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Differential Responses of Wheat Inhibitor-like Genes to Hessian Fly, *Mayetiola destructor*, Attacks During Compatible and Incompatible Interactions

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Abstract Four groups of inhibitor-like genes that encode proteins with diverse structures were identified from wheat. The majority of these genes were upregulated by avirulent Hessian fly, *Mayetiola destructor* (Diptera: Cecidomyiidae), larvae during incompatible interactions, and were downregulated by virulent larvae during compatible interactions. The upregulation during incompatible interactions and downregulation during compatible interactions resulted in four- to 30-fold differences between the expression levels in resistant plants and those in susceptible plants. The increased expression of inhibitor-like genes during incompatible interactions suggested that these genes are part of defense mechanisms in wheat against Hessian fly attacks, whereas the downregulation during compatible interactions suggested that virulent larvae can suppress plant defenses. Both the upregulation of the inhibitor-like genes during incompatible interactions by avirulent larvae and the downregulation during compatible interactions by virulent larvae were through mechanisms that were independent of the wound response pathway.

Keywords cDNA · Diptera · Downregulation · EST · Gene expression · *Mayetiola destructor* · Microarray analysis · Polymerase chain reaction (PCR) · Real-time PCR · Total RNA · *Triticum aestivum* · Upregulation

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Introduction

In response to insect herbivory, plants launch direct and indirect chemical defenses for their protection (Kessler and Baldwin 2002). Direct defense includes the production of various proteinaceous and nonproteinaceous molecules that are either anti-nutritional or are toxic to the insect (Chen 2008). In addition to direct defenses, plants also synthesize volatile organic compounds that can attract natural enemies of the attacking insect (Schnee et al. 2006). The natural enemies suppress the population of the insect and, as a result, reduce plant damage. This type of defense is referred to as indirect defense (Agrawal and Fordyce 2000).

One of the most extensively characterized defensive chemicals is a large class of proteinaceous inhibitors that includes protease and amylase inhibitors (Chen 2008). Numerous proteinaceous inhibitors with diverse structures have been observed with either high levels of constitutive expression in seeds, or high levels of induction in vegetative tissues upon insect herbivory. Detrimental effect of proteinaceous inhibitors on insect growth and development has been demonstrated either through artificial diets or through transgenic plants.

Research on protease and amylase inhibitors in defense against insect herbivores has been focused initially on generalists with chewing mouthparts. Chewing insects cause physical damage (wounding) to plants, and, as a result, plants

have developed mechanisms to downregulate inhibitor genes due to this mechanical wounding (Kessler and Baldwin 2002). Protease/amylase inhibitors also may be important to plant defense against insects with sucking mouthparts. For example, a proteinase inhibitor accumulated at a significantly higher level in barley leaves infested with aphids (Casaretto and Corcuera 1998). Elevated levels of protease inhibitors either through artificial diet or via transgenic plants resulted in the suppression of aphid growth and development (Rahbe et al. 2002, 2003; Ceci et al. 2003).

Despite this progress, little is known about the role and regulation of inhibitor genes during compatible (virulent parasites in susceptible hosts) and incompatible (avirulent parasites in resistant hosts) interactions in plant–insect systems that have a gene-for-gene interaction. The wheat–Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae), system follows a typical gene-for-gene interaction, and provides a good model for studying the scheme of attack and counter-attack between insects and plants (Harris et al. 2003). From a previous analysis of wheat gene expression with Affymetrix microarrays, we identified a group of inhibitor-like genes whose expression was affected by *M. destructor* attack (Liu et al. 2007). In this study, we investigated the dynamic expression of the inhibitor-like genes in wheat seedlings attacked by virulent or avirulent *M. destructor* larvae. We found that the expression of the inhibitor-like genes was suppressed strongly during compatible interactions. On the other hand, dramatic induction of these genes was observed during incompatible interactions.

Methods and Materials

Plants and Insects Two wheat, *Triticum aestivum*, cultivars, Molly and Karl-92, and two *M. destructor* biotypes, Great Plains (*GP*) and *vH13*, were used. Both *GP* and *vH13* were derived from the same population. Karl-92 carries no *M. destructor* resistance (*R*) genes, and was used for maintaining the population and for complementary DNA (cDNA) cloning. Molly carries the *M. destructor R*-gene *H13*, and is compatible to *vH13* but incompatible to *GP* insects (Patterson et al. 1994; Rider et al. 2002). Molly seedlings were susceptible plants (compatible interaction) when they were infested with *vH13*, and were resistant plants (incompatible interaction) when infested with *GP*. *GP* was maintained on two-leaf seedlings of Karl-92, and *vH13* was maintained on Molly seedlings in growth chambers at 20°C and 12:12 hr (L/D) photoperiod.

Cloning and Characterization of Inhibitor-like cDNAs Expressed sequence tags (ESTs) corresponding to specific inhibitor-like genes were identified from a previous microarray analysis (Liu et al. 2007). To clone full-length cDNAs, an individual EST was used to blast the GenBank EST database to identify other ESTs that share high sequence similarity. Contigs were then assembled with all similar EST sequences by using the CAP3 Program (http://pbil.univ-lyon1.fr/cap3.php, Huang and Madan 1999). A pair of primers was synthesized according to the contig sequence, and was used for polymerase chain reaction (PCR) amplification. DNA template for PCR amplification was derived by reverse transcription of total RNA extracted from Karl-92. The PCR fragments were either sequenced directly or ligated into the pPCR2.1-XL-TOPO plasmid by using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). If ligated into the plasmid, individual clones were picked up and plasmid DNA samples for the individual clones were then sequenced. DNA sequencing was carried out either at Kansas State University, Manhattan, KS, USA or at the Jamie Whitten Delta States Research Center of USDA-ARS at Stoneville, MS, USA.

Sequence alignments were generated with ClustalW (www.ebi.ac.uk/Tools/clustalw/index.html). Secretion signal peptides were predicted using SignalP v. 1.1 (Center for Biological Sequence Analysis, Technical University of Denmark, www.cbs.dtu.dk/services/SignalP) or PSORT II (http://psort.nibb.ac.jp).

Experimental Treatments and RNA Extraction Wheat seedlings were grown and infested with different biotypes of *M. destructor* in a growth chamber set at 20±1°C (daytime) and 18±1°C (night) with a 14:10 hr (L/D) photoperiod. Seedlings were infested with a mean of 10 larvae at the two-leaf stage. Larval hatching time was monitored with additional infested plants under the same growth chamber conditions. Specifically, plants for monitoring hatching time were cut off at the base and were put into a test tube. Newly hatched larvae migrated along the leaves and between leaf sheathes. Initial attacking time was recorded when the larvae moved out of the leaf sheath into the test tube.

Wounding was accomplished with a dissecting pin (size 1, Benz Microscope, Ann Arbor, MI, USA) mounted onto a pin-vise (Cat#: 4845, Bioquip, Rancho Dominguez, CA, USA). Ten penetrations per plant were made above the base of a plant, where *M. destructor* larvae feed. For the combinational treatments of wounding plus larval attack, wounding was carried out immediately before larval migration to the feeding site. Unwounded *H13* plants at the same developmental stage were taken as controls.

Tissue samples from 20 plants per replicate were collected and pooled at 4, 12, 24, 48, 72, 96, and 120 hr after initial larval attack. Only those plants with 10 to 15 larvae per plant were chosen for tissue collection by using only the tissue at the feeding site. Approximately 1 cm of leaf-sheath tissue was collected for RNA extraction. The *H13* seedlings infested with v*H13* were taken as plants during compatible interactions, whereas the *H13* seedlings

infested with *GP* were taken as plants during incompatible interactions. Seedlings under the same conditions, but without infestation, were used as controls. Each experiment contained three independent sets of seedlings (biological replicates) that were treated in the same way at different times in different chambers.

Total RNA was extracted from wheat tissue by using TRI reagent™ according to the manufacturer's guidelines (Molecular Research Center, Inc. Cincinnati, OH, USA). The RNA samples were further purified through an RNease kit according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA, USA). The RNA concentrations were measured by using a NanoDrop-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Real-Time PCR Analysis After removing potential DNA contamination by DNase I, total RNA was reverse-transcribed into cDNA by using superscript reverse transcriptase following the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). After removing the RNA in the reaction by DNase-free RNase A, the cDNAs were used as template for real-time PCR (RT-PCR) analysis.

Real-time PCR was performed with iQ SYBR Green Supermix on a iCycler real time detection system (Bio-Rad, Hercules, CA, USA). Each reaction was carried out with 2 μl of a 1/40 (*v*/*v*) dilution of the first cDNA strand, and 0.5 μM of each primer in 25 μl total volume. The cycling conditions were: 95°C for 5 min followed by 45 cycles of denaturation at 95°C for 20 sec, annealing and extension at 62°C to 64.5°C, depending on the primer set, for 45 sec. At the end of the cycles, PCR specificity was verified by obtaining a dissociation curve, derived by cooling the denatured samples to 55°C and raising the temperature 0.5°C for 10 sec for each cycle, for a total of 80 cycles until reaching 95°C. The PCR products were analyzed on 1.5% agarose gels, and subsequently purified and sequenced to confirm specific amplification.

Primers (Table 1) were designed by using the Beacon Designer (v. 2.0) software from Biosoft International (Palo Alto, CA, USA). Plasmid DNA containing the corresponding insert was used to generate a calibration standard curve, where Cycle Threshold (Ct) values were plotted to serve as standard concentrations. The transcript concentration for each sample was calculated based on the standard concentrations. A negative control without template was always included for each primer set. Template concentrations of different samples were normalized against the ribosomal protein gene 21 (AF093630). For each sample analyzed, results represent the mean of values obtained from at least two independent PCR reactions and from at least three independent biological replicates.

Results

cDNAs Encoding Inhibitor-like Proteins Four groups of cDNAs were identified, and all the four groups encode small proteins with 76 to 131 amino acids (Fig. 1). All of the putative proteins contain a secretion signal peptide and have eight to 12 conserved cysteine residues in the mature protein region. However, there is no sequence similarity among members from different groups.

Group I was previously identified as defensins that were specifically induced in wheat during cold acclimation (*AI-L4*, *BAC10287*) (Koike et al. 2002). Sequence comparison revealed that proteins in Group I share high sequence similarity with a sorghum inhibitor (P21924) that inhibited α-amylases from two insect species, a locust, *Locusta migratoria*, and the American cockroach, *Periplaneta americana* (L.) (Fig. 1a) (Bloch and Richardson 1991). Because of this structural similarity, we refer to this group of cDNAs as α-amylase inhibitor-like cDNAs 1 to 4 (*AI-L1* to *AI-L4*). The specific functions of the inhibitors, however, need to be determined since protease and amylase inhibitors share structural similarities (Rawlings et al. 2005), and some can inhibit multiple types of enzymes (Odani and Ikenaka 1976).

Group II encodes proteins with >80% sequence similarity to a wheat Bowman–Birk type trypsin inhibitor (P81713) (Fig. 1b) (Poerio et al. 1994). Accordingly, members in Group II were named trypsin inhibitor-like protein 1 (*TI-L1*) to trypsin inhibitor-like protein 4 (*TI-L4*).

Unlike Groups I and II, which share sequence conservation with functionally defined proteins, Groups III and IV encode proteins that share sequence similarity with GenBank proteins whose functions have not been defined. Group III encodes proteins that belong to a large family referred to as "protease inhibitor/seed storage/LTP" (Fig. 1c) (Marchler-Bauer et al. 2007). This family is composed of protease and amylase inhi-

Table 1 Primers for real-time PCR analysis of wheat, *Triticum aestivum*, inhibitor-like genes

Gene	Sense Primer	Anti-Sense Primer
AI-L1	5′-GTCAGTCAGTGTACTTGTTC	5′-CAACGACCTTGTCACATTAC
AI-L2	5′-CCAAGAGCCACAAGTTCAAG	5′-TTACGGGACACACGATGC
AI-L3	5′-ACAGAGATGGGGACGATGAAGA	5′-AATTGTCAGTGGCATCGTT
AI-L4	5′-GTTCGTGTGGCTGTCTCC	5′-TCCGTCCATACTGAAACAAGG
TI-L1	5′-TGTAAGACCTGTGCCAAGC	5′-CTGAAAGAAGACCGAGAGAAAC
PI-L1	5′-CGTCCTCAACATCGTCCAG	5′-CCTCTCGCACCACTAATCC
TN-L1	5′-GATACACACAAACACAACGAAG	5′-GGCGAACAGGATGACCAG

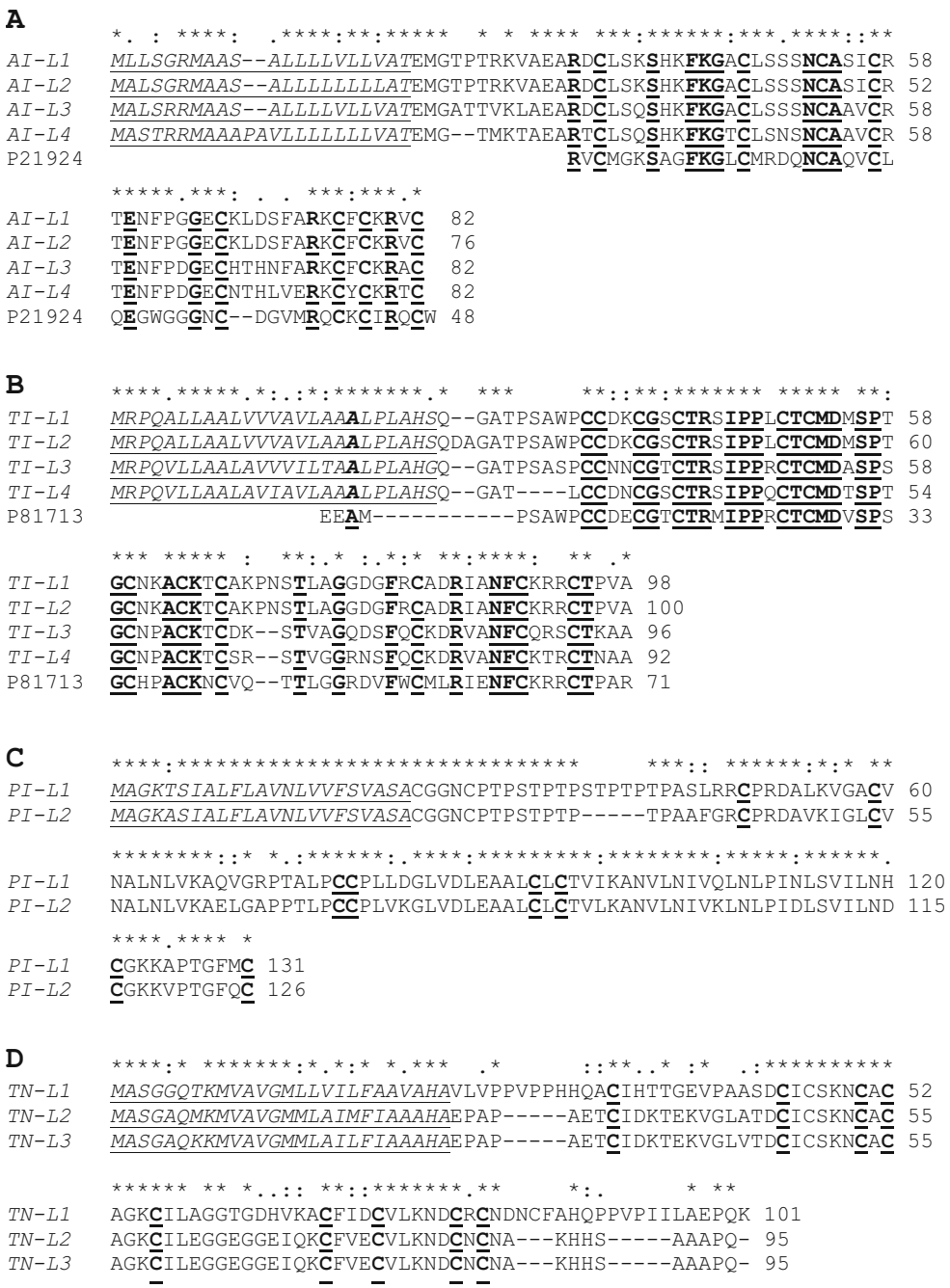

Fig. 1 Sequence alignments of inhibitor-like proteins from wheat, *Triticum aestivum*. The alignments were made by using the program *ClustalW* (see "Methods and Materials"). The *asterisk* represents conserved residues, the symbols ":" and "." represent conserved substitutions, whereas the dash (-) represents a deletion or insertion in the alignments. Bold and underlined letters represent residues that are either identical with a functionally defined protein or cysteines that are representative of the family. Predicted secretion signal peptides at the N-termini are italicized and underlined. (A) A group of proteins with sequence similarity to an inhibitor of insect amylases. (B) A group of proteins with sequence similarity to a characterized Bowman–Birk type trypsin inhibitor. (C) A group of proteins with sequence similarity to a conserved family named protease inhibitor/storage protein/lipid transfer protein (Marchler-Bauer et al. 2007). (D) A group of proteins with sequence similarity to thionins. The nucleotide GenBank accession numbers are EU293126 (*AI-L1*), EU293127 (*AI-L2*), EU293128 (*TI-L1*), EU293129 (*TI-L2*), EU293130 (*TI-L3*), EU293131 (*TI-L4*), EU293132 (*PI-L1*), EU293133 (*PI-L2*), and EU293134 (*TN-L1*). The Affymetrix identification numbers for the original ESTs are: Ta.14568.1 (*AI-L1*), Ta.28550.1 (*AI-L2*), Ta.7756.2 (*TI-L1*), and Ta.5208.1 (*TN-L1*). Other sequences previously published are nucleotide sequences AB089942 (*AI-L3*) and BT008925 (*AI-L4*), and protein sequences AAC49286 (*TN-L2*) and AAP12730 (*TN-L3*)

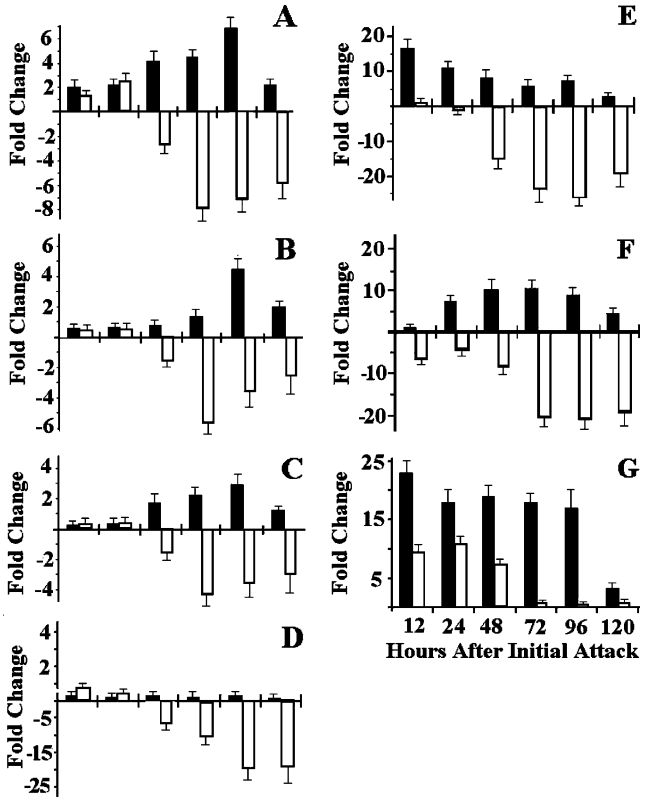

Fig. 2 Differential expression of inhibitor-like genes from wheat, *Triticum aestivum*, during incompatible and compatible interactions with Hessian fly, *Mayetiola destructor*. Solid bars represent fold changes during incompatible interactions, whereas open bars represent fold changes during compatible interactions. Samples were collected at 12, 24, 48, 96, and 120 hr after initial attack by larval *M. destructor*. A to G are for genes *AI-L1, AI-L2, AI-L3, AI-L4, TI-L1, PI-L1*, and *TN-L1*, respectively

bitors, seed storage proteins, and lipid transfer proteins from plants, but the specific function of this family has not been established. Many proteins in this family may have multiple functions such as serving both enzyme inhibitors and storage proteins.

Group IV encodes proteins referred to as thionin-like proteins because of their high cysteine content (Ray et al. 2003). This group of genes was rapidly induced in wheat upon attack by a fungal pathogen. Again, the specific functions of the thionin-like proteins remain to be determined. Many proteins previously characterized as thionins have been found to be protease inhibitors later (Bloch and Richardson 1991).

Upregulation during Incompatible Interactions (GP Insects in H13 Plants) To examine the expression patterns of these genes during incompatible interactions, RT-PCR was carried out with specific primer pairs (Table 1). During incompatible interactions, a similar pattern was observed for the four members of Group I (Fig. 2a–d), except for *AI-L4*, which was not significantly affected by *M. destructor* attacks during incompatible interactions. For the other three genes, significant upregulation began at 24 hr and maximum upregulation was reached at 96 hr. The upregulation declined at 120 hr and returned to normal at 144 hr (data not shown).

Because of high sequence similarity among members in Groups II to IV, primer pairs specific for each cDNA were not found. As a result, a common pair of primers was designed for each gene group. *TI-L1* was strongly (>15-fold) upregulated at 12 hr (Fig. 2e). Upregulation became weaker thereafter and returned to normal at 120 hr. In comparison, *PI-L1* was gradually upregulated beginning at 12 hr and reached maximum at 48 to 72 hr. *TN-L1*, on the other hand, was upregulated strongly (>20-fold) at 12 hr during incompatible and remained high up to 96 hr, followed by a sudden drop at 120 hr (Fig. 2g).

Downregulation during Compatible Interactions (vH13 Insects in H13 Plants) All inhibitor genes except *TN-L1* were strongly downregulated during compatible interactions (Fig. 2a–f). Downregulation began at 48 hr and reached maximum between 72 to 96 hr. *TN-L1* was the only gene that was upregulated during compatible and incompatible interactions (Fig. 2g). However, upregulation was much weaker during compatible interactions than during incompatible interactions, resulting in an incompatible/compatible expression ratio (I/C ratio) of 1.8 to 42 at different time points (Table 2).

Wound-induced Expression Since inhibitor genes responsive to insect feeding are also inducible by wounding (Ryan and

Table 2 Ratios between the expression levels of wheat, *Triticum aestivum*, inhibitor-like genes during incompatible and compatible interactions with Hessian fly, *Mayetiola destructor* (*I*/*C* ratio)

Gene	12 Hours	24 Hours	48 Hours	72 Hours	96 Hours	120 Hours
AI-L1	3.5±0.81	0.91±056	13±3.2	32±8.7	46±6.9	11±2.1
AI-L2	1.8±0.31	1.1±0.24	2.3±0.33	10±1.8	20±4.2	2.4±0.62
AI-L3	1.5±0.22	1.2±0.17	3.5±0.35	8.8±1.4	9.8±1.3	3.3±0.35
AI-L4	0.38±0.18	0.46±0.22	2.2±0.15	4.6±0.18	25±2.1	19±2.3
TI-L1	43±3.4	28±3.5	68±5.4	83±6.5	128±10	59±3.8
PI-L1	10±0.69	32±2.8	100±8.3	180±9.2	160±8.5	78±2.4
TN-L1	2.3±0.13	1.8±0.22	3.3±0.18	36±2.5	42±2.8	6±0.36

Moura 2002), we examined the effect of wounding on the expression of the five inhibitor-like genes, *AI-L3, AI-L4, TI-L1, PI-L1*, and *TN-L1*. All five genes exhibited higher expression levels at 12, 24, and 72 hr following wounding (Fig. 3). Induction by wounding reached maximum (1.5 to 3.5 fold) at 12 hr for *TI-L1* and *TN-L1*, and at 24 hr for *AI-L3, AI-L4*, and *PI-L1*. The wound effect on all five genes declined to baseline levels after 72 hr.

Since both wounding and avirulent larvae upregulated inhibitor-like genes, we then examined possible synergistic effect on gene upregulation. Gene upregulation by wounding and by avirulent larvae appeared additive at some time points (Fig. 3b), but no synergistic effect was observed between these two factors. During compatible interactions, the wound-induced upregulation was suppressed by virulent larvae (Fig. 3c).

Discussion

This research represents the first systematic analysis of the expression patterns of inhibitor-like genes in a plant–insect system that follows a typical gene-for-gene interaction. Several groups of diverse inhibitor-like genes were found to be strongly downregulated by insect feeding during compatible interactions, whereas the same genes were upregulated during incompatible interactions. The combination of upregulation during incompatible and downregulation during compatible interactions resulted in dramatic differences in gene expression between resistant and susceptible plants (Table 2), which strongly suggests inhibitor-like genes are part of defense mechanisms in wheat plants against *M. destructor* attacks. There are diverse proteases including various trypsins and chymotrypsins, cysteine proteases, and different carboxypeptidases in the *M. destructor* digestive system (Zhu et al. 2005; Liu et al. 2007). Elevated inhibitors along with other toxic substances may reduce nutrient uptake and absorption by *M. destructor* larvae.

For chewing insects, upregulation of inhibitor genes is achieved, at least in part, through wounding (Ryan and Moura 2002). For *M. destructor*, the mechanism for upregulation of the inhibitor-like genes appeared independent of the wounding pathway. First, wound-induced upregulation exhibited similar expression for all inhibitor-like genes examined (Fig. 3a), whereas avirulent larva-induced upregulation during incompatible interactions exhibited different patterns for different genes. Second, wound-induced upregulation was relatively weak (two- to three-fold) and for a short time. These differences suggest that the stronger upregulation for longer time is associated with avirulent larval attacks and might be mediated through an alternative pathway(s) mediated by the *R*-gene present in the wheat plants. Further research is required to delineate the mechanism(s) of the avirulent larva-induced upregulation.

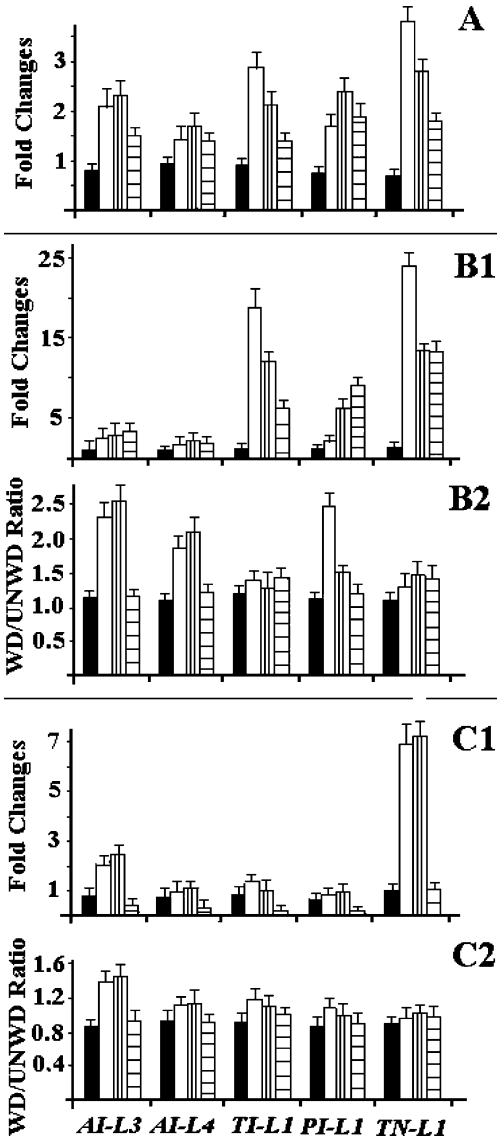

Fig. 3 Impact of wounding on the expression of inhibitor-like genes from wheat, *Triticum aestivum*. Solid, open, vertically striped, and horizontally striped bars represent data obtained from 4, 12, 24, and 72 hr after wounding or initial larval attacks by the Hessian fly, *Mayetiola destructor*. WD/UNWD represents ratios of expression levels between wounded and unwounded samples. (A) Fold changes of gene expression after wounding. (B, upper portion) Fold changes of gene expression after wounding and avirulent (biotype *GP*) larval attacks. (B, lower portion) Ratios between the expression levels of plants that were wounded mechanically and also attacked by avirulent larvae, and the corresponding expression levels of plants that were only attacked by avirulent larvae. (C, upper portion) Fold changes of gene expression after wounding and virulent (biotype *vH13*) larval attacks. (C, lower portion) Ratios between the expression levels of plants that were both wounded mechanically and attacked by virulent larvae, and the corresponding expression levels of plants that were only attacked by virulent larvae

In comparison with various patterns of gene upregulation during incompatible interactions, the downregulation of the inhibitor-like genes by virulent larvae during compatible interactions was similar for all the inhibitor-like genes (Fig. 2a–f).

Significant downregulation was at 48 hr, maximum downregulation at 72 hr, and it remained strong thereafter. The timeframe for the downregulation of the inhibitor-like genes was similar to that of the upregulation of nutrient metabolism genes during compatible interactions (Zhu et al. 2008). The same time frame for the downregulation of defense genes and the upregulation of nutrient genes indicated that gene regulation during compatible interactions was achieved by a common, coordinated mechanism(s). This coordinated mechanism might be related to the ability of virulent larvae to manipulate wheat plants for the creation of nutritive cells at the feeding site (Harris et al. 2006; Anderson and Harris 2006). Hessian fly larvae inject a large number of proteins and other substances into host plants during feeding (Haseman 1930; Hatchett et al. 1990; Chen et al. 2004, 2006, 2008). These injected proteins might target primary and secondary metabolic pathways, resulting in the suppression of plant defense and induction of nutrient supply. The suppression of defenses and formation of nutritive cells provides conditions favorable for a larva to grow and develop.

Host plant resistance is an effective way to control *M. destructor* damage. The challenge with the host plant resistance strategy is that *M. destructor* can develop new biotypes that can quickly overcome deployed resistance (Ratcliffe et al. 2000). The upregulation during incompatible interactions and downregulation during compatible interactions suggests strongly that the inhibitor-like genes are part of the mechanism of *R*-gene mediated defense against *M. destructor*. Further research on the regulatory pathways of defense genes may lead to a more durable resistance by by-passing the major dominant *R*-genes, which are easy to be overcome by new, virulent biotypes.

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