified (Table 3).

In addition, other classes of genes responsible for cell metabolism and development were identified, such as glutamine synthetase, glycine decarboxylase and cytochrome c oxidase. A total of 6 up-regulated clones were identified as glutamine synthetase, in which 4 clones belonged to Contig 18 and 2 clones to Contig 25. These two contigs covered about 80% of gene sequence, are identical to *H. vulgare* glutamine synthetase (GenBank accession no. P13564) (93% and 100% respectively) but did not overlap as only 4 aa are missing between the two contigs. However, 80 aa are missing at the 5' end and 15 aa are missing at the 3' end. Two full length genes, glycine decarboxylase (TaBs105E9 clone, GenBank accession no. AAM92707) and cytochrome c oxidase (Contig 8, GenBank accession no. AAM92706), were also identified (Table 2, 3).

Down-regulated genes differentially expressed, which consisted of 6 contigs and 14 singletons, appeared to be predominantly involved in plant signal transduction and stress responses (Table 2, 3). Of the 25 down-regulated BLAST sequences, 9 clones exhibited higher identity to sequences originating from wheat or barley salted-stressed or ABA-treated cDNA libraries but their functions remain to be ascertained, whereas 9 clones where homologous to genes involved in cell metabolism/development and 3 were involved in biotic/abiotic stress response. A down-regulated ns-LTP clone (TaBs111G2, Contig 23) appeared different from most predicted ns-LTP grouped under Contig 10 as up-regulated clones (Table 2). However, this clone was nearly identical (94%) to one up-regulated sequence from the same contig. Two clones (TaBs101E6 and TaBs114B7) represent a contig of a metallothionein-like protein (Table 2, Contig 2) and one clone (Tabs112F6) had homology to a germin-like protein (Table 3).

Time-course expression of defense-related genes The infection levels in a portion of the T1 inoculated Neepawa (compatible) and BW553 (incompatible) plants allowed to proceed to maturity, were 77% and 0%, respectively, indicating that disease pressure was adequate to permit expression of defense-related responses in plants. The real-time PCR and REST analyses demonstrated that the expression levels for three selected genes (lipase, LTP-1, and LTP-2) originating from the SSH library and three wheat PR-proteins (Glu 3, PR-1.1, and ChiA) were globally higher in

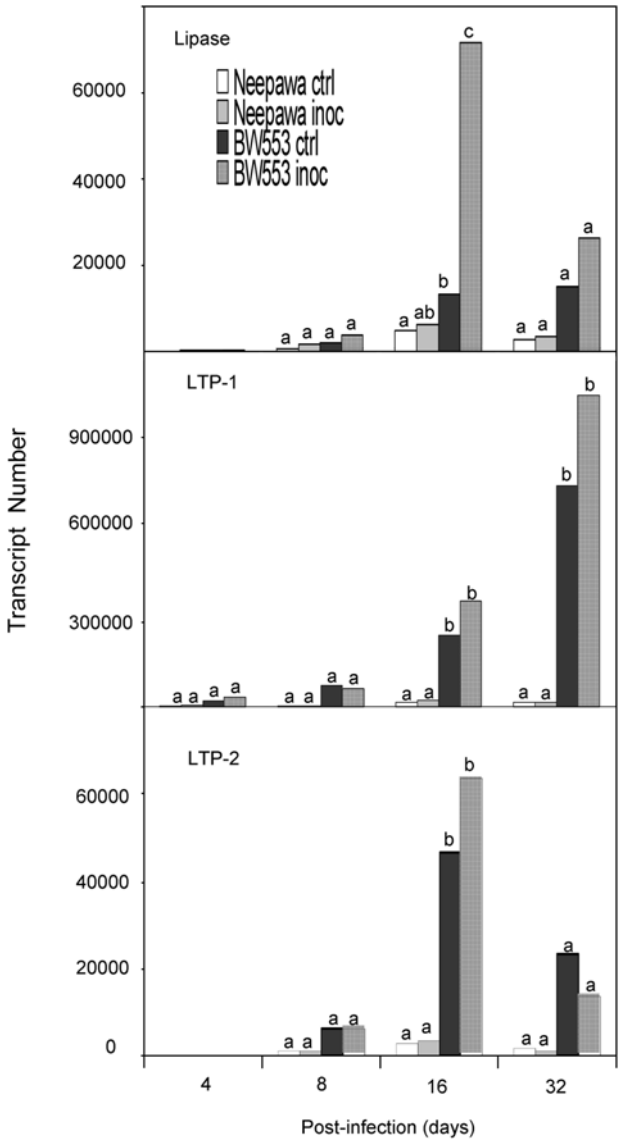

Fig. 1. Time-course expression for lipase, LTP-1 and LTP-2 genes homologues from the SSH cDNA library in inoculated and non-inoculated Neepawa and BW553 wheat cultivars 4 to 32 days post-inoculation using real-time PCR. Treatments within post-inoculation dates followed by the same letter are not significantly different using Fisher's LSD test ($p < 0.05$).

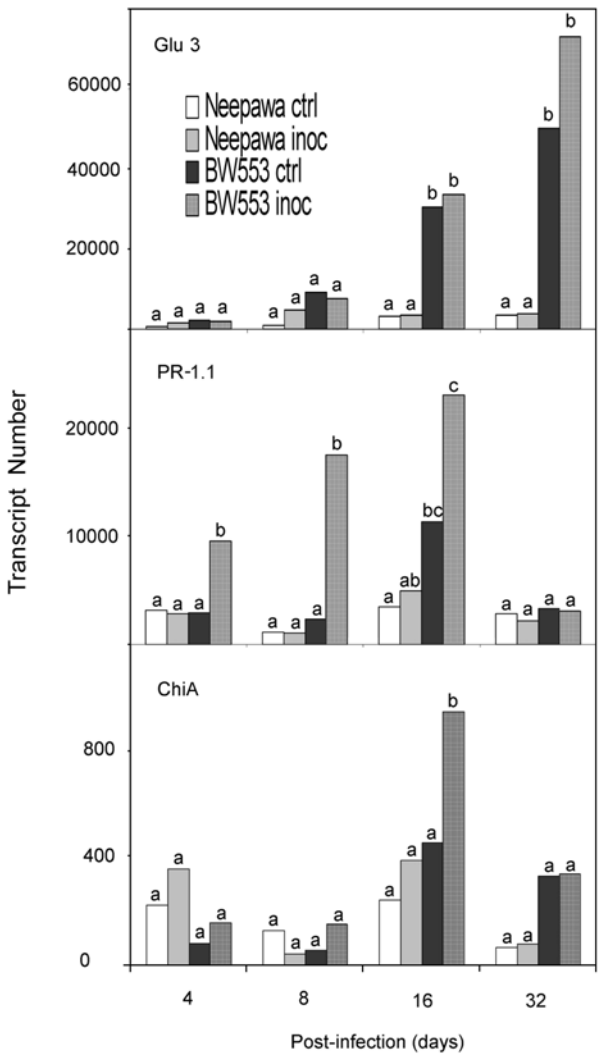

Fig. 2. Time-course expression the wheat PR-proteins (*Glu 3, PR-1.1, and ChiA*) in inoculated and non-inoculated Neepawa and BW553 wheat cultivars 4 to 32 days post-inoculation using real-time PCR. Treatments within post-inoculation dates followed by the same letter are not significantly different using Fisher's LSD test ($p < 0.05$).

both inoculated and non-inoculated resistant BW553 than in both corresponding treatments of the isogenic sib line Neepawa, susceptible to race T1 (Fig. 1, Fig. 2). Additionally, the highest expression levels were observed following 16 and 32 days post-inoculation for all genes except PR-1.1 (Table 4; Fig. 1, Fig. 2) and PR-1.2 that both exhibited earlier expression especially following 4 and 8 days post-inoculation, suggesting an earlier induction of these two genes.

Expression patterns for LTP-1 and LTP-2 homologues were similar although gene transcript numbers were approximately 10-fold higher for LTP-1 compared to LTP-2 (Fig. 1). Additionally, gene transcript numbers fell more than half in the 32-day treatment for LTP-2 compared with LTP-1. The expression of either LTP genes in the incompatible (BW553) interaction was similar in both the inoculated and the non-inoculated treatments. A 4-fold difference ($p < 0.05$) in the lipase gene transcript numbers was observed between the incompatible and compatible interaction after 16 days post-inoculation. Expression of the lipase in the non-inoculated BW553 was similar to expression in both non-inoculated and inoculated Neepawa (Fig. 1). Among PR-proteins, approximately 3, 8, and 2 fold higher PR-1.1 gene transcript numbers ($p < 0.05$) were observed between incompatible compared with the compatible interaction 4, 8, and 16 days post-inoculation treatments, respectively. Additionally, 2 fold higher ChiA gene transcript numbers were observed between the incompatible and the compatible interaction in the 16-day

post-inoculation treatment. These differences implicate the lipase, PR-1.1 and ChiA in the expression of the incompatible interaction involving *Bt-10* and T1.

Discussion

Common bunt and other related smut fungi have long infection times and sparse development in the seedling during the early infection stages (Woolman, 1930; Swinburne, 1963). In fact, the mycelium that develops in infected seedlings remains sparse until a massive proliferation of the fungus occurs later growth stages of the host, in floral tissues (Swinburne, 1963; Fernandez *et al*., 1978). In this study, wheat plants carrying the *Bt-10* gene for resistance responded to invasion by the avirulent race T1 with an array of transcriptional changes, including both the up-regulation and down-regulation of transcripts involved in plant metabolism and plant defense-related genes. A time-course expression of genes selected for further study from the SSH cDNA library showed that expression of ns-LTPs, a lipase, and a series of wheat PR-proteins was higher in resistant BW553 carrying the *Bt-10* gene, than in its isogenic sib, Neepawa. Expression of the lipase, PR-1.1 and ChiA, was higher in the incompatible interaction than in the compatible interaction. These results are among the first to characterize, at the molecular level, the wheat defense reactions for the host-parasite interaction involving common bunt, a unique and economically important plant pathogenic fungus.

Two putative R genes and a transcription factor were differentially expressed in the cDNA SSH library. The R genes were homologous to barley powdery mildew resistance gene *Mla* (Zhou *et al*., 2001; Wei *et al*., 2002; Shen *et al*., 2003) and to the *A. thaliana* putative disease resistance gene *RPP5* (Bevan *et al*., 1998), respectively. The *Mla1* is classified as CC-NBS-LRR type (Shen *et al*., 2003), a class of cytoplasmic R genes, and *RPP5* belongs to TIR-NBS-LRR type, one of the major R gene classes in *Arabidopsis* (Parker *et al*., 1997). Morphological studies on wheat plants have shown that resistance responses are expressed prior to establishment of fungal hyphae in the apical meristems, which occurs 14-35 days post-inoculation (Swinburne, 1963; Fernandez *et al*., 1978). We applied the real-time PCR to explore differential expressions of the *Mla1*-like, the *RPP5*-like, and transcription factor genes based on BW553 and Neepawa cDNAs in both the inoculated and non-inoculated treatments, and the PCR amplification results indicated that all three genes were expressed at very low levels compared to wheat lipase, ns-LTPs, and PR-proteins. The high Ct value (over 30) and low gene transcript number (less than 200) of the *Mla1*-like, the *RPP5*-like, and transcription factor genes (data not shown) were beyond the real-time PCR reliability; therefore, it was not possible to establish their expression patterns from our real-time PCR data. Additional studies would be necessary to determine whether two putative R genes and the transcription factor play an important role in the resistance reaction against common bunt.

The predominant defense-related transcripts up-regulated during expression of the *Bt-10* resistance gene in the wheat SSH library were a lipase and ns-LTPs, which accounted for 8% and 5% of the differentially up-regulated clones, respectively. Expression of both ns-LTP genes was highest in the resistant BW553 compared to susceptible Neepawa, 16 and 32 days post-inoculation, but gene transcript numbers were similar in both inoculated and non-inoculated controls. The results suggested that these ns-LTPs are developmentally and genetically regulated and that similar, though not identical, mechanisms regulate their expression. A similar co-regulation of different families of ns-LTPs following exposure of winter wheat to low above-freezing temperatures, was recently reported (Gaudet *et al*., 2003a). Non-specific LTPs, reported in various organs and tissues in many mono- and dicotyledonous species, are involved in the extracellular transport of lipids, and are considered important in several types of stress responses including attack by plant pathogens (García-Olmedo *et al*., 1998; Douliez *et al*., 2000). Their role in plant defense is not clearly understood but ns-LTPs have been implicated in plant defense against viral, bacterial, and fungal plant pathogens (García-Olmedo *et al*., 1998; Sohal *et al*., 1999). Certain ns-LTPs appear to be involved in the formation of cutin and suberin layers in the plant epidermis, thereby strengthening structural barriers in organs against mechanical disruption and pathogen attack (Douliez *et al*., 2000, Guiderdoni *et al*., 2002). Elevated ns-LTP transcript or protein levels have been observed following infection with the fungal pathogens, *Blumeria graminis, Rhynchosporium secalis* (Molina and García-Olmedo, 1993) and tobacco mosaic virus (Park *et al*., 2002). Recently, a ns-LTP was implicated in SAR signaling in *Arabidopsis* (Maldonado *et al*., 2002). Additionally, elevated transcripts of genes involved in fatty acid metabolism and transport such as lipases and lipid transfer proteins, may be important in priming the synthesis of plant defense-signaling molecules such as JA and other oxylipins (Graham and Eastmond, 2002; Howe and Schilmiller, 2002). It is not possible to conclude that either of the ns-LTPs genes are involved in the specific defense or resistance responses in the incompatible interaction involving *Bt-10*. However, the consistently higher expression of these genes from 8 to 32 days post-inoculation in the resistant BW553 compared to the closely related, susceptible Neepawa suggests that they may have a role in a more generalized defense responses. Alternatively, it is possible that these genes or associated regulatory elements are physically linked to the *Bt-10* gene and co-incorporated into BW553, resulting in the developmentally based constitutive expression of these genes. The lipase clones, grouped into two non-overlapping contigs, were homologous to a full length sequence of a lipase-like protein in *O. sativa*. The time-course expression study suggested that the lipase was specifically associated with the incompatible interaction 16 days post-inoculation of BW553

with the T1 race of bunt. Lipases have been implicated in pathogen resistance responses in plants because of their role in the synthesis and perception of SA that is critical for pathogen- and wound-induced systemic defense responses (Howe and Schilmillar, 2002). The *Arabidopsis* resistance-related genes *EDS1* and *PAD4* have also been shown to be homologous to eukaryotic lipases and to play an important role in SA signaling (Falk *et al*., 1999; Jirage *et al*., 1999). Additionally, lipids have been implicated as elicitors of defense responses, in plant-herbivore (Lait *et al*., 2003) and plant-*Rhizobium* interactions (den Hartog *et al*., 2003). Lipids in wheat bunt fungi can also serve as bio-regulators of teliospore formation and sporidium formation (Trione and Ross, 1988).

Transcripts of the PR-proteins (PR-1.1 and PR-1.2) exhibited a rapid up-regulation as early as 4 days post-inoculation, reached maximum levels after 16 days, followed a down-regulation after 32 days. Conversely, maximum expression of the ChiA and the Glu 3 was observed following 16 and 32 days post-inoculation, respectively. Additionally, both ChiA and PR-1.1 were expressed at higher levels in the inoculated BW553 compared to the non-inoculated treatment and both Neepawa treatments. Chitinases are reported to play a dual role in the host-parasite interaction; apoplastic chitinases degrade fungal chitin following initial penetration of the intercellular spaces by the pathogen (Collinge *et al*., 1993; de A. Gerhardt *et al*., 1997). The released chitin may then elicit a more generalized defense response resulting in the up-regulation of both apoplastic and vacuolar chitinases, and other defense responses including the hypersensitive response (de A. Gerhardt *et al*., 1997). Plants also produce an array of PR-1 proteins that exhibit differential toxicities to various plant pathogens (Niderman *et al*., 1995). The expression of PR-1.1 after 4 days post-infection and rising to maximum levels after 8 days suggested that up-regulation of this defense-related protein is among the first defense response effected towards pathogen infection in the incompatible interaction involving *Bt-10*.

None of the original PR-proteins where represented in the SSH library. Similar results were observed in other SSH libraries after plant exposure to low temperature and leaf rust (Gaudet *et al*., unpublished results). This could be explained by a similar up-regulation in PR-protein transcripts occurred in inoculated Neepawa (compatible) treatment that served as the driver in the subtraction process. Because a 10X excess of driver cDNA was used in the SSH procedure, it is likely that differences in up-regulation in PR-proteins less than 10 times would not be detected. Even in susceptible reactions, up-regulation of PR-protein genes tends to occur later than in resistant reactions and at lower levels; delays in up-regulation of defense responses have been observed in compatible interactions involving other pathogens (Maleck *et al*., 2000; Martinez de Ilarduya *et al*., 2003).

Collectively, the up-regulation of defense-related proteins including the R genes, ns-LTPs, the lipase, reaching maximum expression levels 16 and 32 days post-inoculation, was expected since the SSH cDNA library was constructed from RNA isolated at 16 days post-inoculation. With the exception of PR-1.1 and PR-1.2, the expression of the PR-proteins also followed a similar expression pattern. Spore germination under optimum conditions takes 4-10 days (Lowther, 1950; Russell and Mills, 1994). Additionally, published histological studies have demonstrated that both infection of the coleoptiles in resistance and susceptible varieties was similar after 7 days post-inoculation and that resistance expression results in the failure of fungal hyphae to become established in the growing point of resistant varieties 14-35 days post-inoculation (Woolman 1930; Fernandez *et al*., 1978). Therefore, expression of defense-related genes would be expected with 2-4 weeks post-inoculation. The very rapid expression of PR-1.1 and PR-1.2, 4 and 8 days post-inoculation, likely coincided with the earlier stages of spore germination and the early stages of penetration. A similar rapid up-regulation of chitinases and glucanases was observed in wheat prior to penetration of stomata by *Puccinia graminis* f.sp. *tritici*, the stem rust pathogen and was associated with the release of a pathogen elicitor (Munch-Garthoff *et al*., 1997). These defense response patterns are clearly different from those observed by Caldo *et al*. (2004) for incompatible and compatible interactions of involving *Mla1*-like genes for resistance to powdery mildew on barley; build-up of transcripts of defense-related genes in both compatible and incompatible interactions were identical except that transcript levels decreased during later stages of infection in the compatible interactions whereas they remained elevated in the incompatible interactions.

In summary, a SSH cDNA library originating from the wheat line BW553 carrying the *Bt-10* gene for common bunt resistance yielded several resistance- and defense-related genes including R genes, ns-LTPs, and a lipase. A time-course study during infection of the seedling with race T1 of common bunt showed that these defense-related and other wheat PR-proteins were expressed at higher levels in incompatible interaction in BW553 than its closely related susceptible sib Neepawa. Additionally, differential up-regulation of a lipase, PR-1.1 and ChiA specifically in response to infection with the avirulent T1 race of the pathogen was observed. Studies are currently underway to determine if the up-regulation of these genes and other sequences originating from the SSH cDNA library are an expression of defense responses to the common bunt pathogen in the wheat carrying *Bt-10*.

Acknowledgments We thank Dr. Isobel Parkin from the Saskatoon Research Centre, AAFC for arraying clones on nylon membranes. The partial funding from the Agriculture and Agri-Food Canada Matching Investment Initiative is acknowledged.

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