Gene-for-Gene Defense of Wheat Against the Hessian Fly Lacks a Classical Oxidative Burst

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Genetic similarities between plant interactions with microbial pathogens and wheat interactions with Hessian fly larvae prompted us to investigate defense and counterdefense mechanisms. Plant oxidative burst, a rapid increase in the levels of active oxygen species (AOS) within the initial 24 h of an interaction with pathogens, commonly is associated with defenses that are triggered by gene-for-gene recognition events similar to those involving wheat and Hessian fly larvae. RNAs encoded by Hessian fly superoxide dismutase (*SOD*) and catalase (*CAT*) genes, involved in detoxification of AOS, increased in first-instar larvae during both compatible and incompatible interactions. However, mRNA levels of a wheat NADPH oxidase (*NOX*) gene that generates superoxide (O_2^-) did not increase. In addition, inhibiting wheat *NOX* enzyme with diphenyleneiodonium did not result in increased survival of avirulent larvae. However, nitro blue tetrazolium staining indicated that basal levels of O_2^- are present in both uninfested and infested wheat tissue. mRNA encoded by wheat genes involved in detoxification of the cellular environment, *SOD*, *CAT*, and glutathione-S-transferase did not increase in abundance. Histochemical staining with 3,3-diaminobenzidine revealed no increases in wheat hydrogen peroxide (H_2O_2) during infestation that were correlated with the changes in larval *SOD* and *CAT* mRNA. However, treatment with 2',7'-dichlorofluorescein demonstrated the presence of basal levels of H_2O_2 in the elongation zone of both infested and uninfested plants. The accumulation of a wheat flavanone 3-hydroxylase mRNA did show some parallels with larval gene mRNA profiles. These results suggested that larvae encounter stresses imposed by mechanisms other than an oxidative burst in wheat seedlings.

Additional keywords: flavonoid, oxidative stress, quantitative real-time polymerase chain reaction, SYBR Green.

Many plant species initiate an oxidative burst as the first step in a complex cascade of resistance events leading to pathogen death (Doke 1985; Kepler et al. 1989). The oxidative burst begins within a matter of minutes to a few hours after initial attack. The rapid response is possible because preexisting NADPH oxidase (*NOX*) enzyme complexes, capable of generating active oxygen species (AOS), reside in the plasma membrane (Lamb and Dixon 1997) or are exocellular (Bolwell and Wojtaszek 1997). Although the oxidative burst is initiated too quickly to depend on de novo synthesis of enzymes (Doke 1983), increases in RNAs encoding oxidant-generating enzymes probably maintain the response (Lamb and Dixon 1997). The one-electron reduction of molecular oxygen to form the superoxide anion (O_2^-) is catalyzed by NADPH oxidase ($O_2 + NADPH \rightarrow O_2^- + NADP^+ + H^+$) (Lamb and Dixon 1997). Superoxide anions then undergo either spontaneous dismutation (Sutherland 1991) or are catalyzed by superoxide dismutase (*SOD*) to form hydrogen peroxide (H_2O_2) in the apoplasts, cytosol, chloroplasts, or mitochondria (Scandalios 1993).

The accumulation of AOS is harmful to the host plant cells; consequently, antioxidant enzymes such as catalases (*CAT*) and glutathione S-transferases (*GST*) commonly are involved in scavenging AOS from the cellular environment (Levine et al. 1994). The AOS (superoxide, hydroxyl radicals, and hydrogen peroxide) act directly to kill pathogen cells (Dixon et al. 1994), as well as cross-linking plant cell wall proteins for reinforcement (Cosgrove et al. 2000), signaling up-regulation of defense genes (Levine et al. 1994), and triggering plant cell death through a hypersensitive response (HR) that restricts pathogen growth (Dixon et al. 1994).

Other mechanisms are capable of generating AOS. For example, the mitochondrial Krebs cycle generates AOS as natural byproducts of respiration (Bolwell and Wojtaszek 1997). In addition, some flavonoids can act as pro-oxidants to generate AOS (Hodnick et al. 1989). Certain flavonoids become activated only after ingestion, so that AOS are produced inside the insect rather than in plant tissues (Ahmad and Pardini 1990).

Although herbivorous insects utilize mechanisms for nutrient acquisition that differ greatly from those of microbial pathogens, certain feeding strategies result in similar defense responses. Evidence for an insect-induced oxidative burst has been reported for sorghum plants infested with the greenbug aphid (*Schizaphis*

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graminum (Rondani)). This interaction results in differential expression of sorghum *GST* and *CAT*, suggesting AOS accumulation and detoxification (Zhu-Salzman et al. 2004). AOS-associated HR surrounds pathogens with dead tissue that serves as a barrier to limit lesion expansion. HR also has been effective in limiting nutrient acquisition by sessile insects like bark beetles (Berryman and Ferrell 1988) and gall midge larvae (Fernandes and Negreiros 2001). The Hessian fly, *Mayetiola destructor* (Say), is a member of the gall midge family, although it does not induce true gall formation and becomes sessile after the first larval molt. Interactions of Hessian fly larvae with wheat (*Triticum aestivum* L. em Thell.) can result in extensive areas of cell collapse, resembling an HR, within 2 days of egg hatch (Grover 1995).

Unlike the larvae of many phytophagous insects, Hessian fly larvae do not destructively consume wheat tissue by chewing, or bore holes in stems. Instead, they make only small wounds in a few cells and are believed to inject salivary compounds that initiate interactions with the plant (Grover 1995; Hatchett et al. 1990). This type of attack is similar to interactions initiated by fungi, nematodes, and many other pathogens that, like Hessian fly larvae, trigger a gene-for-gene recognition by a resistant host (Hatchett and Gallun 1970) and cause physiological rather than extensive physical damage to a susceptible host. Components of the larval saliva (Liu et al. 2004) are hypothesized to subvert the development of the susceptible host, leading to alterations in mRNA levels (Puthoff et al. 2005; Sardesai et al. 2005; Subramanyam et al. 2006) and plant cell fate (Harris et al. 2006) that are necessary for insect development.

During compatible interactions with developing seedlings, Hessian fly larvae establish feeding sites among the leaf sheaths near the crown at the base of the plant. Larval feeding on susceptible plants induces a generalized increase in leaf sheath cell permeability within 5 days of the initiation of the interaction (Shukle et al. 1992). This permeability allows nutrients to move to the surface of the leaf sheath for larval consumption and is associated with the formation of gall-like nutritive tissue (Harris et al. 2003, 2006). Feeding by virulent larvae not only stunts plant growth but also causes seedlings to accumulate more chloroplasts (Cartwright et al. 1959; Robinson et al. 1960). Second-instar larvae continue to feed but are incapable of injecting secretions into the plant (Hatchett et al. 1990). The irreversible changes in wheat tissues create a satisfactory environment to allow larval growth by delivering nutrients and protecting the larvae among the stunted leaf sheaths.

During incompatible interactions, larval salivary components trigger recognition, and the resulting activation of wheat genes involved in defense (Williams et al. 2002) can be detected within 6 to 12 h of egg hatch (Sardesai et al. 2005; Subramanyam et al. 2006). However, the first-instar larvae do not die immediately. Rather, they continue to probe and move down the plant for 3 to 4 days (Gallun 1977; Grover 1995) and are unable to alter plant physiology and development to establish permanent feeding sites (Grover 1995). Avirulent larvae die later than the 2-day time point in which Grover (1995) observed plant cell necrosis. The cause of larval death, however, may be due to any combination of factors, not limited to a localized oxidative burst-associated HR. Other factors may include ingestion of lectins that block absorption of nutrients or feeding deterrents that lead to starvation. This incompatible interaction appears to have little impact on the plant because infested, resistant wheat plants exhibit relatively normal growth (Gallun et al. 1961).

When plants are attacked by a population of both virulent and avirulent larvae, as occurs in many field infestations, both larval genotypes develop to maturity (Grover et al. 1989) even

if avirulent larvae hatch and begin probing before virulent larvae (C. E. Williams and J. A. Nemacheck, *unpublished data*). The form of resistance induced by avirulent larvae does not kill virulent larvae that are only a few millimeters away; therefore, plant resistance mechanisms appear to be very localized, involving modes of action such as the production of feeding deterrents and defense molecules primarily in a few cells at the point of contact. However, induced susceptibility in the plant appears to be somewhat systemic because avirulent larvae of mixed infestations survive due to their proximity to the nutritive tissue-inducing virulent larvae.

Many insects are able to overcome the effects of host-plant AOS via an antioxidant defense mechanism consisting of the enzymes SOD, CAT, glutathione reductase, and thioredoxin reductase (Missirlis et al. 2001). In a mechanism parallel to the AOS-scavenging pathway of plants, SOD of virulent cabbage looper larvae (*Trichoplusia ni* Hübner) catalyzes the dismutation of O_2^- to H_2O_2 , which then is converted to H_2O and O_2 by the action of larval CAT (Ahmad and Pardini 1990). In that study, a twofold increase in SOD enzyme activity is the primary response of the insect to dietary AOS.

Plant production of AOS, associated with an HR that limits pathogen growth, is a commonly observed defense mechanism triggered by gene-for-gene interactions. Because well-defined mechanisms of resistance have not been identified in the gene-for-gene interactions that trigger the resistance of wheat against the Hessian fly, we initiated a search for evidence of a classical oxidative burst. Hessian fly larval *SOD* and *CAT* mRNAs became more abundant during the initial days of the interaction, suggestive of plant-imposed stress. We then assayed infested wheat plants for evidence of oxidative burst-associated AOS and for increased levels of mRNAs that encode enzymes involved in generating or in scavenging AOS. However, no evidence was found of oxidative stress occurring within the plant tissues. In contrast, flavanone 3-hydroxylase (*F3H*) RNAs increased rapidly in incompatible but slowly during compatible interactions. Although a classical oxidative burst was eliminated as a mechanism responsible for changes in larval *SOD* and *CAT* mRNA levels, the causal mechanisms of plant defense are yet to be identified.

RESULTS

Accumulation of Hessian fly *SOD* and *CAT* mRNA.

Gene-specific primers (Table 1) for quantitative real-time polymerase chain reaction (q-RT-PCR) were designed for Hessian fly *SOD* (*mdesSOD-1*) and *CAT* genes (*mdesCAT-1*). mRNA levels were quantified during development of Biotype L Hessian fly larvae on wheat plants containing the *H9* resistance gene *Iris-H9* (incompatible interaction) or on the susceptible nearly isogenic wheat line Newton (compatible interaction). A gradual increase was seen for both *SOD* and *CAT* mRNA levels from larvae involved in compatible interactions (Fig. 1A and B, dashed lines; arbitrary expression values [AEV] shown in log scale), suggesting a growing response to environmental stress over time. However, throughout the experiment, larval *SOD* mRNA remained significantly lower in incompatible than in compatible interactions. For example, 4 days after hatch, larval *SOD* mRNA was 4.61-fold ($P = 1.6E-09$) more abundant in compatible than in incompatible interactions; the fold change was calculated from non-log scale AEV. In addition, levels of larval *CAT* mRNA increased earlier in incompatible than in compatible interactions. One day after hatch, larval *CAT* mRNA was 2.08-fold ($P = 7.1E-07$) more abundant in incompatible interactions. These data suggest that larvae involved in both compatible and incompatible interactions were responding to some type of stress.

Accumulation of mRNA from a wheat gene associated with production of AOS.

Wheat *NOX* mRNA levels were quantified by q-RT-PCR to determine whether the accumulation of wheat *NOX* mRNA was responsive to feeding by first-instar Hessian fly larvae and, thus, may be a source of oxidative stress. *NOX* was chosen because it encodes an enzyme believed to sustain O_2^- production after the initial oxidative burst that utilizes preexisting enzymes (Doke 1983). We cloned a partial cDNA sequence of the wheat *NOX* gene that included 794 bp of the predicted 2,782-bp mRNA and was 89% identical and 95% similar to the rice *NOX* gene *RbohA*. The wheat *NOX*-predicted amino acid sequence was 92% identical to the *RbohA*-deduced protein sequence, which is similar to the mammalian *gp91^{phox}* respiratory burst *NOX* gene (Groom et al. 1996). Therefore, we concluded that we had cloned a sequence from a wheat *NOX* gene.

The observed increases in larval *SOD* and *CAT* mRNA were independent of wheat *NOX* mRNA abundance during the first 24 h of the interaction. *NOX* message levels remained low in uninfested controls, compatible interactions (Iris-*H9* wheat with *vH9* larvae), and incompatible interactions (Iris-*H9* wheat with Biotype L larvae) (Fig. 2A). Likewise, no change was seen in samples collected 2, 3, 4, or 8 days after egg hatch (all *P* values >0.05; data not shown). The first through fifth leaves were measured 17 days after egg hatch, confirming that the plants had reacted correctly to the larvae (compatible produced stunted plants, incompatible and controls produced plants of normal height) (Fig. 2B). The low levels of *NOX* mRNA in all samples suggested that changes in *NOX* RNAs were not a response of wheat to Hessian fly larval feeding.

Inhibition of NADPH oxidase complexes.

Although changes in wheat *NOX* mRNA levels were not detected in response to Hessian fly larvae, levels of the NADPH complex present in the plasma membrane prior to infestation may be sufficient to activate an oxidative burst. Diphenyleneiodium (DPI) treatment of nearly isogenic wheat lines '97211' and 'Len' was performed to assess the involvement of superoxide-producing NOX complexes in defense against Biotype L Hessian fly larvae. Wheat seedlings growing under hydroponic conditions were treated with DPI (Auh and Murphy 1995; O'Donnell et al. 1993), which binds to the flavoprotein component of the NOX complex (Cross and Jones 1986). If NOX complexes were essential to the resistance response, inhibition would decrease plant production of O_2^- , resulting in increased survival of larvae on resistant 97211 plants.

Larval counts 5 days after egg hatch indicated that DPI inhibition of NOX complexes under hydroponic conditions did not result in increased larval survival. In the presence of DPI, resistant plants still were able to mount defenses leading to 100% larval death, with the exception of one plant harboring two larvae that were believed to be genetically virulent genotypes contaminating the Biotype L stock (in all, 25 larvae were present, only 2 of which were virulent) (Fig. 2C, resistant white bar). The remaining resistant plants (97211) treated with DPI were able to mount a normal defense response against the avirulent larvae, as indicated by the presence of dead, red larvae. Low-level contamination was confirmed

Fig. 1. mRNA levels for two Hessian fly genes involved in active oxygen species-scavenging. mRNA levels were quantified by quantitative real-time polymerase chain reaction (q-RT-PCR) 1 through 4 days after egg hatch in Biotype L larvae feeding on Iris-*H9* wheat plants (incompatible interaction, dotted line) and Newton wheat (compatible interaction, dashed line). Log of the arbitrary expression values (Log AEV) is shown. Error bars represent standard error from the analysis of variance test. Each graph represents three independent experiments, each subjected twice to q-RT-PCR. **A**, Hessian fly *SOD* and **B**, Hessian fly *CAT* mRNA levels.

Table 1. Wheat and Hessian fly gene-specific primers used in quantitative real-time polymerase chain reaction

Gene	Accession	Origin	Primer sequence ^a
<i>CAT</i>	X94352	Wheat	(F) 5' TCGGACACCGAGGACCTATC 3'
	...	...	(R) 5' CCGTGCATGAACAACACGTT 3'
<i>F3H</i>	DQ208192	Wheat	(F) 5' GTCGTGGATGAGGCAAGCTT 3'
	...	...	(R) 5' ACTACAAGAAGTTCTGGAGCAGGAA 3'
<i>GST</i>	AF109714	Wheat	(F) 5' AGTCAACATGCGTGAAGAAG 3'
	...	...	(R) 5' AATCTCACTGGTGGTGAATCGAT 3'
<i>NOX</i>	AY561153	Wheat	(F) 5' ATGTTCCGCAACTTGGTGACT 3'
	...	...	(R) 5' CGTCTGCTCTAAGAAGACCACTTTT 3'
<i>SOD</i>	CB307850	Wheat	(F) 5' CCGAGGTCTGGAACCATCAC 3'
	...	...	(R) 5' AGCCGAAATCCTTCTCGATCT 3'
<i>UBQ</i>	X56803	Wheat	(F) 5' GGTGTTCCGGTATCCTCCAA 3'
	...	...	(R) 5' TGCTCCACACCAGCAGAAGT 3'
<i>ACT</i>	AF017427	Hessian fly	(F) 5' ATGTGTGACGACGAAGTTGCT 3'
	...	...	(R) 5' GGCAACATACATGGCTGGTG 3'
<i>CAT</i>	DV752667	Hessian fly	(F) 5' ATGTTTGGGATTTTATTTCATTG 3'
	...	...	(R) 5' GGAGATGGTTTCGATTCTGCTGTTACCA 3'
<i>SOD</i>	DV752666	Hessian fly	(F) 5' ATGGTTAAAGCAGTTTGTGTTATTG 3'
	...	...	(R) 5' ATACCAATGAACACCGCAACCAAT 3'

^a F = forward and R = reverse.

when four Biotype L larvae survived on three resistant 97211 plants grown in vermiculite without DPI (on 12 plants, a total of 328 larvae were present, only 4 of which were virulent) (Fig. 2C, resistant black bar). Both groups of susceptible Len

plants, those treated with DPI and untreated plants, exhibited normal compatible interactions (Fig. 2C, white and striped bars). However, an average of 2.5 larvae on Len plants treated with DPI did die (Fig. 2C, susceptible white bar), suggesting a small

Fig. 2. Superoxide production in wheat. **A**, Levels of mRNA for a *NOX* gene involved in generation of active oxygen species. Log of the arbitrary expression values (Log AEV) is shown for uninfested Iris-H9 plants (solid line), vH9-infested Iris-H9 plants (compatible interaction, dashed line), and Biotype L-infested Iris-H9 plants (incompatible interaction, dotted line). Error bars represent standard error from the analysis of variance test. Tissue was harvested for total RNA isolation 1 day before hatch (0 time point) and at 2, 6, 12, 18, and 24 h after egg hatch. The graph represents two independent experiments, each subjected twice to quantitative real-time polymerase chain reaction. **B**, Average length of the first through the fifth leaves, 17 days after egg hatch, from uninfested Iris-H9 wheat plants (solid line), vH9-infested Iris-H9 plants (compatible, dashed line), and Biotype L-infested Iris-H9 plants (incompatible, dotted line). **C**, Average percentage of larvae surviving per plant on nearly isogenic resistant (97211) and susceptible (Len) wheat plants in the diphenyleneiodium (DPI) experiment designed to inhibit the superoxide-producing NADPH complex. Error bars represent standard error of the means. White bars represent the percentage of larvae surviving on DPI-treated resistant and susceptible plants grown in hydroponic solution. Striped bars represent the percentage of larvae surviving on resistant and susceptible plants grown in vermiculite without DPI. Black bars represent percentage of larvae surviving on resistant and susceptible plants grown in vermiculite without DPI. Nitro blue tetrazolium staining showed superoxide production in all tissues at the crown of **D**, uninfested wheat seedlings as well as **E**, susceptible 'Len' wheat seedlings 12 h after hatch of virulent Biotype L larvae (compatible interaction). Bars represent 1 mm.

Fig. 3. Presence of hydrogen peroxide in wheat crown tissues. **A** through **C**, mRNA levels of wheat genes involved in cellular protection from active oxygen species. Log of the arbitrary expression values (Log AEV) is shown for uninfested Iris-H9 plants (solid line), vH9-infested Iris-H9 plants (compatible interaction, dashed line), and Biotype L-infested Iris-H9 plants (incompatible interaction, dotted line). Error bars represent standard error from the analysis of variance test. Tissue was harvested for total RNA isolation 1 day before hatch (0 time point) and at 2, 6, 12, 18, and 24 h after egg hatch. Each graph represents two independent experiments, each subjected twice to quantitative real-time polymerase chain reaction. mRNA profiles for **A**, *SOD*, **B**, *CAT*, and **C**, *GST*. **D** through **H**, Iris-H9 wheat crowns were stained with 3,3-diaminobenzidine during the first 96 h of interaction with virulent and avirulent first-instar Hessian fly larvae. Bars represent 1 mm. **D**, Incompatible interaction, 12 h after hatch of Biotype L Hessian fly larvae. **E**, Incompatible interaction, 24 h after hatch of Biotype L Hessian fly larvae. **F**, Incompatible interaction, 4 days after hatch of Biotype L Hessian fly larvae. **G**, Compatible interaction, 12 h after hatch of vH9 Hessian fly larvae. **H**, Iris-H9 wheat infected with powdery mildew (*Blumeria graminis* f. sp. *tritici*) to show the typical brown precipitate from accumulation of H₂O₂ (compatible interaction). **I**, H₂O₂-dependent 2',7'-dichlorofluorescein fluorescence of leaf-three crown tissue 24 h after egg hatch. Photos of Iris-H9 with vH9 (compatible) and Iris-H9 with Biotype L (incompatible) crown tissue were taken in the elongation zone where larvae feed, 1 cm above the root/crown junction. Photos were taken distal to the elongation zone for the negative control (uninfested Iris-H9), 3 cm above the root/crown junction. Bars represent 0.1 mm.

toxic effect. Although DPI did not increase survival of Hessian fly larvae, it did cause some yellowing of wheat plants and a noticeable decrease in root mass. Both are symptoms characteristic of plants exhibiting DPI inhibition of the NOX complexes (Foreman et al. 2003).

Detection of superoxide radicals.

Resistant, susceptible, and uninfested control plants were stained with nitro blue tetrazolium (NBT) to detect the presence of O_2^- that may have been produced by mechanisms other than those involving NOX. The wheat lines used for these

studies were nearly isogenic for resistance (line 97211) and susceptibility (line Len) to Hessian fly Biotype L. Reduction of NBT to formazan yields a bluish precipitate in the presence of O_2^- (Doke 1983). A bluish color was observed throughout the crown tissue with an equal intensity regardless of whether the plants were uninfested controls (Fig. 2D) or involved in compatible (Fig. 2E) or incompatible (not shown) interactions. The extensive staining at the crown of the uninfested control plants indicated that O_2^- is produced during normal cell division and growth in wheat seedlings, as was reported earlier (Selote et al. 2004). Thus, no correlations could be made between accumulation of O_2^- and plant resistance. However, this basal level of O_2^- could be a source of oxidative stress to both virulent and avirulent Hessian fly larvae.

Accumulation of wheat *SOD*, *CAT*, and *GST* mRNA.

The mRNA levels of wheat genes encoding antioxidative enzymes that minimize the effects of oxidative stress on the plant (*SOD*, *CAT*, and *GST*) (Hammond-Kosack and Jones 1996) were analyzed during the first 24 h of compatible (*Iris-H9* wheat with *vH9* larvae) or incompatible (*Iris-H9* wheat with Biotype L larvae) interactions. Comparisons of plants involved in compatible and incompatible interactions with uninfested control plants showed no differential accumulation of mRNA (Fig. 3A to C). Specific q-RT-PCR primers for all genes (Table 1) were designed from available wheat sequences.

Profiles for *SOD*, *CAT*, and *GST* mRNA resembled those described earlier for the AOS-producing *NOX* gene, which were derived from the same tissue samples (Figs. 2A and 3A to C). These RNAs did not increase following Hessian fly attack (all *P* values >0.05), further supporting the earlier experiments indicating that neither oxidative burst nor AOS scavenging are induced in wheat defense against this insect pest.

Assay for presence of H_2O_2 in wheat seedlings.

Although we were unable to detect increased mRNA levels for one gene that generates and three that scavenge plant AOS, genes other than those that we evaluated may be involved in AOS production. Thus, we used histochemical staining methods to determine whether AOS in the form of H_2O_2 could be detected in crown tissues of wheat plants involved in compatible (*Iris-H9* with *vH9* larvae) or incompatible (*Iris-H9* with Biotype L larvae) interactions. Oxidation of 3,3-diaminobenzidine (DAB) stains tissues brown in the presence of H_2O_2 (Orozco-Cárdenas and Ryan 1999; Thordal-Christensen et al.

1997). No Hessian fly-induced increase in H_2O_2 was detected in wheat tissues with DAB after the initiation of larval feeding (0, 2, 6, 12, 18, 24, 48, 72, or 96 h) in either compatible or incompatible interactions (Fig. 3D to G). In contrast, a typical brown color originating from precipitated DAB was observed clearly in leaves of *Iris-H9* wheat infected with a virulent race of powdery mildew, *Blumeria graminis* f. sp. *tritici*, included as a positive control for staining (Fig. 3H).

Additional controls (not shown) demonstrated that, when exogenous H_2O_2 (final concentration of 1.5% vol/vol) was added to DAB, uninfested intact wheat crown tissue and tissue homogenate stained an intense brown color, whereas DAB with added H_2O_2 but lacking wheat tissue turned light brown. These controls indicate that peroxidase was present in the tissues but H_2O_2 levels were undetectable until exogenous H_2O_2 was added. Thus, oxidative burst-associated H_2O_2 is not present in wheat crown tissue under Hessian fly attack. Finally, the cut ends of leaves stained dark brown due to production of H_2O_2 during wounding (Fig. 3F, lower right corner). Low constitutive levels of H_2O_2 were detected at the crown of both infested and uninfested plants by the more sensitive 2',7'-dichlorofluorescein (DCFH) method (Fig. 3I). This H_2O_2 was associated with the zone of cell elongation rather than with resistance.

Accumulation of a wheat *F3H* mRNA.

The previous experiments failed to associate increases in Hessian fly *SOD* and *CAT* mRNA during infestation with an endogenous oxidative burst in crown tissues in resistance mechanisms. Consequently, a mechanism capable of producing AOS inside the insect rather than within plant tissues was sought. Wheat *F3H* mRNA levels were quantified because *F3H* is a member of a gene family involved in biosynthesis of myriad pro-oxidant compounds. Some of these compounds function as insect feeding deterrents (Estiarte et al. 1999) and are capable of generating AOS upon ingestion by insects (Ahmad and Pardini 1990).

Specific q-RT-PCR primers for *F3H* (Table 1) were designed from cDNA sequence obtained from a GeneCalling experiment (C. E. Williams, unpublished data). During incompatible interactions (*Iris-H9* plants with Biotype L larvae), *F3H* mRNA was most abundant 1 day after egg hatch (Fig. 4, dotted line, 10.52-fold above control, *P* = 0.004). During compatible interactions (*Iris-H9* plants with *vH9* larvae), the levels of wheat *F3H* mRNA gradually increased through the end of the experiment, 8 days after egg hatch (Fig. 4, dashed line). The most significant difference in *F3H* mRNA levels between the control and compatible interaction were seen 3 and 4 days after hatch (5.2-fold, *P* = 0.031 at day 3 and 7.35-fold, *P* = 0.011 at day 4).

DISCUSSION

The experiments reported here were initiated as part of a program to determine the involvement of standard plant defense mechanisms in the resistance of wheat plants against first-instar Hessian fly larvae. Our previous work demonstrated that, during incompatible interactions, a few unusual lectin-like wheat genes may be involved in the production of feeding deterrents or products that block absorption of nutrients within the larval midgut (Subramanyam et al. 2006; Williams et al. 2002) (Giovannini et al., submitted for publication). In addition, some of the most Hessian fly-responsive wheat genes also react to applications of the defense-signaling molecule, salicylic acid (Sardesai et al. 2005; Subramanyam et al. 2006). However, larvae fail to elicit a robust response from several standard wheat-defense and wound-response genes (Sardesai et al. 2005, and N. Sardesai, S. Subramanyam, and C. E. Williams,

Fig. 4. Accumulation of a wheat flavanone 3-hydroxylase mRNA. Log of the arbitrary expression values (Log AEV) is shown for uninfested *Iris-H9* plants (solid line), *vH9*-infested *Iris-H9* plants (compatible interaction, dashed line), and Biotype L-infested *Iris-H9* plants (incompatible interaction, dotted line). Error bars represent standard error from the analysis of variance test. Tissue was harvested for total RNA isolation 1 day before hatch (0 time point) and 1, 2, 3, 4, and 8 days after egg hatch. The graph represents two independent experiments, each subjected twice to quantitative real-time polymerase chain reaction.

unpublished data). Because wheat resistance to Hessian fly is triggered by a gene-for-gene recognition (Hatchett and Gallun 1970) and a related HR-like cell death was reported earlier (Grover 1995), we investigated the involvement of an oxidative burst due to its association with these components of resistance during plant interactions with microbial pathogens.

In three independent experiments with Biotype L larvae on resistant and susceptible nearly isogenic lines of wheat, the levels and rates of increase for larval *SOD* and *CAT* RNAs differed between compatible and incompatible interactions. These results suggested that both interactions imposed stresses on larvae. However, the larval mRNA responses associated with the presence of the wheat *H9* resistance gene during the incompatible interaction represent profiles of larvae that may be consuming very little while in the process of dying. In contrast, the high levels of *SOD* and gradually increasing levels of *CAT* mRNA during the compatible interaction represent profiles of healthy Hessian fly larvae effectively dealing with the basal defenses of susceptible plants while consuming nutrients and growing.

The response of *T. ni* to O_2^- -generating dietary pro-oxidants is similar to that of healthy Hessian fly larvae during compatible interactions with wheat. *T. ni* *SOD* activity rapidly increases only twofold whereas *CAT* activity stays relatively constant (Ahmad and Pardini 1990). This *T. ni* response is efficient because *SOD* activity catalyzes dismutation of O_2^- radicals 10^{10} -fold faster than the spontaneous dismutation that would occur in the absence of the enzyme (Fridovich 1983), and the constitutive level of *CAT* is sufficient to deal with large and rapid increases in H_2O_2 (Ahmad and Pardini 1990).

Under normal growth conditions, both O_2^- radicals and H_2O_2 are generated at the crown of wheat seedlings where cell elongation is occurring. Because Hessian fly larvae feed in this region of the plant, they were exposed to AOS. Superoxide radicals are produced during mitochondrial electron transport (Lamb and Dixon 1997) and were found in healthy wheat seedlings at a concentration of 0.75 mM/g of dry weight (Selote et al. 2004). H_2O_2 is present at a low concentration of approximately 0.02 μ M/g of protein in healthy wheat plants (Agarwal et al. 2005). Using NBT and DCFH, O_2^- and H_2O_2 staining was indistinguishable in intensity and distribution among crowns of uninfested plants, as well as infested plants involved in compatible and incompatible interactions.

Although AOS were produced at the crown of both healthy and infested wheat plants, a classical oxidative burst appeared to play no role in resistance against the Hessian fly. DAB staining detected no difference in H_2O_2 accumulation during the first 4 days of compatible and incompatible interactions. Wheat mRNAs encoding enzymes involved in either AOS generation (NOX) or detoxification (*SOD*, *CAT*, and *GST*) did not vary in abundance during the first 8 days. Furthermore, DPI inhibition of preexisting NOX complexes did not increase the survival of larvae during incompatible interactions.

In examples where an oxidative burst has been well documented, specific characteristics are evident. An extended oxidative burst is detectable within 3 h of initial contact during incompatible interactions between soybean cells and avirulent *Pseudomonas syringae* pv. *glycinea* (Orlandi et al. 1992). The initiation of the oxidative burst is correlated with activation of NOX catalytic subunits associated with microsomal plasma membrane fractions in *Phytophthora infestans*-infected potato tuber slices (Doke and Miura 1995). Once initiated, an oxidative burst may be sustained by pathogen-induced changes in RNAs encoding oxidant-generating enzymes (Lamb and Dixon 1997). Evidence of an oxidative burst in plant defense against insects is suggested in experiments where mRNA from the tomato *gp91^{phox}* homolog (similar to the human gene encoding the

heme-binding subunit of the superoxide-generating NOX complex) accumulates both locally and systemically in response to whiteflies (Walling 2000). However, none of these components of an oxidative burst were detected in our experiments.

An alternative mechanism for generating oxidative stress in Hessian fly larvae without induction of AOS production within wheat tissues may be involved in resistance. Plants produce a variety of phenolic compounds, such as quinones and flavonoids, some of which are activated to produce AOS once ingested by insects (Ahmad and Pardini 1990). The most abundant flavonoid in healthy wheat is isoorientin (Estiarte et al. 1999), a compound also found in corn silks that is extremely effective in inhibiting growth of corn earworm larvae (Widstrom and Snook 1998). A flavonoid profile has not yet been generated for wheat during interactions with Hessian fly larvae. Consequently, we can only speculate that increased production of ingestion-activated AOS-generating flavonoids plays a role in defense. However, the rapid 10.52-fold ($P = 0.004$) increase in wheat *F3H* mRNA within 24 h of attack by avirulent larvae (compatible) suggests that flavonoids may contribute to defense. In contrast, the slow increase in *F3H* mRNA during compatible interactions may lead to production of compounds at a rate that allows the virulent larvae to adapt through the gradual increases in larval *SOD* and *CAT* mRNA and their encoded detoxifying enzymes.

The lack of an oxidative burst within wheat tissues suggested that the Hessian fly-induced cell death reported by Grover (1995) is due to other causes. True HR induced by another gall midge (*Dasineura marginemtorquens* Bremi) on resistant willow (*Salix viminalis* L.) spread across six cell layers within 12 h and led to insect death at 40 h after egg hatch (Ollerstam et al. 2002). However, the reaction described by Grover does not fit the criteria for an HR, because the cell collapse was evident 2 days after egg hatch in both incompatible and compatible interactions, and death of avirulent larvae occurred several days later. No morphological characteristics distinguish HR from other causes of cell death (Heath 1998). HR typically induces systemic changes throughout the plant, including induced resistance to a variety of pathogens (Kombrink and Somssich 1995). In contrast, the resistance mechanisms of wheat against avirulent Hessian fly larvae appear to be highly localized around the attempted feeding sites; thus, nearby virulent larvae are able to survive and initiate systemic changes in the plant leading to survival of both virulent and genetically avirulent larvae (Grover et al. 1989). Further work is underway to identify genes and mechanisms involved in the resistance and susceptibility mechanisms in the Hessian fly-wheat interactions.

MATERIALS AND METHODS

Plant growth and Hessian fly infestations.

Purified Hessian fly stocks were kept in a 4°C cold room and maintained by the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) Crop Production and Pest Control Research Unit in West Lafayette, IN. Hessian fly Biotype L is virulent to wheat lines carrying resistance genes *H3*, *H5*, *H6*, and *H7/8*, which were used to verify fly genotype in these studies, and to wheat lines Len and Newton, which carry no resistance gene. Biotype L is avirulent to both line 97211 (Ratcliffe et al. 2002), which is nearly isogenic to Len, and to the *Iris-H9* line of wheat (Patterson et al. 1994), which is nearly isogenic to Newton. The *vH9* Hessian fly stock is virulent to *Iris-H9*.

Wheat seeds were sown, 10 to 12 per 10-cm pot, in vermiculite with time-release fertilizer. Pots were kept at 4°C for a week to synchronize germination and then transferred to a growth chamber at 18°C with a 24-h photoperiod so as to not induce

temperature-sensitive or light-sensitive genes. When plants reached the one-leaf stage, they were covered with a plastic cup and infested with six to eight females and three to four males per pot. After 6 h, adult flies were brushed off the plants. Time zero was estimated by closely monitoring egg hatch and dissecting plants periodically to determine when larvae had reached the crown of the plant and were attempting to feed. Infestations of the wheat differential lines carrying resistance genes *H3*, *H5*, *H6*, and *H7/H8* plus the *Iris-H9* line were included as controls in each experiment to verify the genotype of flies; for example, experimental plant tissue for use in q-RT-PCR was destructively harvested days before symptoms of plant susceptibility or resistance could be evaluated, but the survival of Biotype L larvae on the *H3*, *H5*, *H6*, and *H7/H8* control plants and their death on *Iris-H9* confirmed that the previously harvested experimental plant tissue had been treated correctly with the larval genotype that would trigger the desired molecular response. The phenotype of each infested differential control plant (stunted or unstunted) was evaluated 11 days after egg hatch. In addition, a subset of the experimental plants from each infested treatment (compatible and incompatible) was dissected 5 days after egg hatch, and numbers of live and dead larvae were recorded to determine infestation levels and to verify whether the expected interaction (compatible or incompatible) had occurred. Eighteen days after egg hatch, 10 plants from each treatment were measured from soil level to each ligule and to each leaf blade tip to verify stunting of susceptible plants.

NBT staining for superoxides.

Crown tissue was harvested from resistant line (97211), susceptible line (Len), and uninfested control plants from both lines at 2, 6, 12, 18, and 24 h after egg hatch (Biotype L) and immediately immersed in stain consisting of 0.2% (wt/vol) NBT powder (Sigma-Aldrich, St. Louis) in deionized H₂O (the experiment was done twice, each time with 5 to 10 plants per time point for each treatment). The crown tissues in stain were vacuum infiltrated and held for 5 to 6 h at room temperature, protected from light. The NBT solution was removed from the vials and stained tissues were fixed by vacuum infiltration with 2% formaldehyde and 2% glutaraldehyde in 50 mM PIPES, pH 7.2, and held overnight at 4°C. The next day, tissues were rinsed two times for 15 min in 50 mM PIPES, then washed in 95% EtOH until tissue appeared clear. Stained tissues were stored in 70% EtOH. Samples were examined under a dissecting microscope (Model EMZ-TR, Sony Meiji, Tokyo) and photographed with a digital camera (Model MVC-FD88, Sony Digital Mavica, Tokyo).

DPI treatment to block NADPH oxidase activity and superoxide production.

Plants used in this experiment were from two nearly isogenic wheat lines differing in their reactions to Hessian fly biotype L; line 97211 was resistant and Len was susceptible. Plants were germinated under greenhouse conditions. After the first leaf emerged, six 97211 plants and six Len plants were placed in each of two plastic trays covered with aluminum foil to control algal growth, with roots floating in Hoagland's solution (Hoagland and Arnon 1950) under constant aeration. The trays were placed in an 18°C growth chamber with a 24-h photoperiod. One day after the plants were transferred to Hoagland's solution, each tray was infested with 20 to 30 female and 15 male adult Biotype L Hessian flies under a cheesecloth tent. One day after infestation, the Hoagland's solution was changed and one tray was supplemented with 3 µM DPI (Sigma-Aldrich) and the other tray was left with Hoagland's solution as a control for the DPI treatment. As controls for the hydroponic treatments, 12 plants from each of the two nearly

isogenic lines were grown in vermiculite without DPI and infested as described above. Larval survival was determined by dissecting plants under a microscope and counting the living and dead larvae 5 days after eggs hatched. Living larvae were distinguished from dead red larvae by their whitish appearance and larger size. Of the six experimental Len plants grown in DPI, one plant died and another plant appeared chlorotic, possibly due to the toxicity of DPI to the plants.

DAB assay for hydrogen peroxide accumulation.

The DAB uptake method was used to detect peroxide accumulation (Thordal-Christensen et al. 1997). DAB powder (Sigma-Aldrich) was dissolved in water (1 mg/ml) by adding HCl to reach a pH of 3.8. Harvested crown tissue (five plants per time point per treatment, experiment done twice) was immersed immediately in DAB solution, vacuum infiltrated for 45 s, and held for 8 h under light at 25°C. The reaction was terminated by immersion of tissue in boiling 96% EtOH for 10 min and cleared with 70% EtOH. Crown tissues were preserved in 70% EtOH for photography. Uninfested, stained tissues were used as a negative control. Leaves infected with *B. graminis* f. sp. *tritici* were used as a positive control for the presence of H₂O₂. Infection of wheat leaves with *B. graminis* f. sp. *tritici* occurred naturally in the greenhouse due to high temperatures and humidity.

Additional controls were performed (five plants per treatment) to demonstrate that the addition of exogenous H₂O₂ turned the DAB solution brown and that peroxidase activity was present in wheat crown tissue. The controls included 1 ml of DAB solution mixed with 1 ml of 3% H₂O₂ solution in the absence of wheat crown tissue, DAB plus H₂O₂ with intact or homogenized wheat crown tissue, and DAB minus H₂O₂ with intact or homogenized wheat crown tissue.

DCFH assay for hydrogen peroxide accumulation.

In the presence of hydrogen peroxide, DCFH is oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF) (Allan and Fluhr 1997; Schopfer et al. 2001; Simontacchi et al. 1993). The technique described by Rodríguez and associates (2002) and Schopfer and associates (2001) was followed with some modifications. *Iris-H9* plants were infested at the two-leaf stage with *vH9* (compatible interaction) or Biotype L (incompatible interaction) Hessian flies. One day after eggs hatched, uninfested control and infested plants were dissected to expose the entire third leaf, where larvae were feeding. The tissue was excised 1.5 cm below and 3.5 cm above the root/crown junction to avoid wound-induced fluorescence at the elongation and larval feeding zone (tissue from the root/crown junction to 2 cm above). Crown tissue was placed on a microscope slide and covered with 500 µl of partially cooled agar solution followed by a cover slip. The following solution was heated to solubilize the agar: 1% (wt/vol) Bacto agar (Difco Laboratories, Detroit) in 20 mM potassium phosphate buffer, pH 6.0, with 50 µM 2',7'-dichlorofluorescein diacetate (Sigma-Aldrich). For negative fluorescence controls, leaf-three blade tissue (fully expanded area) and tissue 3 cm above the root/crown junction (distal to the elongation zone) were collected and treated. For a positive fluorescence control, leaf tissue was covered with 1.5% (vol/vol) H₂O₂ before adding the agar. Thirty minutes after placing the cover slip, fluorescence was viewed on a Nikon E800 microscope with excitation and emission wavelengths of 470 ± 20 and 525 ± 25 nm, respectively. Images were captured with a SPOT RT Slider digital camera.

Cloning of the wheat NADPH oxidase partial cDNA.

We cloned a 794-bp cDNA fragment from the *NOX* gene by PCR, using a pair of primers (forward: 5'-TAT ATG TTT GTT

CAA TGT CC-3' and reverse: 5'-GCT CCA CAG TAG AAT ACA CC-3') designed from Rice EST accession: X93301 (Amicucci et al. 1999). The template for PCR cloning was cDNA synthesized from total RNA from crown tissue of an F₁ hybrid (97211/Len) 72 h after hatch of Biotype L larvae. The PCR contained 1 ml of template cDNA (0.1 mg), 0.2 µM each primer, 200 µM dNTPs, 50 mM KCl, 2 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 0.001% gelatin, and 1 U of *Taq* DNA polymerase in a final volume of 50 µl. PCR was initiated with denaturation at 94°C for 2 min, followed by 32 cycles of 94°C for 15 s of denaturation, 43°C for 30 s of annealing, and 72°C for 2 min of extension, followed by a final extension at 72°C for 10 min. PCR products were subjected to electrophoresis on a 0.8% agarose gel and DNA was visualized by ethidium bromide staining. The resulting amplicon was excised from the gel and purified using the Wizard plus SV minipreps DNA purification kit (Promega Corp., Madison, WI, U.S.A.). The purified fragment was cloned into the pCR 4-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). DNA was isolated from transformed colonies using the Wizard Plus SV minipreps DNA purification kit (Promega Corp.). The sequence of this wheat fragment (GenBank accession AY561153) shared a high degree of identity (*e* value = 1e-146) with the rice *NOX* gene *RbohA*. Consequently, this wheat sequence was used in the design of q-RT-PCR primers for the *NOX* gene of wheat (Table 1).

Cloning of the Hessian fly *SOD* and *CAT* partial cDNAs.

A 442-bp cDNA fragment from the Hessian fly *SOD* gene and a 507-bp fragment from the *CAT* gene were recovered from a larval midgut cDNA library (Mittapalli et al. 2005). The Hessian fly *SOD*-deduced amino acid sequence (GenBank accession DV752666) shared 76% identity (*e* value = 1e-59) with the *Musca domestica* (Linnaeus) *SOD*, and the *CAT*-deduced amino acid sequence (GenBank accession DV752667) shared 71% identity (*e* value = 1e-105) with *Bombyx mori* (Linnaeus) *CAT*.

RNA extraction.

Plant tissue containing larvae was harvested and stored at -80°C until it was homogenized to a fine powder in liquid nitrogen. In a mortar, 2.7 ml of TRIzol reagent (Invitrogen) was added to approximately 400 mg of ground crown tissue (pooled from 10 to 15 plants). The extraction procedure followed the protocol described by Puthoff and associates (2005).

Total RNA was extracted from whole larvae, using the RNAqueous-4PCR kit (Ambion, Austin, TX, U.S.A.) following the manufacturer's protocol and Mittapalli and associates (2005).

q-RT-PCR.

Primers were designed from wheat plant and Hessian fly gene sequences (Table 1, sequence accession numbers) using the Primer Express software from Applied Biosystems (Foster City, CA, U.S.A.). Quantification of RNA for q-RT-PCR was conducted according to Spangler and associates (2003) and Puthoff and associates (2003) with modifications. In brief, 4.35 µg of total RNA in 10 µl of water was treated with DNase using the DNA-free kit (Ambion) following the manufacturer's instructions. The reverse transcriptase reaction to generate the cDNA for use in q-RT-PCR was carried out using the SuperScript First Strand cDNA Synthesis kit (Invitrogen) as follows: 1 µl of oligo d(T) primer and 1 µl of dNTPs were added to the 10 µl of total RNA. The mixture was heated at 65°C for 5 min and then placed on ice. The following were added on ice: 2 µl of 10× first-strand buffer, 2 µl of 50 mM MgCl₂, 2 µl of 0.1 M dithiothreitol, 1 µl of RNaseOut, and 1 µl of SuperScript II

reverse transcriptase. In order to monitor cDNA synthesis in a parallel tracer reaction, 5 µl were removed from each sample and mixed with 1 µl of a 1:5 dilution of ³²P-dCTP (Amersham, Piscataway, NJ, U.S.A.) in water. The remaining 15 µl of each reverse transcriptase reaction and its 5 µl tracer reaction were incubated at 42°C for 2 h. Reactions were stopped by heating samples at 70°C for 15 min. After cDNA synthesis was stopped, the 15-µl reverse transcriptase reactions were placed on ice while the ³²P-tracer reactions were used to quantify the amount of cDNA synthesized. From each ³²P-tracer reaction, 5 µl was spotted onto a DE-81 filter (Fisher, Fairlawn, NJ, U.S.A.). Filters were dried for 10 min, then washed four times for 4 min each in 0.5 M sodium phosphate buffer (1 M monobasic and 1 M dibasic in 4 liters of water). Following two 1-min rinses in water, filters were washed in 95% EtOH and allowed to dry. Each filter was placed in a scintillation vial containing 5 ml of ScintiVerse (Fisher) and radiation in each filter from the cDNA was quantified in a scintillation counter. The resulting counts were used to normalize the cDNA from the corresponding reverse transcriptase reactions to a final concentration of 10 ng/µl of sample. Correct normalization for equal concentration of cDNA among samples was confirmed by PCR amplification with ubiquitin primers (Table 1, UBQ) for normalized wheat samples and actin primers (Table 1, ACT) for normalized Hessian fly samples (data not shown).

PCR was conducted on an ABI 7000 using the following mixture: 2 µl of normalized cDNA, 10 µl of 2× SYBR Green Mix (ABI, Foster City, CA, U.S.A.) and 0.25 µM each primer in a 20-µl reaction. Reactions were carried out using the following cycling parameters: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 10 s and 60°C for 1 min. At the end of each run, a melt curve analysis was conducted to ensure primer specificity and purity of the PCR product. Relative mRNA levels were calculated by the standard curve method (User Bulletin 2, ABI PRISM 7700 Sequence Detection System) as described here. Briefly, an aliquot was taken from each cDNA sample used in an experiment to construct a pooled sample (for example, cDNA aliquots were taken from samples for times 0, 2, 6, 12, 18, and 24 h from uninfested controls as well as from plants undergoing compatible and incompatible interactions; equal aliquots were pooled from each of the 18 samples) (Fig. 2A and B). This pooled sample was serially diluted and subjected to q-RT-PCR. The threshold cycle for each dilution was plotted against its cDNA concentration (with an arbitrary starting quantity for the undiluted pooled sample assigned the value of 1), and used as the standard curve regression equation to generate the AEV for the experimental samples. A standard curve was generated for each target gene on the same PCR plate that held the time series of experimental samples. Linear standard curves, with a slope between -3.5 and -3.2 and *R*² value >0.99, were required for all primers used in this study. For quantification of plant mRNA levels, two independent sets of plants were grown (time series was collected twice) and each set of cDNA samples was subjected to q-RT-PCR twice for each gene that was analyzed. Replicates for larval gene quantification were handled the same way, except that the time series was collected from three independent experiments.

Statistical analysis of q-RT-PCR data.

Quantification of the mRNA levels for all genes used in this study is shown in log (base 10) scale (log AEV) so that data for treatments with high levels of mRNA can be viewed on the same graph with data for treatments showing little change. Error bars calculated from the analysis of variance (ANOVA) test were the same for all samples and time points due to the nature of the analysis. Significance of the data was determined for

each gene by ANOVA using the PROC MIXED procedure of SAS (SAS Institute 1999). The analysis model included treatments (compatible, incompatible, and uninfested), time points, and interaction between treatments and time points as fixed effects. Data from two biological replicates, each composed of two technical replicates, were combined and included as a random effect in the analysis model. Orthogonal contrasts were used to evaluate whether the effects of treatment or treatment-time point interaction on log value of the AEV were significant. The *P* values from the ANOVA test are indicated in the results only for time points of interest. Two treatments were considered to have statistically different AEVs at a particular time point if the *P* value associated with the contrast was less than 0.05. Fold changes were calculated from AEV before the data were log-transformed.

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