Expression of two wheat defense-response genes, Hfr-1 and Wci-1, under biotic and abiotic stresses

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Abstract

Wheat plants respond to attack by Hessian fly in a gene-for-gene manner resulting in either a compatible or an incompatible interaction depending on the genotype of the infesting larvae. We previously reported a Hessian fly-responsive wheat gene (Hfr-1), up-regulated during incompatible interactions, that showed high sequence identity to a wheat gene (Wci-1) known to be induced by benzothiadiazole (BTH). Here, we analyze the temporal expression of these genes under biotic (Hessian fly/aphid/virus) and abiotic (mechanical wounding/water-deficit) stresses, as well as during treatments with the global signaling molecules, salicylic acid (SA), methyl jasmonate (MeJa) and abscisic acid (ABA) using northern hybridization and quantitative real-time PCR. Virulent Hessian fly infestation increased the expression of both Hfr-1 and Wci-1 in the crown tissue, while low systemic induction was observed in the leaf blade tissue. Although both genes were up-regulated to higher levels during incompatible interactions in multiple wheat genotypes, Hfr-1 transcripts accumulated to a higher level than Wci-1 transcripts. In response to infestation by non-viruliferous and viruliferous bird cherry-oat aphids, Wci-1 was up-regulated, while Hfr-1 was not. SA and BTH treatments up-regulated both genes, whereas MeJa and ABA up-regulated only Wci-1. When leaves were mechanically wounded, Wci-1 mRNA was up-regulated but Hfr-1 was not, while water-deficit stress up-regulated Hfr-1, but not Wci-1. Our results show that despite high sequence identity, Hfr-1 and Wci-1 exhibit differential expression profiles in response to various stresses and are regulated through separate signaling pathways. Hfr-1 responds to defense mechanisms elicited by feeding of Hessian fly larvae and water stress, while Wci-1 shows characteristics of a general defense-response gene in most of the biotic and abiotic stresses we investigated.

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1. Introduction

Crop plants are constantly exposed to a wide array of environmental stresses that cause major losses in productivity. Resistance and susceptibility to these biotic and abiotic stresses are complex phenomena, in part because stress may occur at multiple stages of plant development and often more than one stress simultaneously affects the plant. To cope with various environmental challenges, plants execute a number of physiological and metabolic responses [1]. One of the mechanisms involved in plant defenses is the induction of specific defense and pathogenesis-related (PR) genes. These stress-responsive genes are regulated by multiple signaling networks [2,3] with significant overlap between the patterns of gene expression that are induced in response to different stresses [4–6]. However, induced defense mechanisms can be costly to the plant, diverting resources that would otherwise be utilized toward reproduction [7,8]. Consequently, resistance mechanisms that prioritize the involvement of specific members of a gene family against the stress at hand, may result in less costly modes of defense.

In a previous report, we isolated and characterized a Hessian fly-responsive wheat gene (Hfr-1; GenBank accession no.
AF483596), a defense gene that is a member of a small gene family. Hfr-1 was up-regulated rapidly in incompatible interactions between wheat (Triticum aestivum L., em Thell.) and first-instar larvae of the Hessian fly, Mayetiola destructor (Say) [9]. Database searches and sequence comparisons revealed that Hfr-1 showed high sequence identity (68% amino acid and 83% nucleotide) to a wheat chemically induced gene (Wei-1; GenBank accession no. U32427). Wei-1 was previously shown to be up-regulated by a systemic acquired resistance (SAR) inducer and analog of salicylic acid (SA), benzo (1,2,3), thiadiazole-7 carboxthioic acid S-methyl ester (BTH), and by the fungal pathogen Erysiphe graminis [10]. Both genes appear to encode jacalin-like mannose-binding lectins [9], suggesting that Hfr-1 may act as a feeding deterrent to Hessian fly larvae or may coat the larval mid-gut and interfere with nutrient absorption. The high degree of sequence identity between Hfr-1 and Wei-1 suggested that their roles in defending the plant against various stresses may be similar.

Among the many stresses that wheat plants endure during development is infestation by Hessian fly, one of the most destructive insect pests of wheat, belonging to the gall midge family (Diptera: Cecidomyiidae) [11]. Within a few hours of hatching from the eggs, Hessian fly larvae crawl down the leaf blade to the base of the plant (crown) where they feed on the abaxial surface of the youngest leaf sheaths [12]. Larvae that infest resistant plants die within 3–5 days of hatching [13]. However, the successful establishment of larvae on susceptible plants is associated with a wide range of plant developmental changes, including increases in leaf sheath cell permeability [14], formation of nutritive tissue around the feeding sites [15], plant stunting [16] and accumulation of chloroplasts [17]. These symptoms become irreversible after 4–5 days of virulent larval feeding [18].

Biotypes of the fly are defined by their virulence or avirulence to wheat cultivars possessing specific resistance genes. Thirty-two Hessian fly R genes have been identified so far [19–23]. The interactions between wheat and Hessian fly larvae operate in a very specific gene-for-gene manner [24]. The response of a given plant genotype may result in either a compatible interaction (susceptible to the insect) or an incompatible interaction (resistant to the insect), depending on the genotype of the infesting larvae. It has been postulated that during compatible interactions, the first-instar Hessian fly larvae inject salivary substances [25] that result in plant stunting and physiological changes required for larval survival. Incompatible interactions are characterized by recognition of larval salivary components by the plant and induction of defense genes that lead to larval death.

In the present work, we report the first detailed comparison of two closely related defense genes as they respond to multiple biotic and abiotic stresses. Expression of Hfr-1 and Wei-1 was quantified by quantitative real-time PCR (qRT-PCR) and response to elicitors of defense pathways was monitored. We hypothesized that the high degree of sequence identity between Hfr-1 and Wei-1 would be reflected by similar expression profiles under different stresses. However, our results clearly show striking differences in the expression of the genes under the various treatments investigated in the present study. We show that Hfr-1 responds to Hessian fly infestation and water-deficit stress unlike Wei-1, which is more of a general defense-response gene. We now establish conclusively that although these genes show a high degree of similarity in terms of their nucleotide and encoded amino acid sequences, their applications in defense appear to be quite distinct and prioritized.

2. Materials and methods

2.1. Plants, insects and infestations

2.1.1. Hessian fly infestation

Expression studies were carried out on ‘Iris’ wheat (T. aestivum), harboring the H9 Hessian fly-resistance gene. This genotype of wheat is susceptible to Hessian flies (M. destructor) with the vH9 (virulence to H9) allele and resistant to Biotype L flies containing the avrH9 (avirulence to H9) allele. Biotype L and vH9 flies were maintained as purified laboratory stocks in a 4 °C cold room at the USDA-ARS Crop Production and Pest Control Research Unit, Purdue University.

H9 wheat seedlings were grown, in Ray Leach Conetainers (Portland, OR; 21 cm length × 4 cm diameter) filled two-thirds with Turface MVP soil conditioner (Profile Products, Buffalo Grove, IL) for easy root removal, and topped with soil. The plant growth chamber was maintained at 18 °C and 24 h photoperiod with a light illumination supplied at 250–300 μmol m⁻² s⁻¹ throughout the experiment. Each conetainer contained three to five wheat seedlings. The plants were watered as required and fertilized with Peter’s fertilizer (Peter’s Fertilizer Products, W.R. Grace and Co., Allentown, PA). At the two-leaf stage (7–10 days after germination) approximately 750 plants were covered with a cheesecloth tent and infested with about 1000 newly emerged adult female and male Hessian flies of Biotype L or vH9, which were allowed to mate and ovipost for 24 h. Three days after infestation, five plants from each treatment were sampled throughout the day and inspected for hatching larvae. Time of hatch was noted and time zero was designated once multiple larvae reached the crown of the plants. Leaves of randomly selected plants were inspected after infestation and number of eggs and larvae were counted to determine infestation levels. For time-course experiments, presence of living or dead larvae were noted and leaf length was recorded to confirm compatible versus incompatible interactions 11–15 days after egg hatch. No other insects or pathogens were apparent on the experimental plants. Root, crown (extending from the junction of the root and aerial portion of the plant to about 1 cm below the ligule of the first leaf) and leaf blade tissue were harvested at 24, 48 and 72 h after egg hatch as well as from the uninfested control plants. In a separate experiment, crown tissue was also collected from plants 2, 6 and 12 h after egg hatch to compare the expression of Hfr-1 and Wei-1 during early stages of larval establishment. Tissue for this and all other experiments was harvested into liquid nitrogen and stored at −80 °C until further use.

Crown tissue was harvested from wheat lines ‘Karen’ and ‘Molly’, harboring the H11 and H13 Hessian fly-resistance
genes, respectively. In the interaction of Hessian fly with wheat containing the H11 resistance gene, Biotype L was used as the virulent fly and Biotype E was used as the avirulent fly. In the interaction with wheat harboring the H13 resistance gene, vH13 and Biotype L were used as the virulent and avirulent fly types, respectively. Plant growth and infestation conditions were similar to those described for wheat containing the H9 resistance gene. Tissue was collected 24, 48 and 72 h after egg hatch from the infested and uninsected plants.

All wheat genotypes were homozygous for their respective Hessian fly-resistance genes. Eight days after egg hatch, compatible interactions for all three (H9, H11 and H13) genotypes showed symptoms associated with infestation by virulent flies (stunted seedlings harboring white second-instar larvae). Incompatible interactions for all three wheat genotypes showed the presence of dead, red first-instar larvae and plants comparable in growth to the uninsected controls. The purity of the biotype stocks was confirmed by infesting differentials (H3 ‘Monon’, H5 ‘Magnum’, H6 ‘Caldwell’, H7H8 ‘Seneca’, H13 ‘Molly’ and H9 ‘Iris’). To limit variability among samples, 15–35 plants were pooled for each time point and plants within each replicate time series (compatible, incompatible and control) were closely monitored for hatch and infestation level to ensure comparable conditions.

2.1.2. Compatible interactions of H9 wheat with bird cherry-oat aphids and two viruses

H9 wheat plants were grown in 6-in. pots (seven seeds per pot) filled with soil and vermiculite, in a growth chamber at 18 °C with 24 h photoperiod. Wheat seedlings were grown to the two-leaf stage and infested with non-viruliferous bird cherry-oat aphids (Rhopalosiphum padi) and two viruses (NY-RPV, New York isolate of virus). The plants were divided into three treatment groups—uninfested plants, plants infested with bird cherry-oat aphids and two viruses (NY- RPV, New York isolate of virus) and cereal yellow dwarf virus (CYDV; NY- isolate of virus) and cereal yellow dwarf virus (NY- isolates of virus). The plants were divided into three treatment groups—uninfested plants, plants infested with aphids alone and plants infested with the viruliferous aphids. Infestation was carried out as described earlier [26]. Tissue comprising the leaf blades and crown was harvested at day 0 and 1, 2, 5, 7, 9, 12 and 15 days post-infestation. This time scale was chosen based on the work done by Balaji et al. [26]. According to this study, virus RNA levels peaked between 5 and 8 days post-infestation and returned to basal levels by day 15. Virus transmission was confirmed by observing symptoms of chlorosis in numerous locations on the leaves and sheaths of all virus-infected plants at day 15 post-infestation.

2.2. Treatment of plants with abiotic stresses and elicitors

2.2.1. Mechanical wounding

H9 wheat plants grown in 6-in. pots (five plants per pot), as described earlier, were subjected to mechanical wounding by pinching all along the leaf blades at intervals of approximately 5 mm using a pair of surface-sterilized blunt-tip forceps. Leaf blade tissue was harvested from the wounded and unwounded plants 24, 48 and 72 h after injury.

2.2.2. Water-deficit stress

H9 wheat plants were grown in 6-in. pots (five plants per pot) filled with Turface MVP soil conditioner and watered to saturation twice daily and fertilized once with Peter’s fertilizer. When the plants reached the two-leaf stage, water-deficit stress was initiated by withholding water. The watering schedule for control plants was invariant. Complete aerial tissue comprising the crown and leaf blade was harvested from water-deficit and control plants 24, 48 and 72 h after the stress was initiated.

2.2.3. Signaling molecules SA, BTH, MeJa and ABA

H9 wheat plants were grown as described in the above section in 6-in. pots (five plants per pot). Approximately, 50 ml of aqueous solutions of salicylic acid (SA) (10 mM; Sigma, St. Louis, MO) benzo-thiazole (BTH) (10 mM; Novartis Crop Protection Inc., Greensboro, NC), methyl jasmonate (MeJa) (45 μM; Sigma) or abscisic acid (ABA) (100 μM; Biomedicals Inc., Aurora, OH) were uniformly applied per pot as a fine mist from an aerosol spray bottle, covering the above-ground surfaces of all plants. At 24, 48 and 72 h after treatment, complete aerial tissue including the leaf blades and crown from chemically treated and untreated plants was harvested.

2.3. RNA isolation and Northern blot analysis

RNA was isolated from frozen tissue with TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. Total RNA concentration was measured with an Ultraspec 3300 UV Spectrophotometer (Amersham Biosciences, Piscataway, NJ). The extracted RNA samples (15 μg per lane) were separated in 1% formaldehyde-denaturing agarose gels, blotted onto a Hybond™-N membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and cross-linked to the membrane with ultraviolet light [27]. The Hfr-1 probe was a near full-length cDNA clone UPW1Hfr1a ([9]; GenBank accession no. AF483596) used for the preparation of the Hfr-1 probe. The near full-length cDNA clone Wci-1 ([10]; GenBank accession no. U32427), encoding the wheat chemically induced gene, was obtained from Novartis Crop Protection Inc. A 28S barley rRNA clone, pJMA-45 (650 bp fragment, provided by Dr. Joseph Anderson, USDA-ARS, West Lafayette, IN), was used as a control probe for equal loading among lanes. DNA probes were labeled with 32P-dCTP (Amersham) by random priming using the Prime-It II kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions.

After the RNA blots were prehybridized for 4 h at 65 °C in PerfectHyb Plus hybridization buffer (Sigma), they were hybridized for 18–22 h at 65 °C with 32P-labeled cDNA probes (2.1–3.1 × 106 cpm probe/ml buffer). Following hybridization, the membranes were washed at a high stringency (to prevent cross hybridization to orthologous genes) with a final wash of 0.5 × SSC and 0.1% SDS at 65 °C for 10 min. The blots were exposed to film (BioMax MR, Eastman Kodak Company, Rochester, NY) using Biotech L-Plus intensifying screens (Fisher Scientific, Fair Lawn, NJ) at −80 °C.
Quantitative real-time PCR (qRT-PCR) was conducted as previously described [28,29] with the following modifications. RNA samples were treated with RNase-free DNase at 37 °C for 30 min using the DNA-free kit (Ambion Inc., Austin, TX) prior to cDNA synthesis, to ensure that the amplicon template originated from RNA and not DNA. First-strand cDNA synthesis from RNA of all samples belonging to one stress/treatment was carried out simultaneously using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) with oligo-d(T) primers following manufacturer’s guidelines. Three micrograms of DNA-free total RNA were denatured at 65 °C for 5 min in the presence of 1 μl oligo-d(T) primer and 1 μl 10 mM dNTP mix and placed in ice. To this were added 2 μl 10× RT buffer, 2 μl 50 mM MgCl2, 2 μl 0.1 M dithiothreitol, 1 μl RNase OUT Recombinant RNase Inhibitor and 1 μl SuperScript II Reverse Transcriptase to make a final volume of 20 μl reverse transcription (RT) mix. From this, 5 μl of the mix were removed and 1 μl of 32P-dCTP was added to monitor as a parallel tracer reaction for cDNA synthesis. The 32P-labelled mix and the remaining 15 μl of the RT mix were incubated at 42 °C for 2 h. Using the 32P-labelled reactions as a quantification guide, first-strand cDNA from each sample was diluted to ensure equal amounts of cDNA template for quantification of gene expression by qRT-PCR.

To detect and quantify gene expression we employed qRT-PCR using gene-specific primers. Primers for Hfr-1 and Wci-1 genes were designed using the Primer Express Software Version 1.5 (Applied Biosystems, Foster City, CA). Specific primers for Hfr-1 gene amplification were designed on the basis of the known sequence of Hfr-1 (GenBank accession no. AF483596). The forward Hfr-1 primer sequence was 5’-CTTAAGACCTCTGTTTCTCTAGGTA-3′ and the reverse primer sequence was 5’-GATGTTGATGCGTTAACAAGC-3′. Wci-1 gene-specific primers were designed based on the known sequence of wheat Wci-1 (GenBank accession no. U32427). The forward Wci-1 primer sequence was 5’-GTTGTCGCCTTCCACCTTCTATGT-3′ and the sequence of the reverse primer was 5’-CTTGCTGATGAAACATCACACT-3′. The Hfr-1 primers amplified product from only Hfr-1 templates and not from Wci-1 templates, and vice versa, as demonstrated by the following: (i) Hfr-1 and Wci-1 primers were shown to generate amplicons from only their corresponding Hfr-1 or Wci-1 clone when used as template, (ii) Hfr-1 and Wci-1 primers were shown to amplify only their respective sequences from a cDNA time point sample (comprising transscripts of both Hfr-1 and Wci-1) which were then cloned into the pCR-4 TOPO vector (TOPO-TA Cloning Kit for Sequencing; Invitrogen) and sequenced (ABI PRISM DYE Namic ET Terminator Cycle Sequencing Kit; Amersham) on a ABI PRISM 3700 sequencer (Applied Biosystems) and (iii) melting curve analysis after each qRT-PCR experiment indicated that only a single product was amplified, as described below.

The qRT-PCR was carried out on an ABI PRISM 7000 Sequence Detector (Applied Biosystems) using the SYBR Green I dye-based detection system. The total qRT-PCR volume of 20 μl contained 10 μl SYBR Green I PCR Master Mix (Applied Biosystems), 500 nM each of forward and reverse primers and 2 μl of the cDNA samples (10 ng/μl). All reactions were set up in triplicate. No-template controls were included in each PCR plate to ensure purity of reagents and minimal carryover contamination. No-reverse transcription controls were included in the PCR runs to ensure negligible contamination of the total RNA samples with genomic DNA. In addition, qRT-PCR was carried out with ubiquitin, as a control gene, to verify equal amounts of target cDNA in all samples. All cDNA samples of each treatment were amplified simultaneously in one PCR plate. PCR was initiated with a pre-incubation at 50 °C for 2 min and denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Immediately after the final PCR cycle, a melting curve analysis was done to determine the specificity of the reaction by observing the melting temperature of the product. This was done by incubating the reaction at 95 °C for 15 s, annealing at 60 °C for 20 s and then slowly increasing the temperature to 95 °C over 20 min. Threshold cycle (Ct) number used in the real-time PCR quantification is defined as the PCR cycle number that crosses an arbitrarily chosen signal threshold in the log phase of the amplification curve.

Quantification of Hfr-1 and Wci-1 gene expression, displayed as arbitrary expression value (AEV) was based on the Relative Standard Curve Method (User Bulletin 2: ABI PRISM 7700 Sequence Detection System). Briefly, a serial dilution was constructed from a pooled sample that contained an aliquot from each cDNA sample in the experiment. To calculate AEV, the Ct for each dilution was plotted against the log of its concentration and Ct values for the experimental samples were plotted onto this dilution series standard curve. Input amounts were calculated from separate standard curves generated for each target gene. Only qRT-PCR assays that resulted in standard curves with the following parameters [30] were considered: (1) linear standard curve throughout the measured area, (2) standard curve slope between −3.7 and −3.2 and (3) R² above 0.99. The qRT-PCR results are represented in the form of two types of graphs. One type shows the AEV for each sample at the respective time point with error bars calculated from raw data. For calculations of significance, the log of the AEVs for each gene were analyzed by Analysis of Variance (ANOVA) using the PROC MIXED procedure of SAS (SAS Institute Inc. SAS/STAT User’s Guide, Version 8, 1999). The analysis model included treatment, time points and interaction between treatments and time points as fixed effects. ANOVA output yielded p-values that are indicated in the results. Data from two biological replicates (each replicate assayed three times in independent qRT-PCR experiments) were combined and included as a random effect in the analysis model. Orthogonal contrasts were used to evaluate differences between treatments at each time point. Arbitrary expression was considered statistically significant if the p-value was <0.05. Since both biological replicates showed similar trends, we depict results for one of the biological replicates. The
second type of graph shows fold-change in gene expression for the experimental samples with respect to the untreated plant sample at the respective time points. Fold change was calculated by dividing the AEV of an infested sample by the AEV of the uninfested control at that same time point. Fractions of 1 (indicating down-regulation in the infested versus the uninfested control sample) were plotted as inverse negative values (e.g. 1/5 was plotted as –5). Error bars representing the variance in technical replicates were calculated from raw data.

3. Results

3.1. Specificity of real-time primers

Hfr-1 and Wei-1 qRT-PCR primers specifically amplified their respective target sequences. Hfr-1 amplified an amplicon of 124 bp from the plasmid containing the Hfr-1 gene but not from the plasmid containing the Wci-1 gene, whereas, Wci-1 amplified an amplicon of 104 bp only from the plasmid containing the Wci-1 gene (Fig. 1). The sequence of the products amplified by the Hfr-1 primers from the cDNA time point samples containing transcripts of both Hfr-1 and Wei-1, was identical to the published Hfr-1 gene sequence (GenBank accession no. AF483596), whereas the sequence of products amplified by the Wei-1 primers was identical to the Wci-1 gene (GenBank accession no. U32427). Melting curve analysis at the end of the qRT-PCR experiments confirmed single products specific to each gene.

3.2. Preferential expression of Hfr-1 and Wci-1 in crown tissue as a response to Hessian fly infestation

Hfr-1 and Wci-1 transcript levels were studied in root, leaf blade and crown tissue during compatible and incompatible interactions of H9 wheat plants with Hessian fly larvae. Northern blot analyses detected no Hfr-1 or Wci-1 mRNA in roots of Hessian fly-infested and uninfested plants (data not shown); hence, qRT-PCR was not performed in these samples. In the leaf blades, expression levels of Hfr-1 and Wci-1 were low but detectable (Fig. 2A), with up-regulation of Hfr-1 in H9 plants infested with avirulent Biotype L (incompatible interaction) being slightly higher as compared to the H9 plants infested with virulent vH9 fly (compatible interaction) and the uninfested plants. Levels of Wci-1 transcript accumulation in leaf blade were much lower than those of Hfr-1, since the autoradiogram of the membrane probed with Wci-1 required 14 times longer exposure to reach comparable intensity. Since both genes showed barely detectable levels of expression in the leaf-blade tissue, qRT-PCR was not conducted on these samples. Autoradiogram exposure times indicated that the levels of Hfr-1 and Wci-1 transcripts were higher in the crown tissue than in leaf blade (Fig. 2B and C). It was observed that 48 h after hatch, in the incompatible interaction, Hfr-1 was up-regulated (Fig. 2B) 4.2-fold (p < 0.0001) higher than control and 1.7-fold (p = 0.003) higher than the compatible interaction. By 24 h post-hatch Wci-1 was up-regulated 11.5-fold (p < 0.0001) higher than the control and 5-fold (p = 0.0018) higher than the compatible interaction (Fig. 2C). Although in this experiment the fold-change was higher for Wci-1, Hfr-1 message is much more abundant (over 14-fold as indicated by autoradiogram exposure time). A low level of Hfr-1 and Wci-1 transcripts was detected in the crown tissue of control plants, indicating their constitutive expression.

3.3. Hfr-1 expression is up-regulated during early stages of Hessian fly infestation but Wci-1 expression is not affected

The first significant up-regulation of Hfr-1 was observed in the incompatible interaction just 2 h after egg hatch (1.5-fold above control, p = 0.03) indicating a very early response (Fig. 3A). By 6 h after egg hatch, the level of Hfr-1 transcript was higher in the incompatible than in the compatible interaction (1.8-fold above compatible, p = 0.0049), and by 12 h Hfr-1 up-regulation had reached the same level that was observed at the 24 h time point in the previous experiment. In contrast, only a small elevation in Wci-1 transcript levels was detected during the first 12 h in the infested plants (Fig. 3B).

3.4. Wheat genotypes containing different Hessian fly-resistance genes show similar patterns of Hfr-1 and Wci-1 expression

To verify that Hfr-1 and Wci-1 are also up-regulated during the defense responses of other wheat genotypes against the Hessian fly larvae, the levels of Hfr-1 and Wci-1 mRNA were quantified in wheat lines containing H11 and H13 genes for Hessian fly resistance. In the incompatible interactions of both wheat genotypes (H11 with Biotype E and H13 with Biotype L), Hfr-1 and Wci-1 showed similar expression profiles, with peak transcript accumulation 24–48 h post-hatch and decreasing by 72 h (Fig. 4A–D). The maximum level of up-regulation of Hfr-1 during these incompatible interactions with H11 and H13 wheat reached 161.2-fold at the 48 h time point (p < 0.0001) and 31.5-fold at the 24 h time point (p < 0.0001), respectively, as
3.5. Viruliferous bird cherry-oat aphid infestation triggers up-regulation of Wci-1 but not of Hfr-1

To determine whether both genes were general defense-response genes or responded specifically to Hessian fly, we investigated the expression of Hfr-1 and Wci-1 in wheat plants (containing the H9 resistance gene) infested with bird cherry-oat aphid, R. padi. Viruliferous bird cherry-oat aphids are vectors for the Barley and Cereal Yellow Dwarf Viruses. Analysis of qRT-PCR indicated no significant change in Hfr-1 mRNA levels in response to either aphid infestation alone (p = 0.48 was the lowest for any time point) or aphid plus virus infection (p = 0.16 was the lowest) during compatible interactions as compared to the uninfested control (Fig. 5A). However, both aphid infestation and aphid plus virus infection resulted in a sharp increase in the Wci-1 mRNA levels during compatible interactions (Fig. 5B). Aphids carrying the viruses (Fig. 5B) up-regulated Wci-1 more potently (maximum change compared to control was 15 days, 7331.4-fold, p < 0.0001) than non-viruliferous aphids alone (12 days, 2433.3-fold, p < 0.0001). Avirulent strains of R. padi or the viruses were not available, so responses of Hfr-1 and Wci-1 were not studied during incompatible interactions.

3.6. Mechanical wounding triggers up-regulation of Wci-1 while water-deficit stress elicits slight up-regulation of Hfr-1

In response to mechanical wounding, which in some ways mimics wounding by insect attack, Wci-1 was sharply up-regulated showing a 270.9-fold (p < 0.0001) increase in transcript levels by 48 h, whereas the Hfr-1 gene was not responsive (p = 0.8) to this treatment (Fig. 6A). Under conditions of water deprivation, Hfr-1 was up-regulated 2–2.4-fold by 24 (p = 0.01) and 72 h (p = 0.005) post-treatment, respectively (Fig. 6B). At 48 h Hfr-1 transcript levels did not show significant increases (p = 0.9). No difference in Wci-1 transcript levels was observed in plants subjected to water-deficit stress (at no time point was the p < 0.6), as compared to plants that were watered regularly (Fig. 6B).
3.7 SA and BTH elicit expression of both Hfr-1 and Wci-1 while MeJa and ABA up-regulate Wci-1

SA, a signaling molecule in systemic acquired resistance (SAR) of dicotyledonous plants, caused a 96.9-fold ($p < 0.0001$) increase in Wci-1 transcript level within 24 h of treatment that gradually decreased by 48 (43-fold, $p < 0.0001$) and 72 h (32-fold, $p < 0.0001$) post-treatment (Fig. 7A). On the other hand, Hfr-1 was up-regulated only 2-fold ($p < 0.0001$) at 48 h as compared to the untreated plants. Treatment with BTH, a synthetic analog of SA, sharply increased Hfr-1 and Wci-1 transcripts within 24 h (Fig. 7B). Hfr-1 and Wci-1 were up-regulated 164.2- ($p < 0.0001$) and 611.4-fold ($p < 0.0001$), respectively, at 72 h post-treatment, relative to the levels in untreated plants (Fig. 7B). Both MeJa (Fig. 7C) and ABA (Fig. 7D) treatment slightly down-regulated Hfr-1 (for all time points $p < 0.0007$). In contrast, Wci-1 was up-regulated strongly in response to both the treatments. As seen in Fig. 7C, MeJa resulted in a 174.6-fold ($p < 0.0001$) up-regulation of Wci-1 by 24 h. ABA treatment caused a 13.1-fold ($p < 0.0001$) increase in Wci-1 mRNA at 24 h post-treatment (Fig. 7D). Maximum fold-change of Hfr-1 and Wci-1 expression in response to various biotic/abiotic stresses and signaling molecules are summarized in Table 1.

### Table 1

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<td>$H9$</td>
<td>Wounding</td>
<td>NA</td>
<td>1.0</td>
</tr>
<tr>
<td>$H9$</td>
<td>Water stress</td>
<td>NA</td>
<td>2.4</td>
</tr>
<tr>
<td>$H9$</td>
<td>SA</td>
<td>NA</td>
<td>2.0</td>
</tr>
<tr>
<td>$H9$</td>
<td>BTH</td>
<td>NA</td>
<td>164.2</td>
</tr>
<tr>
<td>$H9$</td>
<td>MeJa</td>
<td>NA</td>
<td>0.63</td>
</tr>
<tr>
<td>$H9$</td>
<td>ABA</td>
<td>NA</td>
<td>0.43</td>
</tr>
</tbody>
</table>

HF: Hessian fly; SA, salicylic acid; BTH, benzo thiadiazole; MeJa, methyl jasmonate; ABA, abscisic acid, NA, not applicable.

$^a$ Maximum fold change during the time course experiments with respect to untreated controls at the same time point.

### 4. Discussion

In the current study, we have analyzed tissue-specific and stress-induced expression of Hfr-1 and compared it with that of the closely related gene, Wci-1. Tissue-specific expression analyses of $H9$ wheat seedlings during incompatible interactions with Hessian fly larvae revealed that although Hfr-1 mRNA becomes more abundant than Wci-1 mRNA, both genes were most responsive in the crown tissue (Fig. 2B and C) being expressed at higher levels than in the leaf blade (Fig. 2A) or roots. Because at the larval feeding site Hfr-1 mRNA is at least 14-fold more abundant than Wci-1 mRNA, Hfr-1 is more likely to play the more significant role in defense against this insect. We hypothesized that wheat genotypes containing different $R$ genes would show similar defense responses to virulent and avirulent Hessian fly larvae attack. As expected, similar trends in up-regulation were observed during interactions of avirulent larvae with other wheat cultivars, carrying the $H11$ and $H13$ resistance genes (Fig. 4), indicating that response involving...
**Hfr-1** and **Wci-1** is conserved independently of plant and larval genotype or specific *R* gene. Although some up-regulation of both genes was detectable in plants involved in compatible interactions (Figs. 2–4), the level of increase above the low constitutive expression was very small compared to that seen in incompatible interactions. In a similar study, PR proteins, such as peroxidase and chitinase, were more abundant in resistant wheat genotypes than in susceptible ones responding to Russian wheat aphid infestation [31]. Incompatible interactions of Hessian fly larvae with *H9* and *H13* wheat also have higher levels of lipoxygenase mRNA compared to the compatible interactions [32].

A relatively minor increase in mRNA levels of **Hfr-1** and **Wci-1** was detected in the leaf blades of wheat seedlings infested with avirulent larvae, indicating that these genes are systemically regulated to a small degree but the main increase in expression is at the crown where larvae feed. Instances of induced systemic gene expression in response to piercing insects have been observed in other plants, including the *SLW1* and *SLW3* genes during the interaction of silverleaf whitefly (*Bemisia tabaci*) with squash plants [33], the systemic expression of **PR-1** and basic β-1,3-glucanase in tomato in response to potato aphids (*Macrosiphum euphorbiae*) and green peach aphids (*Myzus persicae*) [34], and the induction of β-1,3-glucanase activity in the response of wheat to Russian wheat aphid (*Diuraphis noxia*) [35]. Although systemic induction of defense genes may protect plants from further attack by mobile pests such as whiteflies and aphids, feeding of the relatively sessile Hessian fly larvae is restricted to the base of the wheat plant. Consequently, the plant’s ability to focus expression of
**Hfr-1** and **Wci-1** primarily at the region of contact with Hessian fly larvae may act to conserve resources. These data clearly indicate a prioritized defense at the site where avirulent first-instar Hessian fly larvae initiate interactions with the plant [25].

Variability is inherent to experiments involving interactions between plants and insects, making biological replicates difficult to synchronize [7]. The developmental rate of Hessian fly larvae is altered by many environmental factors including temperature, humidity and season. Although the use of plant growth chambers decreases variability, we were unable to control the number of eggs laid on each plant, asynchrony of hatch and time of initial larval feeding. To limit variability among samples, 15–35 plants were pooled for each time point and plants within each replicated time series (compatible, incompatible) were closely monitored for hatch time and infestation level to ensure comparable conditions. However, biological replicates were commonly grown at different times throughout the year and insect development varied. Consequently, fold changes in up-regulation of **Hfr-1** and **Wci-1** varied among experiments, even though the trends were similar among replicates. In all experiments, repeated with different virulent and avirulent larval genotypes (Figs. 2–4, plus many others not reported here), expression of both genes was higher in incompatible interactions than in compatible or controls. In these experiments, larvae were feeding primarily on leaf two, and in compatible interactions all leaves internal to leaf two in the whorl were stunted. To avoid any regulatory consequences of superimposing a wound response, the leaves were not dissected for individual analysis, even though the constitutive expression of non-responding tissue would partially mask the amplitude of the up-regulation in responding tissue. The inherent variability in larval development and environmental factors did not alter the trends among experiments, indicating that the up-regulation of these two genes is a robust component of the wheat defense response against avirulent larvae.

The initial 96 h after egg hatch is a critical period in interactions between wheat and the Hessian fly. It has been postulated that during this time, first-instar larvae inject salivary

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Fig. 5. Response of **Hfr-1** and **Wci-1** to bird cherry-oat aphids and virus. **H9** plants were susceptible to both aphids and virus. (A) qRT-PCR quantification of **Hfr-1**. Upper panel and lower panels show AEV and fold changes in gene expression, respectively, for uninfested **H9** plants and plants infested with aphids alone or aphids vectoring BYDV and CYDV; (B) qRT-PCR quantification of **Wci-1**. Upper panel shows AEV in uninfested and infested plants. Middle and lower panels show fold change of **Wci-1** expression between uninfested and either non-viruliferous or viruliferous-aphid-infested plants. Time in days (d) are indicated. Error bars represent mean ± S.E. of mean of AEV and fold change in the three replicate PCRs.
components into epidermal cells at the crown of the plant [25,36]. Recently, a group of cDNAs encoding secreted proteins has been identified from salivary glands of the first-instar Hessian fly larvae [37,38], indicating that a complex mixture of potential elicitors may be injected into the plant. Plants have evolved mechanisms to detect substances secreted by insects, resulting in the activation of defense responses [39]. If Hessian fly larvae do not elicit wheat defense responses during the first 4 days of interaction, the salivary components initiate drastic alterations in the physiology and developmental fate of the plant [14,18] such as stunting and development of a nutritive tissue at the feeding sites [15]. However, resistant plants react rapidly (within 2 h of egg hatch) with inducible defenses once larvae are detected, preventing them from developing beyond the red-larval hatching stage. Our time-course experiments demonstrated that up-regulation of \( Hfr-1 \) was detected in incompatible interactions within 2–6 h of larval hatch (Fig. 3A), during the critical period of the interaction. Because the \( Hfr-1 \) gene sequence shows strong similarity to jacalin-like mannose-binding lectins [9], thought to block absorption of nutrients or act as feeding deterrents, the \( Hfr-1 \) protein may be an early component of a defense mechanism that kills larvae via starvation.

In order to determine whether the changes in gene expression were specific to Hessian fly infestation or were a generalized plant stress response, we compared the levels of \( Hfr-1 \) and \( Wci-1 \) mRNA during virulent bird cherry-oat aphid infestation, both viruliferous and virus-free. Our results showed no change in the expression of \( Hfr-1 \) in plants infested with either viruliferous (\( p = 0.16 \)) or non-viruliferous (\( p = 0.48 \)) aphids. In contrast, \( Wci-1 \) transcript levels increased sharply after attack by both viruliferous (over 7331.4-fold, \( p < 0.0001 \)) and non-viruliferous (2433.3-fold, \( p < 0.0001 \)) aphids (Fig. 5), indicating that it participates in a broader spectrum of tested responses. In addition, simultaneous attack by both aphid and virus resulted in a higher \( Wci-1 \) response than from aphids alone. Similar additive effects have been reported in susceptible tomatoes attacked by whitely biotype B carrying tomato mottle virus [40]. Thus, our data indicate that in dealing with various biotic stresses, the role of \( Hfr-1 \) appears to be more specific to Hessian fly infestation, whereas, \( Wci-1 \) seems to have a broader and more general role.

To further investigate the specificity of \( Hfr-1 \) and \( Wci-1 \) responses, we quantified the expression of these genes during two abiotic stresses, mechanical wounding and water deprivation. Plants are often wounded by natural factors such as herbivores or by farm equipment. Response to mechanical wounding involves the induction of numerous genes, including thionin (\( THI2.1 \)), glutathione-S-transferase (\( GST1 \) and \( GST5 \)) and phenylalanine ammonia-lyase (\( PAL1 \)) [41,42]. Our results showed that \( Hfr-1 \) was non-responsive to mechanical wounding. Feeding by chewing insects, such as tobacco hornworm (\( Manduca sexta \)) larvae is known to enhance the expression of wound-response genes in tomato and potato [43,44]. However, unlike insects that consume foliage, Hessian fly larval feeding inflicts minimal cellular damage to wheat plants [25,45]. We have observed that a wound-inducible cysteine proteinase inhibitor is not induced by Hessian fly infestation in wheat.
Fig. 7. Effect of signaling molecules on expression of Hfr-1 and Wei-1. (A) SA treatment; (B) BTH treatment; (C) MeJa treatment; (D) ABA treatment. Upper panels show RNA gel-blot analyses of Hfr-1 and Wei-1 expression in the treated and untreated 99 wheat seedlings with the 28S rRNA probe as a loading control. Autoradiograms for the blots were exposed for 3 days. Middle and lower panels show fold change in expression of Hfr-1 and Wei-1, respectively, quantified by qRT-PCR. Values represent mean ± S.E. of triplicate PCRs.
containing the H9 or H13 resistance genes (Sardesai et al., manuscript in preparation). Thus, it is not surprising that expression of Hfr-1 is independent of the wound-response pathways, which are often responsive to MeJa. Similar results have been observed in Arabidopsis where HEL, a defense gene that encodes a Hevein like-protein (PR4), is induced by cabbage butterfly (Pieris rapae) larval feeding but not by mechanical wounding [42]. Unlike Hfr-1, Wci-1 was strongly up-regulated in response to mechanical wounding (note difference in band intensity between Hfr-1 and Wci-1 in Fig. 6A) and MeJa, supporting other experiments indicating that Wci-1 is a general stress-response gene. Another common abiotic stress that affects plant growth and development is the availability of water [46]. Several defense-response genes are induced by periods of water-deficit [47]. Under conditions of water deprivation, we observed a two-fold \((p = 0.005)\) increase in Hfr-1 transcripts, but no change \((p = 0.6)\) in Wci-1 expression (Fig. 6B). These results suggest that Hessian fly feeding may cause a loss of fluids that triggers mechanisms that are elicited by water-deficit stress. In studies involving silver leaf whitefly feeding on squash plants, fly-induced genes, SLW1 and SLW3, were also up-regulated by water-deficit stress [33]. Thus, Hfr-1 and Wci-1 clearly have unique roles in the wheat response to abiotic stresses and are involved with different defense pathways.

Differential expression patterns between Hfr-1 and Wci-1 in response to various biotic and abiotic stresses prompted us to study the effect of signaling molecules that are known to modulate activation of defense genes in plants. Depending on the type of stress encountered, host plants switch on the appropriate defense pathway or combination of pathways [48]. Complex signal transduction pathways mediated by SA and JA are induced by biotic and abiotic stresses in plants [41,49,50] and accumulating evidence has also established the involvement of ABA as an important stress regulator [51]. It was shown in another wheat line, ‘Kanzler’, that Wci-1 is up-regulated by exogenous SA, BTH and INA (2,6-dichloro-isonicotinic acid) treatment [10]. Our results confirm that Wci-1 is up-regulated after SA and BTH treatment of H9-containing wheat. Hfr-1 was also up-regulated in response to SA and BTH treatments. It is widely accepted that SA and BTH, a synthetic analog of SA, induce very similar defense responses. However, it was observed that SA was only weakly effective in up-regulating the expression of Hfr-1 and Wci-1 as compared to BTH. Similar results have been reported earlier where SA has been shown to be a weaker gene activator than BTH [10]. Thus, both Hfr-1 and Wci-1 seem to be regulated through a common SA-dependent signaling pathway.

Unlike SA, MeJa treatment did not elicit Hfr-1 expression, but it did trigger activation of Wci-1, which has not been previously reported. Moreover, the fact that Wci-1 is strongly up-regulated in response to mechanical wounding correlates with the involvement of jasmonic acid (JA) in the regulation of Wci-1 expression. It is well-established that some responses to wounding are regulated through the octadecanoid pathway, involving JA as the critical signaling molecule [52]. Thus, our results are not surprising as it is becoming increasingly clear that cross-talk between signaling pathways (in particular those involving salicylate and jasmonate as intermediates) is important for fine-tuning of defense responses [3,53]. Increase in SA is correlated with the down-regulation of JA-regulated defense- and wound-response genes [54]. However, cases of sequential, cooperative and synergistic interactions between SA and JA in response to pathogens have also been reported [55]. In Arabidopsis, 55 genes have been shown to be induced by SA and MeJa treatments, thus providing the evidence that antagonism between the two pathways is restricted to specific genes [5]. In contrast, Hfr-1 is neither affected by MeJa treatment nor elicited by mechanical wounding, suggesting a JA-independent regulation.

Evidence is mounting that implicates ABA, a phytohormone involved in plant growth and development [56], in the regulation of responses counteracting plant stress [51]. Genes responsive to salinity [57], drought [58] and low temperature [59] stress have been proposed to be regulated by both ABA-dependent and ABA-independent signaling pathways. Furthermore, components from wound-response signaling mechanisms overlap with plant defense responses involving ABA. In tomato, ABA perception is necessary for the increased expression of wound-induced protease inhibitor II genes [60]. These findings, along with the up-regulation of Wci-1 by MeJa, strengthen the hypothesis that Wci-1 expression may be regulated by ABA through the wound-stress signal transduction pathway. It was interesting to observe that although Hfr-1 is not up-regulated after treatment with ABA, which is a known modulator of water-deficit response genes [58], Hfr-1 transcripts accumulated to higher levels during water-deficit stress. However, not all water-deficit-induced genes are regulated by ABA [58]. Similar results have been reported for the whitefly-response genes, SLW1 and SLW3, in squash. Although both genes are induced by water stress, SLW1 responds to ABA, but SLW3 does not [33]. Thus, water-deficit stress may regulate expression of Hfr-1 through an ABA-independent signaling mechanism.

The differential responses of Hfr-1 and Wci-1 to global signaling molecules, abiotic stresses (wounding and water-deficit) and biotic stresses (Hessian fly, aphid infestation and virus infection) strongly suggest that the two genes perform different functions in defense, even though they have evolved from a common ancestral gene. Hfr-1 and Wci-1 are members of two different sub-families located in non-homoeologous regions of different chromosomes and thus their regulatory elements have diverged (Subramanyam et al., manuscript in preparation). Hfr-1 is not a general stress-response gene since it is not responsive to most of the biotic and abiotic stresses investigated in this study. Wci-1 responds to multiple stresses with greater latitude in regulation, suggesting a role as a general defense-response gene. In contrast, Hfr-1 is elicited by Hessian fly-specific responses and is probably regulated through the SA-dependent cascade of signal transduction. Hfr-1 also shows slight up-regulation under water deprivation. As differential expression of the two genes may partially be due to differences in the intron sequences, further studies in this respect can elucidate the mechanisms of expression. Studies on differences
in structure of the promoter sequences of Hfr-1 and Wci-1 will also reveal enhancers and regulatory elements responsible for differential expression of these two closely related defense-response genes.

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References


