

Aphid Feeding Activates Expression of a Transcriptome of Oxylinin-based Defense Signals in Wheat Involved in Resistance to Herbivory

Charles Michael Smith · Xuming Liu · Liang J. Wang ·
Xiang Liu · Ming-Shun Chen · Sharon Starkey ·
Jianfa Bai

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Abstract Damage by the Russian wheat aphid (RWA), *Diuraphis noxia*, significantly reduces wheat and barley yields worldwide. In compatible interactions, virulent RWA populations flourish and susceptible plants suffer extensive leaf chlorophyll loss. In incompatible interactions, RWA reproduction and population growth are significantly reduced and RWA-related chlorophyll loss in resistant plants is minor. The objectives of this study were to develop an understanding of the molecular and phytochemical bases of RWA resistance in plants containing the *Dnx* resistance gene. Microarray, real-time polymerase chain reaction, and phytohormone assays were conducted to identify transcriptome components unique to RWA-infested *Dnx* plants and

susceptible (*Dn0*) plants, and to identify and characterize putative genes involved in *Dnx* plant defense responses. We found that RWA-infested *Dnx* plants upregulated >180 genes related to reactive oxygen species, signaling, pathogen defense, and arthropod allelochemical and physical defense. The expression of several of these genes in RWA-infested *Dnx* plants increased significantly from 6- to 24-h post infestation (hpi), but their expression in *Dn0* plants, when present, was delayed until 48- to 96 hpi. Concentrations of 16- and 18-carbon fatty acids, *trans*-methyl-12-oxophytodienoic acid, and abscisic acid were significantly greater in *Dnx* foliage than in *Dn0* foliage after RWA infestation, suggesting that *Dnx* RWA defense and resistance genes may be regulated via the oxylinin pathway. These findings provide a foundation for the elucidation of the molecular basis for compatible- and incompatible plant-aphid interactions.

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C. M. Smith (✉) · X. Liu · S. Starkey
Department of Entomology, Kansas State University,
Manhattan, KS 66506, USA
e-mail: cmsmith@ksu.edu

L. J. Wang
Division of Biology, Clemson University,
Clemson, SC 29631, USA

X. Liu
Department of Plant Biology, North Carolina State University,
Raleigh, NC 27695, USA

M.-S. Chen
USDA/ARS Plant Science Laboratory,
Manhattan, KS 66506, USA

J. Bai
Department of Diagnostic Medicine/Pathobiology,
College of Veterinary Medicine, Kansas State University,
Manhattan, KS 66506, USA

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Introduction

Aphids (Order Homoptera) are major arthropod pests of agriculture worldwide, damaging crops by removing photo-assimilates and vectoring numerous devastating plant viruses. The limited tissue damage caused during aphid feeding and the prolonged interactions of aphid mouthparts with plant cells make plant responses to phloem-feeding aphids distinct from those of chewing insects (Walling 2000). Many species of aphids are resistant to insecticides (Devonshire and Field 1991), and some have developed virulence to plant aphid

resistance genes. Environmental concerns linked to insecticide use have led to the development and cultivation of many aphid-resistant crop varieties during the past century (Panda and Khush 1995; Smith 2005).

The Russian wheat aphid (RWA), *Diuraphis noxia*, is a serious pest of wheat, *Triticum aestivum*, and barley, *Hordeum vulgare*, and with the exception of Australia, the aphid has been introduced globally since the early 1900s (Quisenberry and Peairs 1998). In compatible interactions with RWA, susceptible wheat plants react to the injection of RWA saliva by rolling the leaves longitudinally around the main leaf vein to form a tubular refuge that protects aphids from predators. As a result, RWA populations flourish and plants suffer extensive leaf chlorophyll and carotenoid loss (Burd and Elliott 1996; Heng-Moss et al. 2003). These reductions also are manifested as significant reductions in photosynthetic efficiency that results in weakened plants with substantially lower grain yields (Smith et al. 1991). In incompatible interactions that involve plants containing the RWA-resistance genes, RWA reproduction and population growth are significantly reduced, and chlorophyll loss is minor.

Heritable resistance to pest insects has been widely documented in many cereal, forage, fruit, and vegetable crop plants (Smith 1989) and single resistance (R) genes inherited as dominant traits control resistance in both monocots and dicots (reviewed in Smith 2005). Resistance may be categorized as antibiosis (an adverse effect on insect biology—including mortality), antixenosis (an adverse effect on insect behavior), or tolerance (the ability of a plant to withstand insect damage). Many insect-resistant plants, including RWA-resistant barley and wheat, contain multi-category resistance (reviewed in Berzonsky et al. 2003; Smith 2005).

Ten RWA (*Dn*) resistance genes from cereal crops have been identified (Smith 2004; Liu et al. 2005) and are being deployed in the U.S. (Quick et al. 1996) and South Africa (Prinsloo 2000). However, RWA virulence occurs in Africa (Malinga et al. 2007; Tolmay et al. 2007), Asia (Dolatti et al. 2005), Europe (Basky 2003), North America (Burd et al. 2006), and South America (Smith et al. 2004).

An understanding of the molecular bases of plant-aphid interactions is progressing, albeit slowly, based on the identification of plant genes that control aphid resistance. The NBS-LRR gene *Mi-1.2* from *Lycopersicon peruvianum* controls resistance to the potato aphid, *Macrosiphum euphorbiae* (Thomas), and to three species of the root knot nematode, *Meloidogyne* spp. (Kaloshian et al. 1997; Vos et al. 1998). No monocot insect R genes have been cloned, but the transcript levels of NBS-LRR genes are affected in plants infested by aphids (Lacock et al. 2003; Klingler et al. 2005; Botha et al. 2006; Park et al. 2006). Aphid feeding activates plant defense signals similar to those involved in response to pathogen infection (Tjallingii and Hogen-Esch 1993; Walling

2000; Kaloshian 2004), but the origin of aphid elicitors of these signals is poorly understood (Urbanska et al. 1998; Miles 1999; Forslund et al. 2000).

The recognition of aphid probing and sustained feeding results in transmission of defense response signal cascades that involve jasmonic acid (JA), salicylic acid (SA), ethylene (ET), abscisic acid (ABA), and gibberellic acid (GA) (Smith and Boyko 2006). At the onset of aphid-plant interactions, aphid feeding results in the altered activation of peroxidases, intercellular chitinases, and β -1,3-glucanases involved in the release of plant cell wall oligosaccharides (Smith and Boyko 2006). Reactive oxygen species (ROS) elicitors that respond to aphid feeding may also upregulate the activity of these enzymes. Peroxidase (PER), glutathione transferase (GST), catalase (CAT), nitrate reductase, and quinone oxidoreductase genes are upregulated in aphid-infested plants (Martinez de Ilarduya et al. 2004; Zhu-Salzman et al. 2004; Divol et al. 2005; Boyko et al. 2006; Park et al. 2006; Couldridge et al. 2007).

Interactions between aphids and their host plants elicit the activation of both the JA and SA defense response pathways and the related upregulation of genes controlled by each of these plant hormones. Examples of these interactions include greenbug feeding on sorghum (Zhu-Salzman et al. 2004), the peach potato aphid (PPA), *Myzus persicae*, feeding on *Arabidopsis thaliana* and potato, *Solanum tuberosum* (Fidantsef et al. 1999; Moran and Thompson 2001; Moran et al. 2002), and potato aphid feeding on potato and tomato (Fidantsef et al. 1999; Martinez de Ilarduya et al. 2004). Results of experiments with RWA feeding on wheat, and the tobacco aphid, *Myzus nicotianae*, feeding on wild tobacco, *Nicotiana attenuate*, indicate the involvement of JA signaling but only marginal activity of SA signals in these interactions (Voelckel et al. 2004; Boyko et al. 2006).

Ethylene production increases significantly after aphid feeding on the foliage of aphid-resistant barley or wheat plants compared with susceptible plants (Miller et al. 1994; Argandona et al. 2001; Boyko et al. 2006), and sequences that code for proteins involved in ET production are over-expressed in aphid-infested *Arabidopsis* and celery, *Apium graveolens*, plants (Moran et al. 2002; Divol et al. 2005). Jasmonic acid and ET are synergistic in the defense responses of *Arabidopsis* resistance to the peach potato aphid (Dong et al. 2004) and in the induction of defense responses in squash, *Cucurbita moschata*, foliage to feeding by the silver leaf whitefly, *Bemisia argentifolii*, (van de Ven et al. 2000). WRKY proteins (with the conserved amino acid sequence WRKYGQK) modulate JA-SA interactions in *Arabidopsis* pathogen response (Li et al. 2004) and *WRKY* upregulation in tobacco plants infested by the tobacco aphid suggests that JA-SA interactions also play a role in plant defense responses to aphids (Voelckel et al. 2004). Sequences putatively involved in ABA and GA biosynthesis are

upregulated in aphid-infested foliage of celery, sorghum, and wheat (Zhu-Salzman et al. 2004; Divol et al. 2005; Boyko et al. 2006; Park et al. 2006).

The objectives of this study were to develop an understanding of the molecular and phytochemical bases of RWA resistance in wheat plants that contain the *Dnx* RWA resistance gene. Microarray, real-time PCR, and phytohormone assays were used to identify transcriptome components and phytohormones that were differentially regulated in RWA-infested *Dnx* plants and susceptible (*Dn0*) plants. Previous research (Dong et al. 2004; Voelckel et al. 2004; Boyko et al. 2006; Park et al. 2006), led us to hypothesize that unique components of the *Dnx* transcriptome in the ROS, oxylipin, ABA, and terpenoid pathways are activated by RWA feeding, and that these proteins may function to mediate the expression of the *Dnx* plant phenotype.

Methods and Materials

Plant Genotypes and Aphids A wheat landrace from Afghanistan (USDA Plant Introduction 220127) (Harvey and Martin 1990) containing the RWA resistance gene *Dnx* (Liu et al. 2001) was crossed to the RWA-susceptible wheat genotype ‘Sando’s Selection 4040’ (*Dn0*), originally developed at Oklahoma State University. F₂-derived F₃ family plants originating from this cross were bulked into groups of 10 resistant plants and 10 susceptible plants according to their reaction to RWA biotype 1 (RWA1) infestation (Liu et al. 2001). Bulk lines were advanced to the F₄ generation. Seeds of resistant and susceptible F₄ lines were planted in 12 cm diam plastic pots containing Jiffy® potting mix and grown in the greenhouse during July 2005 at 30°C day: 22°C night, and a photoperiod of 14:10 (L:D). Supplemental lighting was provided from sodium halide lamps. Experiments were conducted in the greenhouse under the same conditions. Seed stocks are currently maintained by the Plant Resistance Laboratory, Department of Entomology, Kansas State University (KSU), Manhattan, KS, USA. RWA biotype 1 (RWA1) used in all experiments originated from a culture established with aphids collected from Hays, KS, in 2004, courtesy of Dr. J. P. Michaud, KSU Dept. of Entomology, Hays, KS. The culture was maintained on RWA-susceptible ‘Jagger’ wheat plants at the environmental conditions described above for plant growth.

In total, 140 plants with the *Dnx* gene and 110 plants lacking the gene (*Dn0*) were assessed across five different experiments. These included plant phenotyping experiments to classify plant and aphid responses to each other (20 plants of each genotype), a microarray experiment to assess wheat EST expression (90 *Dnx* plants, 60 *Dn0* plants), northern blot verification of microarray results (9 plants of each genotype), real-time PCR verification of

microarray results (9 plants of each genotype), and phytohormone experiments (12 plants of each genotype).

Phenotype Evaluations The previously reported resistance of *Dnx* and susceptibility of *Dn0* plants to RWA1 (Liu et al. 2001; Boyko et al. 2006) was confirmed in experiments to measure phenotypic damage expression, tolerance resistance, and antibiosis resistance with protocols of Boyko et al. (2006). Pre-germinated *Dnx* and *Dn0* F₄ seeds were planted in pots and allowed to grow to the two-leaf stage. Plants were paired for height and growth (*Dnx* with *Dnx*, *Dn0* with *Dn0*), and one plant of each pair was infested with five RWA1 late stage nymphs. The remaining plant of each pair was not infested and used as a control. All pots were covered with individual nylon-mesh cages, and 10 pairs (replicates) of plants were arranged in a randomized complete block design. When the infested *Dn0* plants showed complete leaf rolling and 95% chlorosis of the youngest leaf (~21 d), cages were removed and leaf chlorosis, leaf rolling and leaf folding damage sustained from RWA1 feeding was rated on a 0–3 scale for each symptom; where 0 = no damage, 1 = <50% symptoms, 2 = >50% symptoms and 3 = 100% symptoms/dead plant) (Smith et al. 1991).

Tolerance resistance, the ability of a plant to withstand arthropod damage and yield significantly greater dry mass than a susceptible plant under similar conditions of infestation (Smith 2005), was measured by calculating the proportional plant dry weight change (DWT) of *Dnx* and *Dn0* plants as $DWT = [(WC - WT)/WC] \times 100$, where WC was the dry weight of the uninfested control plant, and WT was the dry weight of the infested plant (Reese et al. 1994). The shoots from uninfested and infested plants were cut at the soil surface, placed in aluminum foil pouches, dried in an oven at 75°C for 72 h, and tissue weights were determined.

Antibiosis resistance, in which the plant adversely affects the growth and development of the arthropod, was determined by counting the numbers of RWA1 on the infested plants in each of the pairs of plants in the tolerance experiment. Aphids produced on infested plants of each genotype were removed with a camel’s-hair brush, collected on wax paper, funneled into vials of 95% EtOH, and counted. A tolerance index (TI) was calculated by using the equation: plant DWT/number of aphids produced on the infested plants (Reese et al. 1994). The plant TI was determined to compensate for the confounding effect of differing numbers of RWA1 on infested plants. Genotypes with TI values significantly lower than those of the susceptible control plants were considered tolerant. Data for plant damage, plant DWT, plant TI, and RWA1 population development were subjected to ANOVA by using the SAS GLM procedure (SAS 2001). Where significant, treatment means were separated by using the LSD at $\alpha=0.05$.

RNA Extraction For the microarray hybridization experiments, RNA was extracted from leaves of *Dnx* and *Dn0* plants at the two-leaf stage of growth when the 3rd leaf was beginning to unfurl. Plants were grown in the greenhouse as mentioned previously and were not under drought stress. There were three replications of *Dnx* infested plants, three replications of *Dnx* uninfested plants, two replications of *Dn0* infested plants, and two replications of *Dn0* uninfested control plants. Each replicate of each treatment contained 15 plants. Each of the 15 treatment plants was infested with approximately 50 RWA1 late stage nymphs and adults. In each treatment replicate, leaves of all plants were harvested 24 h after infestation, pooled, quick frozen in a freezer at -80°C , and ground in liquid N_2 . Total RNA was extracted from pooled leaves with TRI Reagent™ following the manufacturer's protocol (Molecular Research Center Inc., Cincinnati, OH, USA). RNA samples were purified with a RNease Kit following the manufacturer's instruction from QIAGEN Inc. (Valencia, CA, USA). RNA concentrations were measured by using a NanoDrop-1000 Spectrophotometer from NanoDrop Technologies (Wilmington, DE, USA). RNA quality was checked with an Agilent 2100 Bioanalyzer following the Reagent Kit guide from Agilent Technologies (Foster City, CA, USA).

Microarray Hybridization and Data Analysis The Affymetrix GeneChip® Wheat Genome Arrays (Santa Clara, CA, USA) contain 61,127 probe sets representing 55,052 transcripts for all 42 chromosomes in the wheat genome on a single array. The array includes ESTs from *T. monococcum*, *T. turgidum*, and *Ae. tauschii*, and GenBank® full-length mRNAs from all species through May 18, 2004. Labeling and hybridization of arrays were performed according to the standard protocol provided by Affymetrix. (www.affymetrix.com/support/technical/manual/expression_manual.affx). After hybridization and washing, arrays were scanned with an Affymetrix GeneChip Scanner 3000. Hybridization quality was verified by scaling factor, overall hybridization rate, and signal strength of several bacterial spike controls. The spike controls were hybridized with labeled targets in different concentrations resulting in particular ratios between different spikes.

Image acquisition, image settings, and raw data generation were accomplished with Affymetrix GeneChip® Operating Software (GCOS). After alignment of the image settings for each chip, raw data were extracted and marked as “present” (well above the background), “marginal,” or “absent” (similar to, or below the background) under default settings. The overall target signal intensity of each chip was scaled to 500 and then loaded into GeneSpring GX 7.3.1 software (Agilent Technologies, Palo Alto, CA, USA) for further analysis. The data were pre-normalized to the 50th percentile per chip and then normalized based on per-gene normalization with the median method, from which the signal of each gene in a

particular sample was divided by the median signal of the same gene in all samples.

Normalization per gene and per chip of the log₂ values was performed to allow the comparison of the independent replicates performed in the experiment. Normalization was performed for all measurements by using the flags (“present,” “marginal,” or “absent”) assigned by Affymetrix treatment of the arrays. However, only those transcripts that were declared present or marginal in at least 4 of the 6 microarrays hybridized with RWA-resistant *Dnx* probes and 3 of the 4 arrays hybridized with RWA-susceptible *Dn0* probes were taken into account. This procedure facilitated the elimination of transcripts with very low signals in both treatments (declared “absent”).

The resulting genes that exhibited significant changes in expression in comparisons between treated and control *Dnx* or *Dn0* plants were selected by applying a *t*-test (one-way ANOVA Welch *t*-test, $P > 0.05$ with “Benjamini and Hochberg False Discovery Rate” for multiple comparisons $\alpha = 0.05$). A cutoff value of a 2-fold change, commonly used for microarray analysis, was used to discriminate the expression of genes that were differentially altered in response to RWA feeding. Fold change values were calculated between treatment and control samples based on the normalized average measurement of the signal intensity. The number of sequences examined was further narrowed after eliminating those with raw expression values of < 600 units.

Comparisons of changes in differential up- or down-regulation of gene expression between RWA-infested *Dnx* and *Dn0* plants were discriminated by using the CEDA (Comparative EST Data Analysis) virtual subtraction procedure developed by Wang and Zhang (2004). Genes expressed in uninfested control plants were removed from those in infested plants with the formula: [(*Dnx* infested-*Dnx* control)-(*Dn0* infested-*Dn0* control)] for *Dnx* plants, and [(*Dn0* infested-*Dn0* control)-(*Dnx* infested-*Dnx* control)] for *Dn0* plants. The CEDA output provided a list of candidate up- or downregulated genes, uniquely expressed in *Dnx* infested plants or *Dn0* infested plants, and sorted by the statistical significance of change in gene expression.

The nucleotide sequences identified after probing and hybridization of arrays were clustered by using the CAP3 software tool with default settings (Huang and Madan 1999). From 61,290 array probe sequences, a non-redundant dataset containing 7,511 contigs and 42,863 singletons was obtained that allowed the comparison of the expression of different probes mapped to a single contig. To understand gene function, non-redundant sequences were searched against the UniProt reference database (Bairoch et al. 2005) with the BLASTX program downloaded from the NCBI site (<ftp://ftp.ncbi.nih.gov/blast/>). Queried sequences were annotated by using the best hit in UniProt with an E value threshold of 10^{-5} . Functional annotation included text

description as well as gene ontology terms of each matched reference sequence (Camon et al. 2003), to understand the biological processes involving genes of interest. Unique known transcripts in *Dnx* and *Dn0* plants were compared for similarity to known genes, and these genes were classified by function.

Northern Blot Analyses To validate the expression of ESTs in the microarray hybridization experiment, a separate northern blot experiment was conducted with total RNA isolated from fresh leaf tissue of RWA-infested resistant (containing the *Dnx* gene) and susceptible (containing the *Dn0* gene) wheat plants infested with 20 RWA biotype 1 late stage nymphs per plant. Tissues were collected at 6-, 12-, 24-, 72-, and 120 h post-infestation, and RNA was extracted with TRI reagent™ (Molecular Research Center). Each treatment consisted of RNA pooled from two biological replicates, and each replicate contained leaf tissue from 3 plants, for a total of 6 plants. Three separate uninfested *Dnx* plants and 3 separate uninfested *Dn0* plants served as non-infested controls. Tissues were collected from plants at each time point. Five µg of total RNA from each treatment were subjected to electrophoresis in denaturing 1.5% agarose gels containing formaldehyde and transferred onto a GeneScreen membrane (Perkin-Elmer Life Science Inc., Boston, MA, USA). Gels were stained with ethidium bromide, and rRNA levels were compared to control for equal loading. Membranes were baked at 80°C for 2 h to fix the RNA and then hybridized separately to individual probes. Primers for PCR amplification were generated at the KSU Core Biotechnology Facility by using selected sequences from the Affymetrix GeneChip® Wheat Genome Array EST files (Table 1). Probes were derived by PCR amplification of plant template cDNAs and labeled with ³²P-dCTP by using the random labeling kit from Stratagene (La Jolla, CA, USA). Prehybridization (4 h) and hybridization (overnight) were carried out at 42°C in hybridization buffer (Chen et al. 2004). Membranes then were washed at medium stringency [2x SSC at room temperature for 30 min, 2x SSC, 1% sodium dodecyl sulphate (SDS) at 65°C for 30 min, and 0.1x SSC, 0.1% SDS at room temperature for 30 min]. Blots were placed against X-ray film (Kodak) and hybridization patterns were visualized by autoradiography.

Real-time Quantitative PCR Analyses Real-time PCR was used to confirm the expression of six stress response or cellular metabolism genes in leaf tissues from both *Dnx* and *Dn0* plants. These genes had been shown previously to be highly downregulated in the transcriptome of *Dnx* plants at 24 hpi. Genes included Q9P3N1 (hypothetical stress response protein), Q5ZD81 (calmodulin-like protein), Q6Z1A3 (putative NAC1 stress response protein),

Q6Z1A3 (putative cytochrome P450), Q7XN01 (transcription protein), and Q6I5G9 (mitochondrial ATP synthase). Primers were designed based on Affymetrix™ Gene Chip EST sequences and GeneBank by using the software package Beacon Designer. Primers sequences are shown in Table 1. Wheat actin (AB181991) was used as an internal control. Total RNA was isolated as described for northern blotting. After purification with TURBO™ DNA-free (Ambion, Austin, TX), 2 µg total RNA were reverse transcribed into cDNA by using a SuperScript III First-Strand cDNA Synthesis System (Invitrogen) following the manufacture's protocols.

The change in expression of these genes was determined at 6-, 12-, 24-, 72-, 96-, and 120 hpi in tissues from *Dnx* and *Dn0* plants infested with 20 RWA biotype 1 late stage nymphs per plant. For each post-infestation—genotype treatment, RNA was collected from two biological replicates, each consisting of leaves pooled from 3 plants. Three separate uninfested *Dnx* plants and 3 separate uninfested *Dn0* plants served as non-infested controls. Real-time PCR was performed with iQ™ SYBR Green Supermix (BIO-RAD) by using the following amplification protocol: 5 min denaturation at 95°C, 40 cycles of 30 s at 95°C, 20 s at 53°C, and 45 s at 72°C. This was followed by product melt to confirm a single PCR product. Gene downregulation fold change was calculated as: $2^{\Delta\Delta Ct} [\Delta\Delta Ct = (Ct_{GOI}In - Ct_{GOI}Un) - (Ct_{HKG}In - Ct_{HKG}Un)]$, where GOI = gene of interest, In = infested sample, Un = uninfested sample, HKG = wheat-actin gene. Data for mean downregulation of each gene were subjected to ANOVA by using the SAS GLM procedure (SAS 2001). Where significant, treatment means were separated by using the LSD at $\alpha=0.05$.

Phytohormone Analyses Individual two leaf stage plants containing *Dnx* or *Dn0* were grown in 10 cm diam. plastic pots filled with Pro-MixBx® potting mix, in the greenhouse [24°C day: 20°C night, photoperiod of 14:10 (L:D)]. Six *Dnx* or 6 *Dn0* plants were each infested for 12-, 24-, 48-, or 96 h with 20 to 30 RWA1 late stage nymphs and adults per plant. Six uninfested *Dnx* or *Dn0* plants collected at 24 h post-infestation served as uninfested controls for all infested *Dnx* or *Dn0* treatments. At the end of each infestation period, all above ground foliage from plants in each treatment were harvested, placed in a freezer at -80°C, ground to powder in liquid N₂, weighed, and analyzed.

Plant powders were extracted in 300 µl cold 1-propanol : H₂O : HCl (2:1:0.005), centrifuged for 1 min, the organic layer transferred to a glass tube, and diluted with 20 µl of 2 M trimethylsilyldiazomethane:methylene chloride (1:4). Each extract then was combined with 20 µl of (12:88 acetic acid:hexane) : methylene chloride (1:4) and allowed to stand overnight to quench methylation. Samples were placed in SuperQ columns, where phytohormones were

Table 1 Oligonucleotide primers used for isolation and expression analysis of wheat proteins involved in defense responses to Russian wheat aphid biotype 1

Purpose/Primer name	Sequence (5'—3')
Northern Blot Analyses	
Q9XEN7 β -1,3-glucanase	
5'	CTCTTCAACCCGGACAAATC
3'	TGAAGAATTTGGGCGTTTTTC
Q8W427 Chitinase III	
5'	CGACAACCTGGACTGCTACA
3'	ATGGATCGCACCATTATTCG
Q01482 <i>WIR1A</i> Membrane protein	
5'	CTCCTGCAGATCGCTCTCTT
3'	CCGGTGGTCTACATCCGTAA
Q43212 Peroxidase precursor	
5'	AACACTGTCCGGAACCTTTGC
3'	TGTCGTGCTGGCTAGTATGC
Q8S702 Glutathione S-transferase	
5'	CCTCAGGGACTGCTCTAACG
3'	GTCCAACGATCCGAAGTTGT
Q8H8H7 Flavanone 3-hydroxylase	
5'	TACCGCAGCTACACCTACGA
3'	TGAGTAATGCTGCGTCGTG
Q9AVM3 Cytochrome P450	
5'	CATCATTGACATGTCCTGAAAA
3'	GGGCTTGACAGTAAGCAAAAA
Q5BQ31 Serine/threonine kinase	
5'	AAAAGGCACATAGCGTCCAT
3'	AGTGGTGGAGACCAGGTTTG
P27357 Thaumatin-like protein PWIR2 precursor	
5'	GCAGCACCCAGGACTTCTAC
3'	TGCGACGTATAGAGGCTTCA
P29114 <i>LOX1</i> Lipoxygenase 1	
5'	GATCGAGAGCAAGGTGGTG
3'	TCAGATGGAGATGCTGTTGG
Q5NTH3 Shikimate kinase 2	
5'	ATCCATACACAGCGGCTTTC
3'	GTAGGGCCTCGACAGCAATA
P12940 Bowman-Birk trypsin inhibitor	
5'	GACCCATCCCTCAACGTCT
3'	ACACCTGCTGGCGTATTCAT
Q6Z676 <i>phi-1</i> ABA dehydration signaling	
5'	CACCTGTTCGACCTTGGTGT
3'	GAAAGCCAGTGCAGCAATTT
P93671 <i>XET</i> Xyloglucan endotransglycosylase	
5'	GTGGGTGCAGAGCAACTACA
3'	GGCGTAAATGCCAAAAGAAGA
Q43210 <i>PAL</i> Phenylalanine ammonia lyase	
5'	ACCAGGGTAAGCACATCGAC
3'	ATCTTTGGCAATGGCCTCTA

Table 1 (continued)

Purpose/Primer name	Sequence (5'—3')
Expression Analysis (qRT PCR)	
Q9P3N1 Stress response protein	
5'	CTTCACATCTAACGGGCATC
3'	ATGGAGGTGCTTGAGACG
Q5ZD81 Calmodulin-like protein	
5'	AGGGAAGGGAAAGGATAAAGTG
3'	CGACCTACAGACAGTACGC
Q6Z1A3 NAC1 Stress response protein	
5'	GGAGGTTACATTACATTTGGAGAG
3'	TGGAGTAGCATTGGACTATTGG
Q6YXE1 Cytochrome P450	
5'	TCATGGAGAAGAACAAGCAG
3'	GAGGCGGGTGTAGAAGAG
Q7XN01 Transcription protein	
5'	GCCATTGCGGAGTCACAAG
3'	TGGTTCGCTCTTCACTATGC
Q6I5G9 Mitochondrial ATP synthase	
5'	TCATGGAGAAGAACAAGCAG
3'	GAGGCGGGTGTAGAAGAG
TA1868 Wheat actin	
5'	GAGTCGGTGAAGGTTGTTTAC
3'	CTTAGGCAGCGTTTGAATAC

removed by vapor phase extraction and eluted from columns with 100–200 μ l of methylene chloride into GC vials. The residual solvent from each SuperQ column was removed and added to each sample vial with a N_2 stream. Due to sampling errors from damage to tissues or sample loss during vapor phase extraction, the number of replications analyzed for each phytohormone treatment varied from $N=3$ to $N=6$.

Samples were subjected to quantitative analyses by using an HP 6890 gas chromatograph and HP 5973 mass spectrometer in the KSU Lipidomics Research Center. Total ion counts were acquired and processed by using Agilent Chemstation software, AMDIS (www.amdis.net), and MET-IDEA (Broeckling et al. 2006; <http://www.noble.org/plantbio/ms/MET-IDEA/index.html>). Plant tissue data were normalized by fresh weight, and sample ion counts were expressed as per cent ng/g standard. Spectral data were subjected to log e transformation and analyzed by using SAS PROC GLM with unequal variance (SAS 2001). The procedures used for tissue preparation, extraction, sample acquisition, and GC-MS analyses are detailed in Schmelz et al. (2004).

Samples were evaluated for content of the following phytohormones and fatty acids: methyl abscisic acid (ABA); methyl benzoate (BA); methyl *trans*-cinnamate (CA); methyl salicylate (SA); *cis*- and *trans* methyl jasmonate (JA); *cis*- and *trans*-methyl-12-oxophytodienoic

acid (12-OPDA); the saturated 16:0 and 18:0 fatty acids, and the 16:1, 16:3, 18:1, 18:2, and 18:3 unsaturated fatty acids. Internal standards were as follows: ABA – (*S*)-5-(1-hydroxy-2, 6, 6-trimethyl-4-oxo-2-cyclohexen-1-yl)-3-methyl-(2*Z*,4*E*)-pentadienoic acid; BA—(ring-¹³C₆) benzoic acid methyl ester; CA – (*E*)-3-phenyl-2-propenoic acid methyl ester; SA – 2-hydroxybenzoic acid methyl ester; and JA – 3-oxo-2-(2-pentenyl) cyclopentaneacetic acid. The CA internal standard was used to calculate the amount of each methyl fatty acid, OPDA-Me trans, and OPDA-Me cis. In several previous experiments, the relative instrument response of CA/methyl 19:0 fatty acid was 0.137 ng/ng.

Results

Phenotype Evaluations F₄ plants containing the RWA1 resistance gene *Dnx* displayed limited leaf rolling symptoms (1.8±0.62, mean ± SE) and leaf chlorosis symptoms (1.8±0.13, mean ± SE), compared to F₄ plants lacking *Dnx* (*Dn0*) (mean ± SE leaf rolling=2.9±0.12, mean ± SE chlorosis=3.0±0.34). (Both symptoms rated on a 0–3 scale; 0=no damage, 3=100% symptoms).

Both antibiosis and tolerance function in RWA1 resistance imparted by other *Dn* genes (Hein 1992; Smith et al. 2004; Voothuluru et al. 2006), and in tolerance experiments infested *Dnx* plants sustained significantly ($P<0.009$) smaller changes in proportional plant dry weight (DWT) compared with *Dn0* plants (Fig. 1b). However, when RWA1 population sizes on *Dnx* and *Dn0* plants were used to compute a tolerance index (TI) to compensate for different population sizes, the observed differences in plant

DWT were not significant (data not shown). Nevertheless, leaves of *Dnx* plants exhibited significantly less leaf rolling and leaf chlorosis compared to *Dn0* plants, thus demonstrating their ability to tolerate RWA1 feeding and chloroplast destruction.

***Dnx* and *Dn0* Plant Transcriptomes** There were distinct and dramatic quantitative differences in the unannotated transcriptomes of RWA1-infested plants and uninfested plants containing *Dnx* and *Dn0*. Infested *Dnx* plants upregulated 1,137 genes and downregulated 171 genes that were expressed at significantly ($P\leq 0.05$) greater levels compared to uninfested control plants (Table 2). In contrast, infested *Dn0* plants significantly upregulated only 201 genes and downregulated 16 genes.

The CEDA virtual subtraction procedure (Wang and Zhang 2004) was applied to the data in Table 2 to identify genes uniquely expressed in *Dnx* infested plants or *Dn0* infested plants. This was accomplished by using the formulae [(*Dnx* infested-*Dnx* control)-(*Dn0* infested-*Dn0* control)] for *Dnx* plants, and [(*Dn0* infested-*Dn0* control)-(*Dnx* infested-*Dnx* control)] for *Dn0* plants. CEDA subtraction yielded 551 upregulated genes and 43 downregulated genes in infested *Dnx* plants (≥ 2 -fold expression differences, $P\leq 0.05$). Of these genes, 401 of the upregulated transcripts and 27 of the downregulated transcripts were of unknown function. After ontology and grouping, 161 genes unique to RWA1-infested *Dnx* plants were significantly upregulated and only 17 genes were significantly downregulated (Table 3). After virtual subtraction of the *Dn0* transcriptome, followed by ontology and grouping, 38 genes were found to be significantly upregulated and 14

Fig. 1 Phenotypic responses of Russian wheat aphid biotype 1 (RWA1) and RWA1-resistant (*Dnx*) and susceptible (*Dn0*) plants at 21 d post infestation: (a) Mean ± S.E. RWA population development (differences significant at $P=0.001$); (b) Mean ± S.E. percent proportional leaf dry weight changes as determined by DWT = [(dry wt. of uninfested control plant–dry wt. of infested plant)/dry wt. of uninfested control] × 100 (differences significant at $P=0.009$); $N=10$

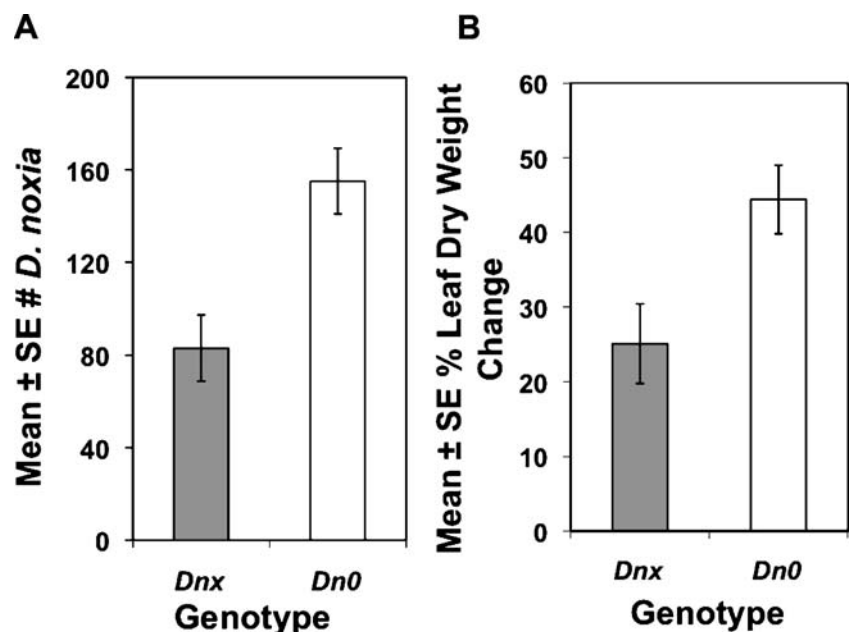


Table 2 Numbers of genes exhibiting significant changes in expression^a in comparisons between infested^b and uninfested plants of wheat genotypes resistant (*Dnx*) and susceptible (*Dn0*) to Russian wheat aphid biotype 1

Genotype/treatment comparison	Number of upregulated genes	Number of downregulated genes
<i>Dnx</i> infested versus <i>Dnx</i> uninfested control	1,137	171
<i>Dn0</i> infested versus <i>Dn0</i> uninfested control	201	16

^a $P \leq 0.05$, minimum 2-fold change^b 24 h post infestation

genes were found to be significantly downregulated (Table 3). The putative functions of affected transcripts in both genotypes were related to plant cell wall disruption; the initiation of defense responses; ROS production; ABA-, ET-, JA-, and SA signaling; phytopathogen defense responses; and arthropod allelochemical and physical defenses (Table 3). We found differences in the *Dnx* and *Dn0* transcriptomes after annotation (Table 3), compared to before annotation (Table 2), as well as before and after application of the CEDA virtual subtraction procedure.

Table 3 Numbers of annotated genes in different functional classes expressed^a in wheat plant leaves containing the *Dnx* resistance gene or *Dn0* susceptibility gene after phloem feeding by Russian wheat aphid

Gene functional class	Number of upregulated genes		Number of downregulated genes	
	<i>Dnx</i>	<i>Dn0</i>	<i>Dnx</i>	<i>Dn0</i>
β -glucanases	7	0	0	0
Chitinases	5	0	0	0
Membrane proteins	7	2	4	1
Protein kinases	12	0	1	1
GST, Ca ⁺⁺ , Fe ⁺⁺ binding	10	7	4	2
Oxidoreductase/Hydroxylase	20	4	3	1
SA signaling	16	3	0	1
JA signaling	8	5	0	0
ABA signaling	4	0	0	0
ET signaling	2	0	0	0
AUX signaling	0	4	0	0
Arthropod allelochemical defense	18	4	0	0
Pathogen defense	15	1	0	0
Arthropod structural defense	37	1	0	2
Metabolism	0	7	5	6
Total number of genes	161	38	17	14

^a $P \leq 0.05$, minimum 2-fold change^b 24 h post infestation^c *Dnx* plants = [(*Dnx* infested-*Dnx* control)-(*Dn0* infested-*Dn0* control)];*Dn0* plants = [(*Dn0* infested-*Dn0* control)-(*Dnx* infested-*Dnx* control)]

Plant Cell Wall Disruption Numerous transcripts with putative functions in the cell wall were 2- to 6-fold upregulated in infested *Dnx* plants (Supplemental Data Table 1). These transcripts include cereal β -1,3-glucanase (*GLG*), chitinase (*Chia*), protein kinase, and WIR1 (*WIR1*) membrane proteins based on sequence similarity. Among them, *GLG* and *Chia* were completely absent in infested *Dn0* plants. On the other hand, infested *Dn0* plants expressed two membrane proteins (Supplemental Data Table 2) that were not expressed in infested *Dnx* plants.

On northern blots, *GLG*, *Chia-3*, and *WIR1B* exhibited a similar expression pattern (Fig. 2a). They were expressed very little in uninfested *Dnx* plants, upregulated slightly at 6 hpi, and strongly at 12 hpi in infested *Dnx* plants. The elevated transcript levels in infested *Dnx* plants gradually decreased after 24 hpi. These three genes were either not upregulated or much less upregulated in infested *Dn0* plants until 72 or 120 hpi, when a significant elevation of the level of these transcripts was observed.

ROS Production Several genes related to ROS metabolism, including *GST*, Ca⁺⁺ and Fe⁺⁺ binding proteins, *CYP450*, oxidoreductases, and peroxidases, were 2- to 8-fold upregulated in infested *Dnx* plants responding to RWA1

biotype 1^b as determined by the Comparative EST Data Analysis virtual subtraction procedure^c

digestion or a combination of both. Plant secondary metabolites suppress insect feeding, and several transcripts associated with secondary metabolite production including cycloartenol synthase, monoterpene synthase, and shikimate kinase were upregulated 2–4 fold in RWA1-infested *Dnx* plants (Supplemental Data Table 1). On northern blots, the transcript coding for shikimate kinase was expressed in infested *Dnx* leaves at 12 hpi but was absent in *Dn0* plants at all post-infestation time points (Fig. 2e). Further, the transcript encoding a UDP-glucose glucosyltransferase, an enzyme that transfers glucose UDP-glucose to terpenes (Xiong et al. 2001, Scharrenberg et al. 2003), was upregulated 2- to 4-fold (Supplemental Data Table 1). Various enzyme inhibitors limit digestion in the insect gut (Gatehouse and Boulter 1983). A transcript encoding a Bowman-Birk trypsin inhibitor was upregulated at 12 hpi in *Dnx* infested plants, but this transcript was not significantly affected by RWA1 feeding in *Dn0* plants until a very late time point (120 hpi) (Fig. 2e). Interestingly, several phytoalexin and pathogen resistance genes underwent 2- to 4-fold upregulation in infested *Dnx* plant foliage, including *ACRE*, *HHT*, *SMM:HSM*, and *SNAP* (Supplemental Data Table 1), but these transcripts were not expressed in *Dn0* infested plants (Supplemental Data Table 2).

Downregulated Transcripts Transcripts downregulated in plants of each genotype possessed various functions (Table 3, Supplemental Data Tables 3 and 4) and several representative downregulated transcripts were chosen for real-time PCR to confirm microarray results (Fig. 3, Supplemental Data Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14.). There were no significant differences in downregulation of the stress response protein in either *Dnx* or *Dn0* tissues at 6-, 12-, 24-, or 72 hpi (Fig. 3a). A calmodulin-like gene displayed similar patterns, and in both *Dnx* and *Dn0* infested tissues, downregulation was significantly greater at 24- and 120 hpi than at 6- or 12 hpi (Fig. 3b). A transcript encoding a NAC domain pathogen response protein (Collinge and Boller 2001) was strongly downregulated in infested *Dnx* plants (Fig. 3c), and was significantly greater in *Dnx* tissues at 72- and 120 hpi than at 6-, 12-, or 24 hpi. There were no differences in expression between any post-infestation times in *Dn0* tissues. Expression of *CYP450 CA619079* was relatively low in *Dnx* and *Dn0* tissues (Fig. 3d), but was significantly greater in *Dnx* infested tissues at 24- and 120 hpi compared to 6- and 12 hpi. There were no differences in expression between *Dnx* infested tissues collected at 72 hpi and any other post-infestation time (Fig. 3d). In *Dn0* infested tissues, there were no differences in the expression of *CYP450* at any post-infestation time. Downregulation of a transcription protein and a mitochondrial ATP synthase in *Dnx* plants was significantly greater in *Dnx* tissues at

120 hpi than at 6 hpi (Fig. 3e, f). There were no significant temporal differences in downregulation of either protein in *Dn0* tissues.

Phytohormone Analyses The concentration of the 16:1 fatty acid in infested *Dnx* and *Dn0* plants increased significantly ($P<0.001$) at 12-, 24-, and 48 hpi, but returned to the control level at 96 hpi (Table 4). The concentration of 16:3 fatty acid increased dramatically in infested *Dnx* and *Dn0* plants at 48 hpi, where it was significantly greater in *Dnx* plants than *Dn0* plants. There were comparatively fewer significant differences between treatments for the 18:1 and 18:3 fatty acids. The 18:1 fatty acid content of both *Dnx* and *Dn0* infested tissues was significantly ($P<0.001$) greater than uninfested controls at 96 hpi. The 18:3 fatty acid content of *Dnx* infested plants was significantly ($P<0.001$) greater than the uninfested control at 12 hpi, and in *Dn0* plants, significantly ($P<0.001$) greater than the control at 12- and 24 hpi. This trend was similar for the 18:2 fatty acid, but the only significant increase in *Dnx* tissues over *Dn0* tissues occurred at 12 hpi (data not shown). There were no significant differences between two genotypes at any time point for 16:0 or 18:0 fatty acids (data not shown).

The *cis*- and *trans*-Me-OPDA content differed significantly ($P<0.001$) between post infestation times in leaves of *Dnx* and *Dn0* plants, and between the genotypes at each post infestation time (Table 5). In *Dnx* plants, *cis*-Me-OPDA content increased dramatically to 2.3% at 24 hpi, and was significantly ($P<0.001$) greater than the uninfested control or any other *Dnx* post infestation time. In contrast, *cis*-Me-OPDA content in *Dn0* plants was reduced at each hpi time compared to that produced in uninfested plants, and reductions were significant ($P<0.001$) at 12- and 24 hpi (Table 5). With the exception of the 24 hpi treatment, *cis*-Me-OPDA content was significantly greater in *Dn0* plants (including uninfested controls) than the corresponding *Dnx* plant treatments. Expression of *trans*-Me-OPDA was more pronounced than *cis*-Me-OPDA. In *Dnx* plants, *trans*-Me-OPDA content was significantly greater at 12-, 24-, and 48 hpi than in uninfested controls, and decreased to the level of the 0 h uninfested control at 96 hpi. As with *cis*-Me-OPDA, production of *trans*-Me-OPDA in uninfested *Dn0* plants was significantly greater than in uninfested *Dnx* plants (Table 5). However, the *trans*-Me-OPDA content of *Dn0* infested plants at all hpi intervals was significantly less than untreated *Dn0* control plants and significantly lower than that of each corresponding *Dnx* plant hpi time. In contrast to Me-OPDA, there were no significant trends in differences in *cis*- or *trans*-methyl jasmonate (meJA) content at different time points or between *Dnx* and *Dn0* plants (data not shown).

Abscisic acid (ABA) content in *Dnx* plant foliage was significantly ($P<0.001$) greater at 12- and 48 hpi than in uninfested plants (Table 6) and was significantly greater in

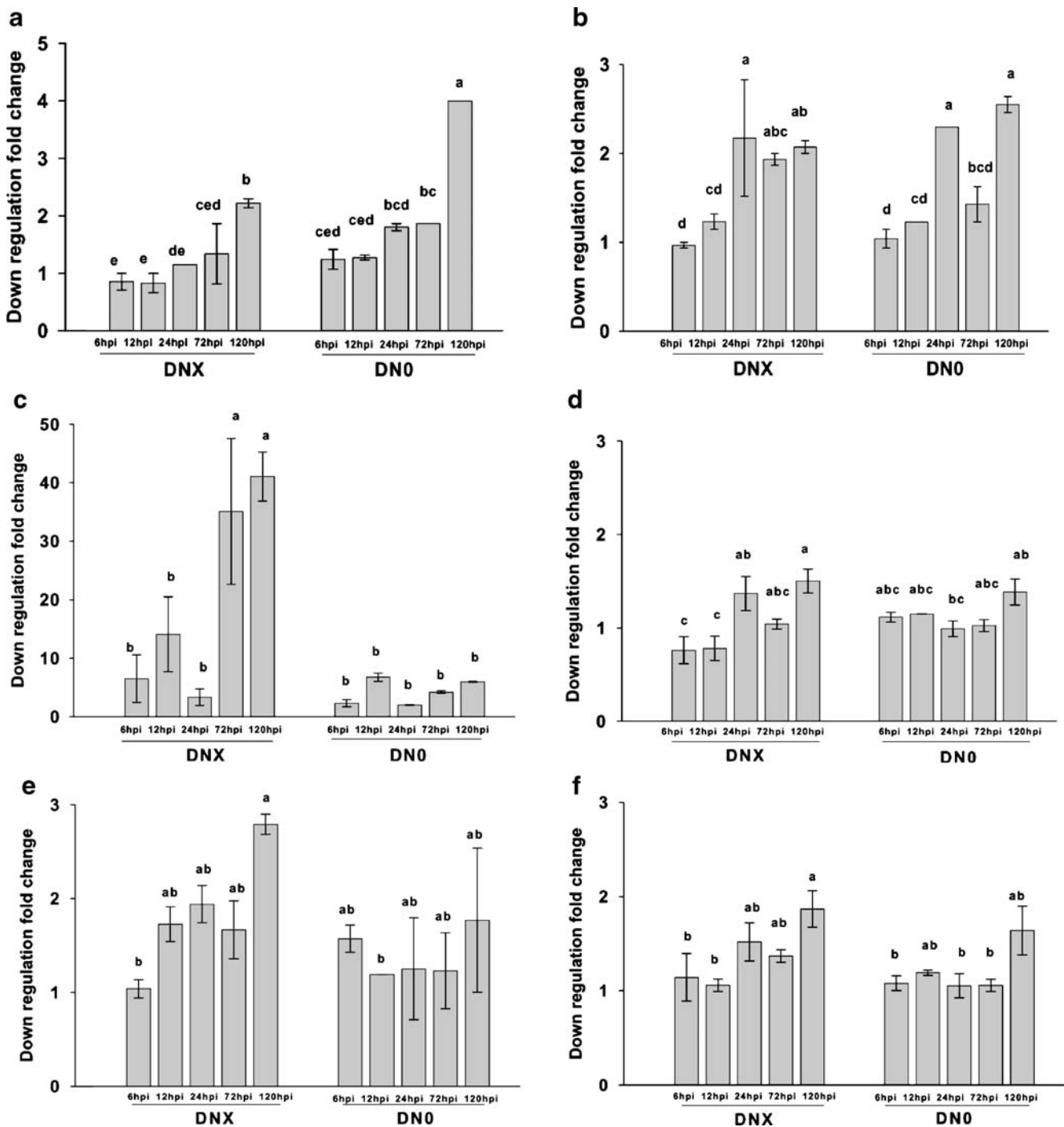


Fig. 3 Temporal downregulation (mean \pm S.E. fold change) of wheat transcripts from plants containing the RWA resistant (*Dnx*) or susceptible (*Dn0*) gene at 6-, 12-, 24-, 72- and 120 h post infestation with RWA biotype 1 adults. (a) Q9P3N1 hypothetical stress response protein; (b) Q5ZD81 calmodulin-like protein; (c) Q6Z1A3 NAC1

stress response protein; (d) Q6YXE1 cytochrome P450 (e) Q7XN01 transcription protein; and (f) Q6I5G9 mitochondrial ATP synthase. Each treatment mean represents two biological replicates, each consisting of leaves pooled from three *Dnx* or *Dn0* plants

Dnx plants than in *Dn0* plants at 48 hpi. ABA content in the foliage of *Dn0* plants was elevated at 12- and 24 hpi, but these amounts were no different from those of the uninfested control. The leaf content of benzoate, a salicylic acid

intermediate, increased significantly ($P < 0.001$) in *Dnx* foliage at 12-, 24-, and 48 hpi compared to the 0 h control, and a similar trend was absent in *Dn0* plants (Table 6). In addition, the benzoate content of infested *Dnx* plants was significantly

Table 4 Mass (% ng/g standard) of unsaturated 16:1 (7-hexadecenoic), 16:3 (7,10, 13-hexadecatrienoic), 18:1 (9-octadecenoic), and 18:3 (9,12,15-octadecatrienoic) fatty acids in leaves of Russian wheat aphid biotype 1—resistant (*Dnx*) and susceptible (*Dn0*) wheat genotypes at 12-, 24-, 48-, and 96 h post-aphid infestation (hpi), and in 0 h uninfested control plants

Post infestation (h)	Mean ± SE (% ng/g)			
	<i>Dnx</i>	<i>Dn0</i>	<i>Dnx</i>	<i>Dn0</i>
	16:1 fatty acid		16:3 fatty acid	
0	0.52±0.03 ab ^a	0.66±0.07 bc	0.04±0.04 a	0.10±0.12 b
12	1.72±0.09 e	1.65±0.05 e	0.08±0.03 b	0.08±0.04 b
24	1.02±0.04 d	1.16±0.06 de	0.14±0.05 b	0.13±0.07 b
48	0.79±0.05 cd	0.36±0.02 a	2.08±0.14 d	0.47±0.23 c
96	0.51±0.08 ab	0.89±0.05 cd	0.03±0.03 a	0.12±0.04 b
	18:1 fatty acid		18:3 fatty acid	
0	1.81±0.11 ab ^a	2.00±0.34 ab	2.28±0.20 a	4.45±2.6 a
12	2.43±0.22abcd	1.88±0.36 ab	14.43±3.11c	11.25±3.01bc
24	2.10±0.13 abc	2.30±0.20abcd	5.08±1.08 a	12.44±3.14bc
48	2.63±0.30 bcd	1.63±0.12 a	7.26±1.47 ab	2.12±0.65 a
96	2.86±0.45 cd	3.06±0.42 d	2.59±0.61 a	2.40±0.42 a

^aFor each fatty acid, means within a column followed by the same letter are not significantly different at $P < 0.001$

greater than in *Dn0* plants at one time point (48 hpi). However, there were no differences in methyl salicylate leaf content between any of the *Dnx* or *Dn0* post infestation treatments (data not shown). The amounts of the related SA intermediate *trans*-cinnamate also increased over time in both *Dnx* and *Dn0* plants, but the quantities were no different from those of uninfested control plants (data not shown).

Discussion

Plant resistance to insects is a complex process, often involving numerous plant biochemical pathways. We hypothesized that the products of unique defense proteins in the *Dnx* plant transcriptome are activated by RWA1 feeding probes, resulting in expression of the *Dnx* plant phenotype. Results

from microarray hybridizations, northern blot and real-time PCR assays, and vapor phase extraction of foliar phytohormones support this hypothesis. The results that we obtained identify unique differences in the molecular responses of resistant *Dnx* plants to RWA attack in comparison with those of susceptible *Dn0* plants, and suggest that 16 carbon membrane fatty acids; oxylipins, ABA, and ET defense response signals; and downstream defense proteins are likely components for *Dnx*-mediated resistance to RWA. Most transcripts potentially involved in resistance were upregulated more rapidly and at a greater magnitude than in susceptible plants, suggesting a more rapid and stronger induction of a *Dnx*-mediated gene network as a likely mechanism for *Dnx* resistance. This mechanism has been observed with several plant resistance responses to various pathogens (van Loon et al. 2006).

Table 5 Mass (% ng/g standard) of *cis*- and *trans*-methyl-12-oxo-phytodienoic acid (12-OPDA) in leaves of Russian wheat aphid biotype 1—resistant (*Dnx*) and susceptible (*Dn0*)wheat genotypes at 12-, 24-, 48-, and 96 h post-aphid infestation, and in 0 h uninfested control plants

Post infestation (h)	Mean ± SE (% ng/g)			
	<i>cis</i> Me-OPDA		<i>trans</i> Me-OPDA	
	<i>Dnx</i>	<i>Dn0</i>	<i>Dnx</i>	<i>Dn0</i>
0	0.21±0.13 ab ^a	1.76±0.04 c	0.08±0.08 a	1.15±0.33 b
12	0.14±0.14 a	0.25±0.07 b	0.90±0.01 b	0.05±0.07 a
24	2.29±0.03 c	0.22±0.16 ab	2.01±0.14 b	0.04±0.09 a
48	0.24±0.06 b	1.28±0.05 c	2.24±0.28 b	0.06±0.10 a
96	0.28±0.11 b	1.52±0.04 c	0.04±0.06 a	0.04±0.08 a

^aFor each OPDA isomer, means within a column followed by the same letter are not significantly different at $P < 0.001$

Table 6 Mass (% ng/g standard) of methyl abscisic acid (Me-ABA) and methyl benzoate (Me-BA) in leaves of Russian wheat aphid biotype 1—resistant (*Dnx*) and susceptible (*Dn0*) wheat genotypes at 12-, 24-, 48-, and 96 h post-aphid infestation, and in 0 h uninfested control plants

Post infestation (h)	Mean ± SE (% ng/g)			
	Me-abscisic acid		Me-benzoate	
	<i>Dnx</i>	<i>Dn0</i>	<i>Dnx</i>	<i>Dn0</i>
0	1.00±0.05 bc ^a	1.79±0.10 cde	0.01±0.04 ab	0.02±0.09 acb
12	2.34±0.05 de	1.98±0.04 de	0.43±0.22 e	0.80±0.31 de
24	1.43±0.05 cd	2.97±0.06 e	0.03±0.09 cd	0.03±0.14 cd
48	2.10±0.07 de	0.54±0.09 a	0.08±0.23 cd	0.02±0.14 ab
96	0.56±0.07 ab	0.61±0.18 a	0.01±0.08 a	0.01±0.11 a

^a For Me-abscisic acid or Me-benzoate, means within a column followed by the same letter are not significantly different at $P < 0.001$

Several results suggest the SA pathway to be less important in mediating *Dnx* resistance to RWA than the JA pathway. Fewer SA-related transcripts were upregulated in *Dn0* plants (3) versus *Dnx* plants (16), and none of those upregulated in *Dnx* plants was expressed in *Dn0* plants. Over five post-infestation time points, there were no differences between tissues of *Dnx* and *Dn0* infested plants in the content of the *trans*-cinnamate or SA. Content of the SA intermediate benzoate in *Dnx* plants was significantly greater than in *Dn0* plants at only 48 hpi.

The levels of expression of the majority of transcripts were similar between *Dnx* and *Dn0* plants. However, approximately 30% of the transcripts contained in the microarray were upregulated in *Dnx* plants, and most of them are plant defense-related genes (Table 3). In comparison, only 19% of the transcripts were upregulated in *Dn0* plants, and the proportion of these upregulated transcripts associated with plant defense was much lower. The transcripts upregulated in susceptible plants, therefore, may be related to general plant stress responses. Most, if not all ABA, ET, and JA signaling genes upregulated in *Dnx* plants were absent in *Dn0* plants. On the other hand, approximately 9% of the upregulated *Dn0* transcripts were related to auxin (AUX) signaling and these were absent in *Dnx* plants (Table 3). The expression of AUX-related transcripts may result in *Dn0* plants increasing their ROS production, as demonstrated by Boyko et al. (2006) and Kawano (2003). An additional 27% of the upregulated *Dn0* transcripts included metabolism genes (Table 3), which were not upregulated in *Dnx* plants. The downregulation of metabolic transcripts in *Dnx* plants at 120 hpi (Fig. 3) may decrease some plant metabolic activities that can enable the production of *Dnx*-related defense compounds. A related downregulation of stress response transcripts in *Dnx* plants could be due to a reduced need for these transcripts, in favor of downstream plant defenses.

At the onset of insect feeding, components of the cell wall membrane such as *GLG* and *Chita*, which were highly expressed in infested *Dnx* plants, participate in insect

defense responses in plants (Kempema et al. 2007; Park et al. 2007; see review of Smith and Boyko 2006). The increased upregulation of defense response transcripts related to ROS metabolism in *Dnx* leaves (Fig. 2b) is similar to that induced by other plant feeding insects (Schmidt et al. 2005; Couldridge et al. 2007). The expression of *GLG*, *Chita*, and ROS-related transcripts in *Dnx* infested tissues suggests their involvement in *Dnx* defense responses to RWA feeding probes.

Further, the putative role of WIR1 membrane proteins in *Dnx* resistance is strengthened by the results of Gaupels et al. (2008), who identified a WIR1A-like protein in barley phloem sap, obtained from stylets of actively feeding *Rhopalosiphum padi* aphids. Our results were similar to those of Zhu-Salzman et al. (2004) and Park et al. (2006), who demonstrated expression of CYP450 monooxygenase (MO) in aphid-resistant sorghum plants. The precise role of the CYP450MOs in *Dnx*-infested plants is difficult to determine, because these compounds function in the synthesis of JA, SA, chemical defenses, and the detoxification of exogenous chemicals such as RWA salivary components.

LOX, a gene whose transcripts are associated with the JA signaling pathway, is strongly induced by foliar feeding of numerous insects (Sardesai et al. 2005; and see Smith and Boyko 2006 review), but plant JA-induced defense responses may be antagonized by those induced by SA (Spoel et al. 2003; Koornneef et al. 2008). For example, silver leaf whitefly herbivory suppresses *LOX2* and *FAD* expression and elevates *PR1* and *PR5* expression (Kempema et al. 2007). However, the upregulation of JA- and SA-related transcripts in *Dnx*-infested plants was similar to that noted by Salzman et al. (2005), in sorghum response to mechanical wounding and by Li et al. (2008) in soybean plant response to aphid feeding. We observed induction of homologs of genes composing much of the JA and SA pathways but expression of SA transcripts was ~ 2x greater than expression of JA synthesis transcripts (Supplemental Data Table 1). Nevertheless, although SA transcripts were more highly expressed in micro-

arrays than JA transcripts, results of northern blot and phytohormone assays indicated that SA does not contribute to *Dnx* resistance (Fig. 2c, Table 6). Liu et al. (2007) reported similar results in Hessian fly resistant wheat plants.

Aphid feeding stimulates *WRKY* expression (Voelckel et al. 2004; Park et al. 2006; Li et al. 2008) (Supplemental Data Table 1), yet *WRKY* transcription factors suppress JA (Kalde et al. 2003; Li et al. 2004; Mao et al. 2007), indicating that *WRKY* expression in *Dnx* tissues may partially explain the comparatively reduced expression of JA-signaling genes. Wound-induced JA production is regulated by the supply of substrate available to allene oxide synthase (Turner et al. 2002). Thus, the minimal foliar damage and related chloroplast loss sustained by *Dnx* plants in our experiments (Fig. 1b), may have limited the release of fatty acids from chloroplast lipids for JA metabolism.

Oxylipin analyses suggest that *LOX*, *trans*-OPDA, and the 18- and 16 carbon OPDA fatty acid precursors function in *Dnx* resistance (Fig. 2d, Tables 4 and 5). The direct role of OPDA in insect defense responses (Stintzi et al. 2001) and the production of OPDA-specific response gene homologs by mechanical wounding (Taki et al. 2005) support these results. Expression of oxylipin metabolites in *Dnx* plants appears to stimulate production of downstream feeding inhibitors and toxins (Fig. 2e), several of which have been reported as resistance factors (Moraes et al. 2000; Miller et al. 2005; Lou and Baldwin 2006; Smith and Boyko 2006; Liu et al. 2007). RWA1 phloem ingestion decreases markedly on *Dnx* plants within 8 hpi (Lazzari et al. 2009), further supporting the possibility that *Dnx* feeding inhibitors and/or toxins contribute to the significant ($P < 0.001$) antibiotic effects exhibited in the reduced RWA1 population development shown in Fig. 1a. These antibiotic effects shown by RWA1 substantiate similar results of Khan et al. (2009) with plants containing *Dnx* in different genetic backgrounds. The ~50% RWA1 population reduction on *Dnx* plants in Fig. 1a is similar to that observed by Boyko et al. (2006) (57%, 66%) and Khan et al. (2009) (34%).

The lack of leaf wilting and interveinal collapse in RWA1 incompatible interactions indicates that *Dnx* resistance also may result from reduced tissue and water loss. For example, *PAL* and *CAD5*, which mediate lignin synthesis, were highly expressed in infested *Dnx* tissues (Fig. 2f), and both have been implicated in insect resistance (Ciepiela 1989; Kempema et al. 2007; Liu et al. 2007). Elevated expression of latex-abundant-, fiber (CA609522), and sorbitol transporter (BT009301) proteins (Supplemental Data Table 1) provide additional evidence of physical components of *Dnx* resistance and an additional explanation of the low tissue dry weight changes occurring in RWA-infested *Dnx* plants (Fig. 1b).

ABA- and ET- dehydration responses were uniquely upregulated in *Dnx* plants during RWA feeding (Fig. 2f,

Table 6), as reported by Salzman et al. (2005) and Park et al. (2006). ABA- and ET over-expression in response aphid-infested plants (Moran et al. 2002; Divol et al. 2005; Boyko et al. 2006) and the upregulation of the ET signaling genes in *Dnx* plants (Supplemental Data Table 1) strongly suggests their role in *Dnx* resistance. Related dehydration response transcripts were highly expressed, and one - *XET*—was differentially expressed in *Dnx* plants (6–72 hpi) and *Dn0* plants (12–24 hpi) (Fig. 2f), also demonstrating their contribution to *Dnx* resistance.

Our results indicating the upregulation of homologs of the pathogen resistance genes *ACRE*, *AP*, *Bet*, *HHT*, and *SNAP* in RWA1-infested *Dnx* tissues is the first report of their expression in response to insect feeding. Validation of the role(s) of these genes and other candidate genes in *Dnx* resistance awaits functional confirmation experiments, likely involving gene silencing. In the interim, more than 400 *Dnx* expressed transcripts are presently of unknown function. Elucidation of their function will provide additional information about putative genes and their expression patterns involved in wheat plant responses to RWA herbivory.

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