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Research

Plant Resistance

# Previous Aphid Infestation Induces Different Expression Profiles of Genes Associated with Hormone-Dependent Responses in Near-Isogenic Winter Wheat Lines

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## Abstract

Hormone-dependent responses in host plants induced by herbivore infestation have species-specific effects. This study focused on determining the relative expression profiles of the genes associated with hormone-dependent pathways in two near-isogenic wheat lines when attacked by cereal aphids. Infestation with *Rhopalosiphum padi* Linnaeus (Hemiptera: Aphididae) and/or *Sitobion avenae* Fabricius (Hemiptera: Aphididae) significantly upregulated the expression of marker genes related to the salicylic acid (SA)- and jasmonic acid (JA)-dependent pathways in the tested lines. In the resistant line 35-E4, previous infestation with *R. padi* significantly increased the relative expression of plant pathogenesis-related protein 1 at all sampling times but did not have a significant effect on the expression of the phenylalanine ammonia-lyase (PAL) gene. In addition, the expression levels of the lipoxygenase (LOX) and allene oxide synthase (AOS) genes immediately increased after the aphid attack. In susceptible line 35-A20, infestation with either *R. padi* or *S. avenae* led to significantly increased expression levels of the AOS and PAL genes. Moreover, sequential aphid infestation induced higher expression of AOS compared with a single-species aphid infestation, whereas the expression of the PAL gene was antagonistically affected by sequential aphid infestation. Overall, these results showed that aphid infestation induced SA- and JA-dependent responses in host plants. However, the expression profiles of these genes in resistant and susceptible host lines were significantly different.

**Key words:** *Rhopalosiphum padi*, *Sitobion avenae*, defense hormone, sequential infestation, expression profile

Crop plants have coevolved intricate and dynamic defense mechanisms that have toxic, repellent, or antinutritional effects on colonizers, thereby alleviating decreases in crop yield and quality (Wu and Baldwin 2009, Rani and Jyothsna 2010, War et al. 2012). Among these plant defense mechanisms, plants release particular volatiles or express genes associated via signal transduction and oxidative defense to control the damage caused by subsequent herbivores (Dudareva et al. 2006, Maffei 2010, War et al. 2012, Dahlin et al. 2015). The signaling molecules jasmonic acid (JA) and salicylic acid (SA) are recognized as major defense hormones against various herbivorous insects (Gill et al. 2010, Shivaji et al. 2010). Once herbivores successfully colonize and begin feeding, previously attacked plants then activate the expression of many genes involved in SA- or/and JA biosynthesis to produce hormone elicitors that initiate defenses against herbivores. These hormone-mediated defense responses induced by herbivore feeding in host plants can have a species-specific effect, and the defense profiles in resistant and susceptible host plants were significantly different (Smith and Boyko 2007, Alvarez et al. 2013). In addition, crop plants in agricultural systems are frequently challenged, either sequentially or simultaneously, by different species of attackers. Under sequential attack, previously infested plants frequently become unsuitable hosts that adversely affect the growth and fecundity of the subsequent colonizers (Rodriguezsaona et al. 2005, Viswanathan et al. 2007, Luo et al. 2016). Thus, determining the expression profiles of hormone-mediated defense genes in plants with different susceptibilities to sequential herbivore attacks is required for a better understanding multiple herbivore–plant interactions.

Piercing-sucking herbivores, such as the bird cherry-oat aphid (*Rhopalosiphum padi* L.) and English grain aphid (*Sitobion avenae*

Fabricius) (Hemiptera: Aphididae), are among the most destructive and common herbivorous insects that either sequentially or simultaneously attack cereal plants (Ni and Quisenberry 2006). Although their stylet-like mouthparts only create small punctures in the cereal plants, the feeding of aphids triggers biochemical and physiological changes in the host. In recent decades, most of the characterized expression profiles of hormone-mediated defense genes in aphid-cereal systems, including in *Schizaphis graminum* (Rondani)-*Sorghum bicolor* (L.), *Diuraphis noxia* (Kurdjumov)-*Triticum aestivum* (L.), and *S. avenae*-*T. aestivum* systems, have only considered one genus of aphid attack (Smith and Boyko 2007, Zhao et al. 2009). Unexpectedly, the expression profiles in cereal seedlings sequential heterospecific aphids attacks have been understudied (Poelman et al. 2008, Snoeren et al. 2011, De Zutter et al. 2017, Kang et al. 2018).

Our previous studies demonstrated that a line derived from a cross between *T. aestivum* (var. Chris) and *Triticum turgidum* (var. *durum* L.) named XN98-10–35 was resistant to *S. avenae* or *R. padi* at the seedling stage and tolerant at the adult stage; moreover, cereal aphid resistance genes were present in this cross (Luo et al. 2014, 2019; Wang et al. 2015; Hu et al. 2016). However, the potential signaling events of genotype XN98-10–35 seedling responses in compatible and incompatible aphid-wheat interactions are poorly understood. Therefore, the present study examined the expression profiles of genes associated with the JA or SA pathways that are induced by infestation with two different species of aphid in near-isogenic winter wheat lines derived from the cross between *T. aestivum* (var. Chris) and *T. turgidum* (var. *durum*) with different resistance levels to *R. padi*. The findings of the present study will contribute to a better understanding of the defense mechanisms involved in multiple wheat–aphid interactions and provide baseline information to guide more effective and environmentally friendly strategies to control *R. padi* and *S. avenae*.

## Materials and Methods

### Test Insect Maintenance

The apterous *R. padi* and *S. avenae* specimens used in all experiments were descendants of aphids that were previously collected in winter wheat fields around Yangling (37°14′N, 108°04′E), Shaanxi province, China. The aphids were maintained on seedlings of the wheat cultivar Aikang 58 in a growth chamber under a 16:8 h (L:D) photoperiod with diurnal temperatures of 25 ± 0.5°:18 ± 0.5°C L:D) and a relative humidity of approximately 70 ± 10%. To ensure the aphid specimens used in the following experiments were the progeny of a single apterous *S. avenae* or *R. padi*, the newly cultured wheat seedlings were exchanged weekly for several months before the experiment.

### Plant Materials and Growth Conditions

The 13 winter wheat lines used in this study are listed in Table 1. Among them, 12 wheat lines were developed or derived from a cross between *T. aestivum* (var. Chris) and *T. turgidum* (var. *durum*) at Northwest A&F University, China. The winter wheat line XN1376 was the control plant because it attracts aphid colonization, whereas wheat line XN98-10–30 derived from this cross was used as a resistant control. Seedling tests were conducted under controlled greenhouse conditions at the State Key Laboratory of Crop Stress Biology, Yangling, Shaanxi Province, China. Seeds of each wheat line with similar size and moisture content were soaked in diluted $H_2O_2$ solution for 2 d prior to germination. The seeds were cultivated in plastic pots (9 × 9 × 10 cm) filled with a soil mixture (organic matter: soil: sand, 1:1:1) and grown in the greenhouse conditions described previously. At the one-leaf stage (7 d after germination), each plant was individually covered with a Perspex tube cage with a mesh screen lid (height 30 cm, diameter 8 cm). Seedlings were watered regularly as needed, and no further fertilizer was used during the experiment. Plants at the three-leaf stage (14 d after germination) were used in the experiments.

### Characterization of the Level of Resistance to *R. padi*

The plants of each genotype were randomly maintained in transparent insect-rearing cages (60 × 60 × 40 cm) covered at the tops with fine mesh to allow for ventilation. Ten individual newly born *R. padi* nymphs (within 24 h after birth) from each aphid-infested treatment were transferred onto the first leaf of the wheat seedlings of each line. When those aphids reached the adult stage, the total number of aphids on each seedling were recorded every 3 d. The number of aphids was counted six times (the susceptible genotype was near death). The aphid resistance levels were ranked according to Painter's method (Painter 1951) from highly resistant to highly susceptible (Supp Table S1 [online only]). Twelve biological replicates were calculated for each genotype.

For the aphid weight-gain experiment, the newly born *R. padi* individuals were weighed within 24 h of birth (W1) using an electronic balance (Mettler Toledo XP26; Mettler Toledo GmbH, Switzerland)

**Table 1.** Pedigrees and levels of resistance to the bird cherry-oat aphid, *R. padi*, in the tested winter wheat genotypes

| Genotype | Pedigree | Aphid index (mean ± SE) | Resistance scale |
|---|---|---|---|
| 35-2A | *T. aestivum* (var. Chris)/*T. turgidum* (var. *durum*) | 1.038 ± 0.025 | LS |
| 35-Q9C | *T. aestivum* (var. Chris)/*T. turgidum* (var. *durum*) | 1.319 ± 0.033 | MS |
| 35-9C | *T. aestivum* (var. Chris)/*T. turgidum* (var. *durum*) | 0.864 ± 0.047 | LR |
| 35-Q12R | *T. aestivum* (var. Chris)/*T. turgidum* (var. *durum*) | 1.079 ± 0.012 | LS |
| 35-Q10R | *T. aestivum* (var. Chris)/*T. turgidum* (var. *durum*) | 0.685 ± 0.019 | LR |
| 35-Q9A1 | *T. aestivum* (var. Chris)/*T. turgidum* (var. *durum*) | 0.545 ± 0.022 | MR |
| 35-A8 | *T. aestivum* (var. Chris)/*T. turgidum* (var. *durum*) | 1.092 ± 0.012 | LS |
| 35-L1 | *T. aestivum* (var. Chris)/*T. turgidum* (var. *durum*) | 0.788 ± 0.041 | LR |
| XN98-10–30 | *T. aestivum* (var. Chris)/*T. turgidum* (var. *durum*) | 0.772 ± 0.024 | LR |
| XN1376 | *T. aestivum* (var. XN84G6)/<br>*T. aestivum* (var. Bi6) | 1.675 ± 0.058 | HS |
| 35-A20 | *T. aestivum* (var. Chris)/*T. turgidum* (var. *durum*) | 1.337 ± 0.037 | MS |
| 35-E4 | *T. aestivum* (var. Chris)/*T. turgidum* (var. *durum*) | 0.509 ± 0.035 | MR |
| 35-3NA | *T. aestivum* (var. Chris)/*T. turgidum* (var. *durum*) | 1.065 ± 0.010 | LS |

and then individually transferred into a clip cage (0.6 cm in diameter and 0.3 cm in height, with sufficient small holes for ventilation) on the second leaf of the tested wheat plants using a fine-hair brush (Gao et al. 2012). After 7 d of consecutive feeding, the aphids were weighed (W2) again. The differences in weight (dW = W2 − W1) were calculated for each aphid. For each genotype, approximately 30 biological replicates were weighed to determine the performance of *R. padi*.

## Aphid Infestations and Phytohormone Treatments

The winter wheat lines 35-A20 and 35-E4 derived from the cross between *T. aestivum* (var. Chris) and *T. turgidum* (var. *durum*) were used in the following experiment. Plants of both lines were individually infested with 20 apterous *R. padi* adults. To elicit the plant defense responses in the aphid-susceptible wheat seedlings, 20 apterous *S. avenae* adults were transferred to the seedlings of line 35-A20. At 48 h after infestation, all aphids were removed. Plants without exposure to aphids served as control plants. To estimate the effects of sequential aphid attacks on induced resistance in wheat line 35-A20, 20 apterous *R. padi* adults were individually transferred to each seedling. After 24 h infestation, these aphids were removed, and 20 apterous *S. avenae* adults were added. The second group of aphids was removed 24 h after their initial infestation. In another treatment, the treatment protocol was followed as previously described except that the order of aphid species exposure was reversed. The leaf tissues were harvested in biological triplicates at 0, 12, 24, 48, and 72 h after aphid removal, and control plant tissues were collected in biological triplicates at the same time points (Fig. 1).

The winter wheat lines 35-A20 and 35-E4 were either exogenously sprayed with 0.1 mM methyl jasmonate (MeJA) or 0.5 mM of SA (containing Triton X-100 at 0.05%) or subjected to 10 mechanical wounds produced with an insect pin (0.38 mm in diameter). The compounds used for the treatments, including MeJA (3-oxo-2-(2-pentenyl) cyclopentane methyl acetate) and SA (2-hydroxybenzoic acid), were purchased from Solarbio Life Sciences, China. The plant tissues were then harvested in biological triplicate at 3 and 24 h. Three biological replicates were grown for each treatment and time point of collection. All collected samples were frozen in liquid nitrogen and stored at −80°C for total RNA extraction.

## Detection of Gene Induction

Total shoot RNA was isolated from 0.1 g of wheat leaves from each treated plant by using TRNzol-A$^+$ total RNA extraction reagent (Tiangen Biotech, Beijing, China). After extraction, the RNA was dissolved in RNase-free water and stored at −80°C until use. The purity of the total RNA was determined by a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA), and the integrity of the total RNA was ascertained by nondenaturing 1.2% agarose gel electrophoresis. The first-strand cDNA was reverse-transcribed by the PrimeScript RT Reagent Kit with gDNA eraser (TAKARA Biotechnology, Dalian, China). The reaction mixture and procedures were used as specified in the reference manual. The enzymes and proteins encoded by the phenylalanine ammonia lyase (PAL) and pathogenesis-related proteins 1 (PR-1) genes were associated with SA synthesis and SA-mediated defense responses, respectively (Wu and Zhang et al. 2012). The enzymes encoded by the lipoxygenase (LOX) and allene oxide synthase (AOS) genes are crucial for the biosynthesis of JA via the oxygenation of free alpha-linolenic acid through the octadecanoid pathway (Schaller 2001). The farnesyl pyrophosphate synthase (FPS) gene is associated with isoprene biosynthesis (Arimura et al. 2000). The full sequences of those five marker genes associated with the JA or SA signaling pathway were identified in silico from the NCBI nucleotide database for primer design: plant pathogenesis-related protein 1 (PR-1, Accession No. AJ007348.1), PAL, Accession No. AY005474.1), FPS, Accession No. JX235717.1), lipoxygenase (LOX, Accession No. U32428.1) and AOS, Accession No. AY196004.1). The primers were synthesized by Tsingke Biological Technology (Beijing, China) and are listed in Supp Table S2 [online only]. Reverse Transcription Quantitative PCR (RT-qPCR) was performed using the SYBR Premix Ex Taq II Kit (TAKARA Biotechnology, Dalian, China). The 20 µl reaction mixtures consisted of 2.0 µl first-strand cDNA reaction product

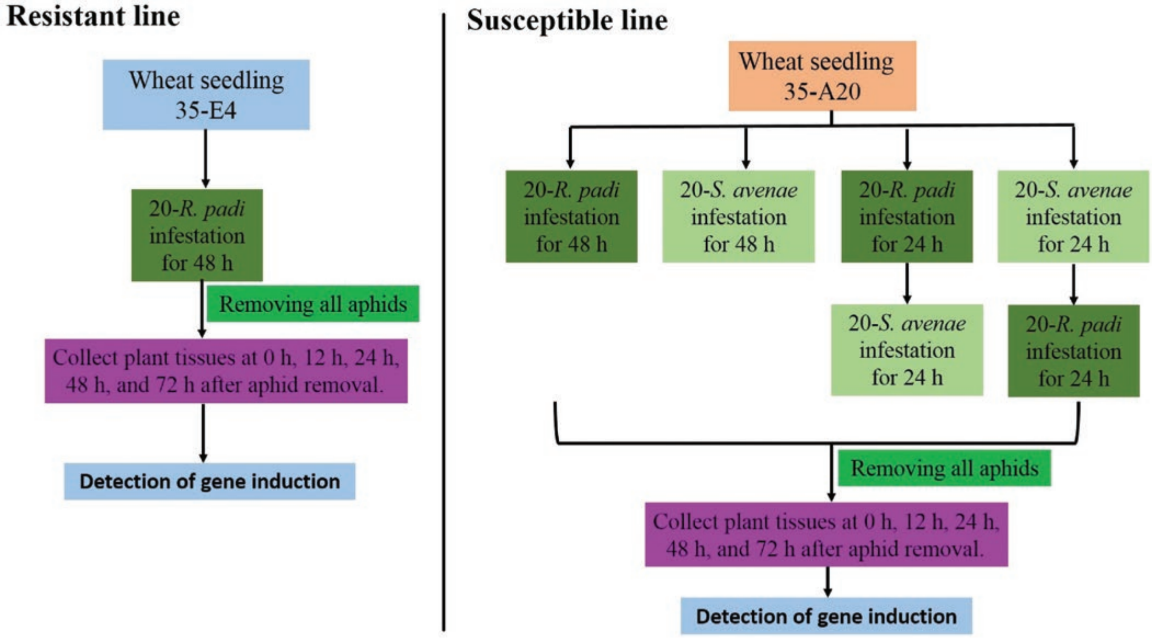

**Fig. 1.** Previous aphid treatments on resistant line 35-E4 and susceptible line 35-A20 to investigate the expression profiles of genes associated with hormone-dependent responses.

(100 ng), 10.0 µl SYBR Premix Ex Taq II, 0.4 µl ROX Reference Dye II, 1.0 µl each primer pair (10 µM), and deionized-distilled (dd) $H_2O$. The fluorescence reading was obtained from the QuantStudio 7 Flex Real-Time PCR System (Thermo Scientific, USA). The *TaActin* gene of wheat was used as an endogenous reference. The relative expression levels of each gene were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The expression level of the control in each line was arbitrarily set to 1. For each gene, the mean and standard error were calculated with data from three independent biological replicates.

### Statistical Analyses

The parameters aphid index and dW of *R. padi* and the expression levels of the marker genes associated with JA or SA pathways in the wheat lines were calculated using Microsoft Excel (version 2010, Microsoft, Redmond, WA). An independent-samples student's *t* test was performed to compare the dW parameters of *R. padi* and the above-described relative expression parameters. The differences were considered statistically significant at $P < 0.05$. These statistical analyses were performed using SPSS software (version 22.0, SPSS Inc., Chicago, IL).

## Results

### Phenotypic Analysis of Wheat Plant Resistance to *R. padi*

Under controlled greenhouse conditions, the aphid index values of 13 winter wheat lines were calculated according to the number of *R. padi* individuals that had colonized the plants (Table 1). Line 35-E4 exhibited the strongest resistance to *R. padi* (aphid index: 0.509, MR), while XN1376 was the most susceptible (aphid index: 1.675, HS). Among the progeny of the cross between *Triticum aestivum* (var. Chris) and *T. turgidum* (var. *durum*), line 35-A20 exhibited the weakest resistance to *R. padi* (aphid index: 1.337, MS).

In the aphid weight-gain experiment, significant differences in the average weight difference of lines 35-E4 and 35-A20 were detected after 7 d of consecutive feeding ($F = 7.715$, $P < 0.05$; $t' = 8.539$, df = 25.927, $P < 0.01$). The amount of *R. padi* weight gain in those two winter wheat lines is shown in Fig. 2. In accordance with the aphid index results, *R. padi* attained the lowest weight increase when reared on line 35-E4, while a higher amount of weight accumulation was detected on line 35-A20. In contrast, *R. padi* fed more on line XN1376, which yielded the largest weight increase, whereas line XN98-10–30 led to a smaller weight accumulation in *R. padi* (data not shown).

The above results revealed that line 35-E4 is a resistant host for *R. padi*, while 35-A20 is a susceptible wheat line. These two lines were used in the following experiment.

### *R. padi* Infestation Induces Multiple Hormone-Dependent Response Pathways

The effects of infestation with *R. padi* for 48 h on the expression levels of marker genes associated with hormone-dependent responses were significantly different between lines 35-E4 and 35-A20 (Fig. 3). In resistant wheat line 35-E4, the relative expression of PR-1, which is involved in SA-dependent responses, was 98.4-fold higher than that in the control plants at 0 h post-aphid removal. Thereafter, although the expression level of PR-1 decreased somewhat, it remained at least 70.0-fold higher than that of the controls and reached 89.0-fold higher at 72 h post-aphid removal (Fig. 3B).

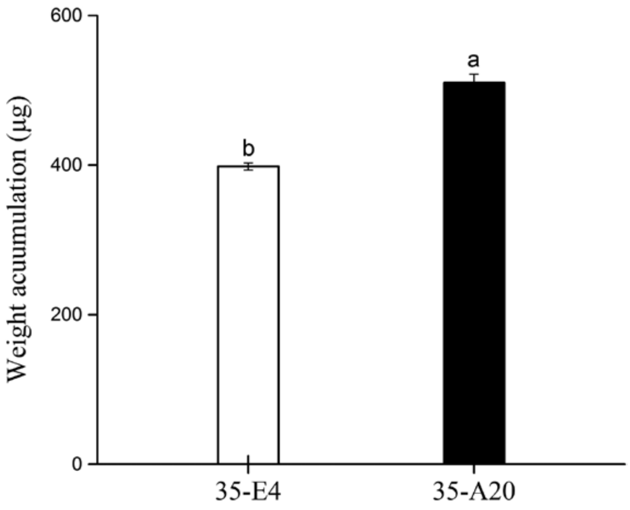

**Fig. 2.** Weight accumulation in the bird cherry-oat aphid, *Rhopalosiphum padi*, after feeding on winter wheat genotypes 35-E4 and 35-A20 for a week. The values are the means ± standard error (SE); the different lowercase letters indicate significant differences ($P < 0.05$).

In addition, the expression levels of the LOX and AOS genes, associated with the JA biosynthesis pathway, were increased 4.5-fold and 10.0-fold, respectively, relative to control plants at 0 h post-aphid removal (Fig. 3C and D). Similarly, the expression levels of these genes decreased several folds from 12- to 72 h post-aphid removal. In contrast, the expression levels of the FPS gene significantly increased at 12 h post-aphid removal, declined rapidly at 24 and 48 h post-aphid removal, and presented the highest expression at 72 post-aphid removal (Fig. 3E). In contrast, the expression levels of the PAL gene remained lower in the treated plants than the controls at all sampling time points (Fig. 3A).

In susceptible line 35-A20, *R. padi* infestation increased the expression level of the AOS gene to 7.5-fold higher than the control level at 0 post-aphid removal (Fig. 3D); thereafter, its expression was restored to the control level from 12 to 72 h post-aphid removal. The expression level of the PAL gene was 4.1-fold and 4.8-fold higher than the control level at 12 and 48 h post-aphid removal, respectively (Fig. 3A). Similar to the findings for resistant line 35-E4, the highest expression of the FPS gene was observed at 12 h after infestation by *R. padi*, with a rapid decline observed from 24 to 72 h post-aphid removal (Fig. 3E). However, the expression levels of the PR-1 and LOX genes were significantly lower than the corresponding control levels (Fig. 3B and C).

### Responses of a Susceptible Line to *S. avenae* Infestation Are Dependent on AOS and PAL Expression

Similar to the profiles observed under *R. padi* preinfestation, after *S. avenae* infestation for 48 h, the expression level of the AOS gene had increased to 8.5-fold that of the control level at 0 h post-aphid removal and then rapidly decreased to 0.6-fold at 12 h (Fig. 4D). Starting at 24 h post-aphid removal, the expression level of the AOS gene increased gradually, reaching 5.7-fold that of the control at 72 h post-aphid removal (Fig. 4D). Similarly, the expression level of PAL was not significantly increased at 0 h, although it increased rapidly at 12 h and reached 11.5-fold and 4.3-fold higher levels compared with the control at 12 and 24 h, respectively, after *S. avenae* removal (Fig. 4A). In contrast, *S. avenae* infestation induced higher expression of the PAL, AOS, and FPS genes than did *R. padi* infestation in

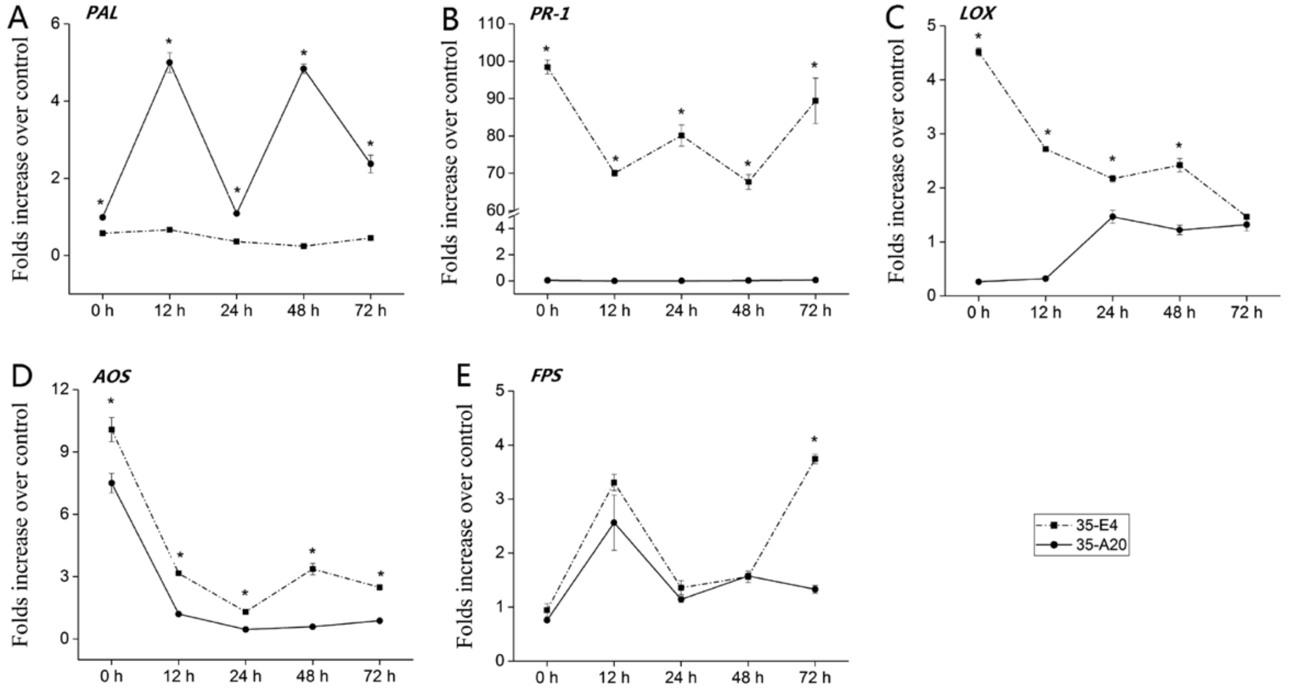

**Fig. 3.** Expression profiles of genes associated with hormone-dependent responses caused by *Rhopalosiphum padi* infestation of winter wheat lines 35-E4 and 35-A20 assayed by RT-qPCR. The relative expression levels of A, the PAL gene; B, the PR-1 gene; C, the LOX gene; D, the AOS gene; and E, the FPS gene. The expression levels in healthy plants were arbitrarily set to 1. The values represent the mean fold increase over control (±SE). *Significant differences in expression between 35-E4 and 35-A20 plants ($P < 0.05$).

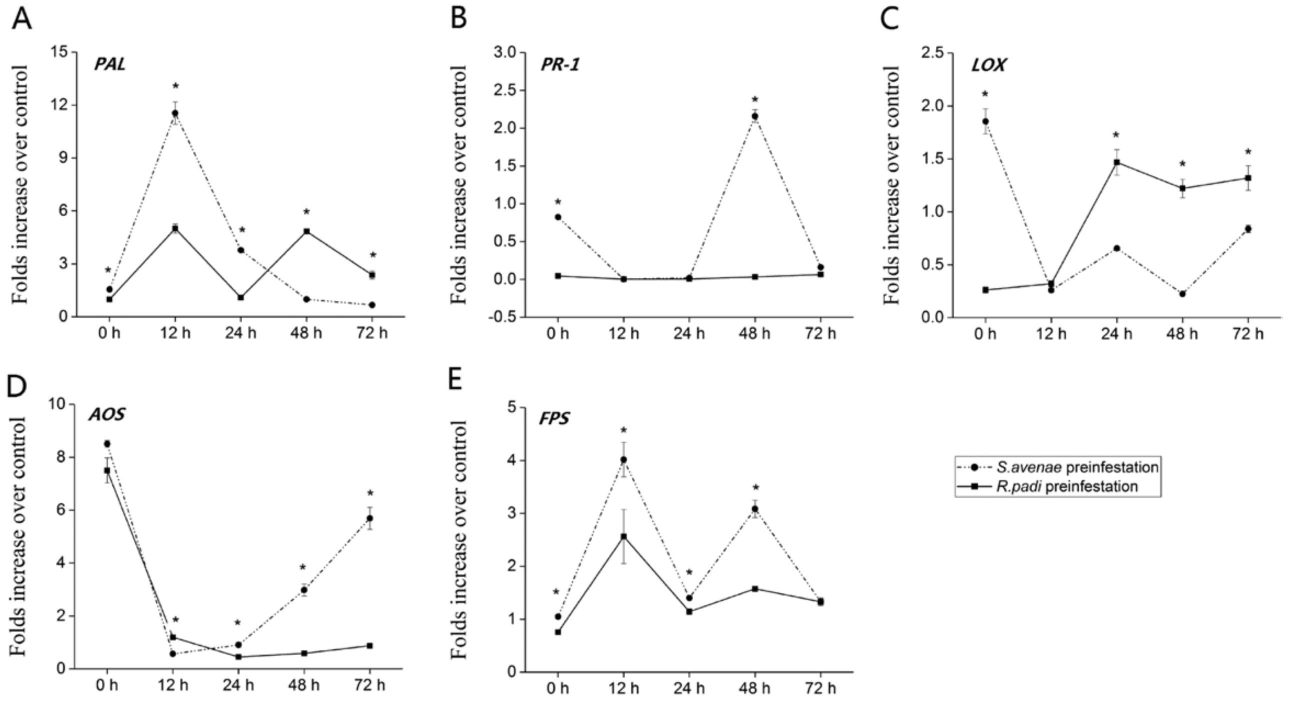

**Fig. 4.** Expression profiles of genes associated with hormone-dependent responses caused by *Rhopalosiphum padi* or *Sitobion avenae* infestation of winter wheat line 35-A20 based on a RT-qPCR assay. The relative expression levels of A, the PAL gene; B, the PR-1 gene; C, the LOX gene; D, the AOS gene; and E, the FPS gene. The expression levels in healthy plants were arbitrarily set to 1. The values represent the mean fold increase over control (±SE). *Significant difference in expression between *Sitobion avenae*- and *Rhopalosiphum padi*-infested plants ($P < 0.05$).

line 35-A20 (Fig. 4A, D, and E). In addition, the FPS gene showed similar expression patterns in both *S. avenae* and *R. padi* infestations of line 35-A20 (Fig. 4E). Although the expression level of PR-1 in the treated plants was higher than that of the control plants at 48 h post-aphid removal, it was significantly lower than that of the control plants at the other sampling times (Fig. 4B).

## Sequential Aphid Infestation Strongly Induces AOS Expression in a Susceptible Line

In the individual aphid infestation experiments, differences in the expression levels of the genes were detected in susceptible line 35-A20 (Fig. 4). Although both the individual and sequential treatments increased the expression level of the AOS gene at 0 h post-aphid removal, the amount of the increase differed (Fig. 5). Sequential aphid infestations induced a higher level of AOS gene expression (Fig. 5D) than did individual infestation with *R. padi* or *S. avenae*. Similar to the individual *S. avenae* infestation, *S. avenae* infestation following an initial infestation with *R. padi* increased the expression level of the PAL gene to 10.0-fold at 24 h post-aphid removal (Fig. 5A). However, *R. padi* infestation following an initial infestation with *S. avenae* showed antagonistic effects, and the expression level of the PAL gene was only 2.0-fold higher than that of the control level at 0 h post-aphid removal (Fig. 5A). In addition, the expression level of the LOX gene was increased to 3.1-fold higher than the control level at 0 h post-aphid removal in the secondary *S. avenae* infestation treatment (Fig. 5C), whereas the expression of the FPS gene was 3.6-fold higher than the control level at 12 h post-aphid removal in the secondary *R. padi* infestation treatment (Fig. 5E). However, neither treatment increased the expression level of the PR-1 gene (Fig. 5B). Thus, the second aphid species introduced had a greater influence than the first on the expression levels of genes associated with induced resistance during sequential infestation in susceptible line 35-A20.

## Induction of Hormone-Dependent Response Genes Following Hormone Spraying or Mechanical Wounding

To determine the direct effects of hormone spraying or mechanical wounding on the expression of induced resistance genes, seedlings were treated with MeJA, SA or mechanical damage. In resistant line 35-E4, exogenous spraying of MeJA and SA significantly increased the expression level of the PR-1 gene to 112.9-fold and 252.5-fold higher than that of the control level, respectively, at 3 h postspraying (Fig. 6B). Similarly, the expression levels of the LOX and AOS were significantly increased at these post-treatment times (Fig. 6C and D). At the 24 h postspraying, the expression levels of the three genes were not markedly influenced by the treatments. However, leaf puncturing induced quantifiable increases in the expression of the PR-1 gene at 24 h (Fig. 6B). Moreover, the treatments did not induce quantifiable increases in the expression of the PAL or FPS genes (Fig. 6A and E).

In susceptible line 35-A20, MeJA spraying significantly increased the expression levels of the AOS and LOX genes to 39.7-fold and 156.2-fold the control levels, respectively, at 3 h (Fig. 7C and D). Meanwhile, PAL mRNA levels increased to 4.9-fold the control levels at 3 h after MeJA treatment (Fig. 7A). Although SA spraying increased the expression levels of these genes, the effects were weaker than those of MeJA spraying. After reaching the levels attained at 3 h, the expression of the genes returned to low levels at 24 h after the treatments (Fig. 7). The mechanical damage treatment affected the expression of only the PAL gene, increasing it to 4.9-fold higher than the control level at 24 h in susceptible line 35-A20 (Fig. 7A).

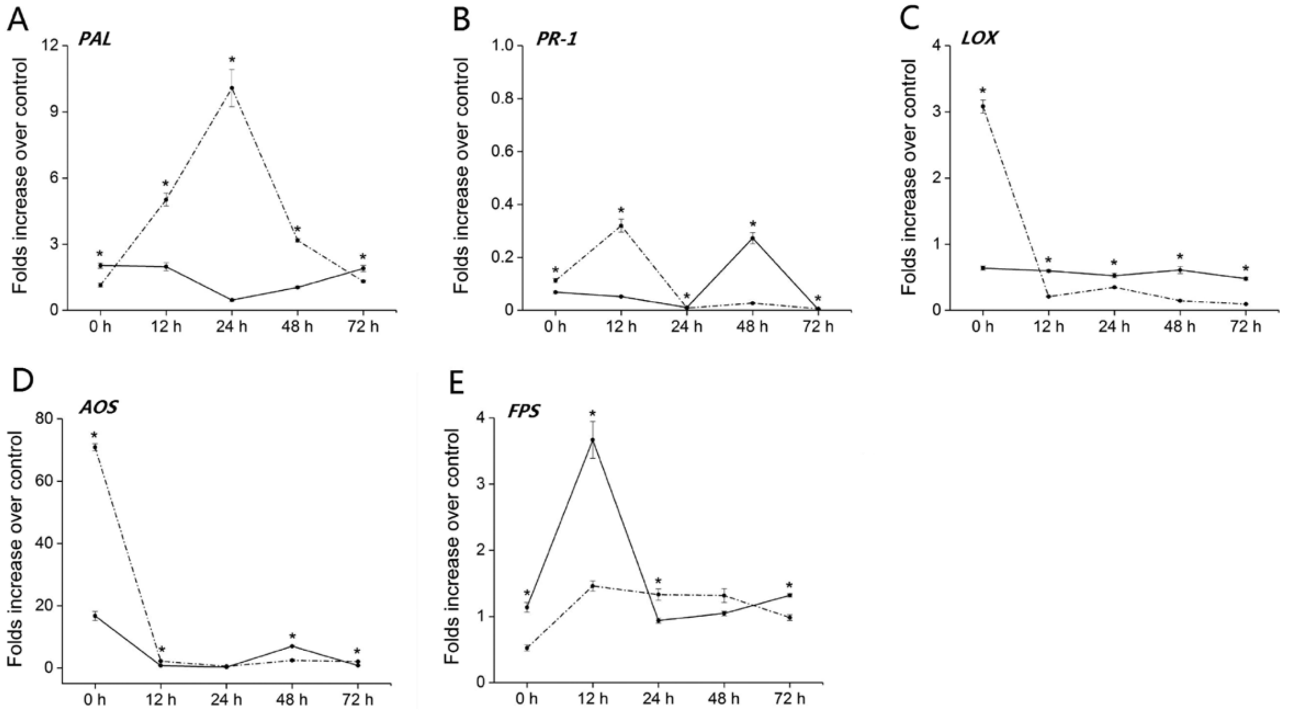

**Fig. 5.** Expression profiles of genes associated with hormone-dependent responses induced by *Rhopalosiphum padi–Sitobion avenae* and *Sitobion avenae–Rhopalosiphum padi* sequential infestations of winter wheat line 35-A20 assayed by RT-qPCR. The relative expression levels of A, the PAL gene; B, the PR-1 gene; C, the LOX gene; D, the AOS gene; and E, the FPS gene. Dashed/dotted lines, *Rhopalosiphum padi* initial infestation for 24 h, followed by *Sitobion avenae* secondary infestation for 24 h; solid lines, *Sitobion avenae* initial infestation for 24 h, followed by *Rhopalosiphum padi* secondary infestation for 24 h. The expression levels of healthy plants were arbitrarily set to 1. The values represent the mean fold increase over control (±SE). *Significant difference in expression between the two kinds of sequential infestation in plants of line 35-A20 ($P < 0.05$).

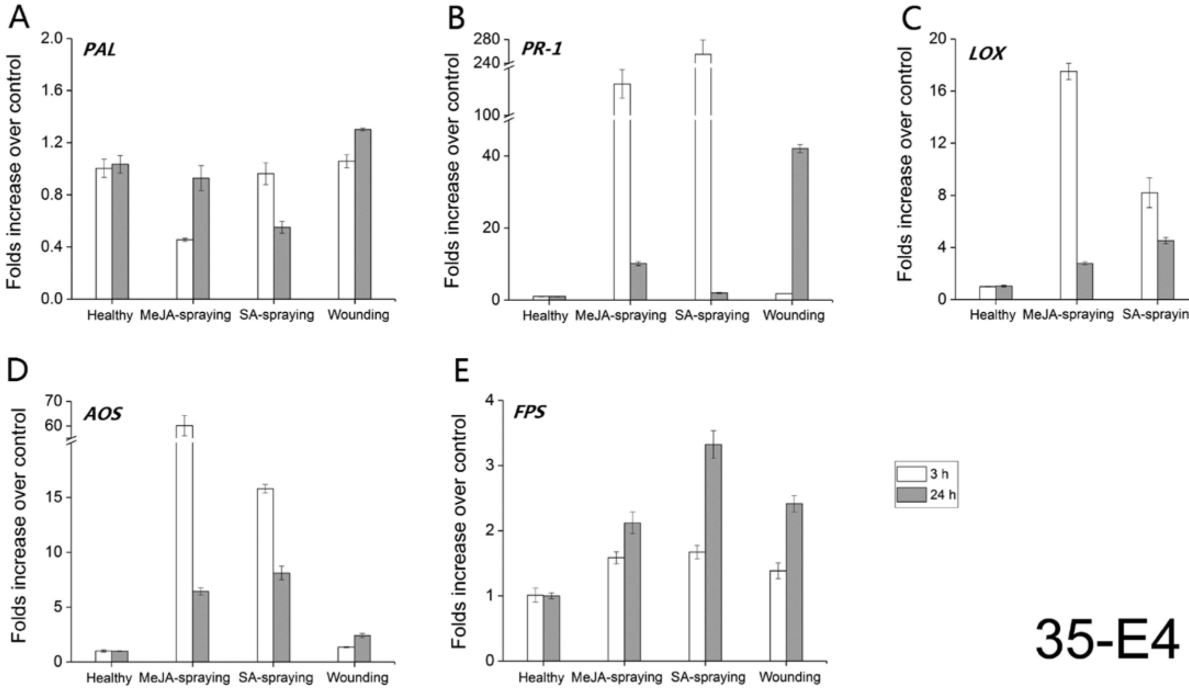

**Fig. 6.** Expression profiles of genes associated with hormone-dependent responses caused by hormone spraying and mechanical wounding in winter wheat line 35-E4 based on a RT-qPCR assay at 3 h and 24 h after treatment. The relative expression levels of A, the PAL gene; B, the PR-1 gene; C, the LOX gene; D, the AOS gene; and E, the FPS gene. The expression levels of healthy plants were arbitrarily set to 1. The bar values represent the mean fold increase over control (±SE).

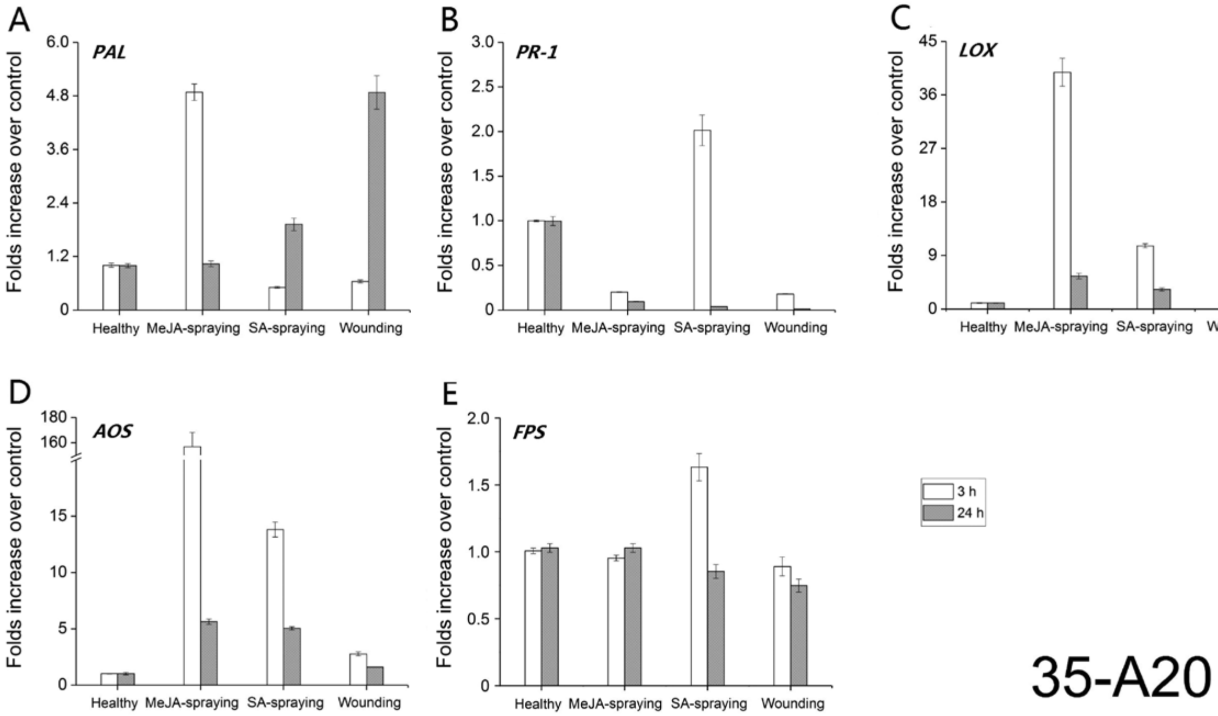

**Fig. 7.** Expression profiles of genes associated with hormone-dependent responses caused by hormone spraying and mechanical wounding in winter wheat line 35-A20 based on a RT-qPCR assay at 3 h and 24 h after treatment. The relative expression levels of A, the PAL gene; B, the PR-1 gene; C, the LOX gene; D, the AOS gene; and E, the FPS gene. The expression levels of healthy plants were arbitrarily set to 1. The bar values represent the mean fold increase over control (±SE).

## Discussion

During aphid infestation, the expression of genes related to the SA- and JA-dependent pathways were significantly regulated in winter wheat lines and the expression patterns of these genes were significantly different between the resistant and susceptible lines (Fig. 8). It was demonstrated that the SA is the primary phytohormone in plant defense against piercing-sucking insects, and it activates the expression of both direct and indirect defenses (Walling 2000,

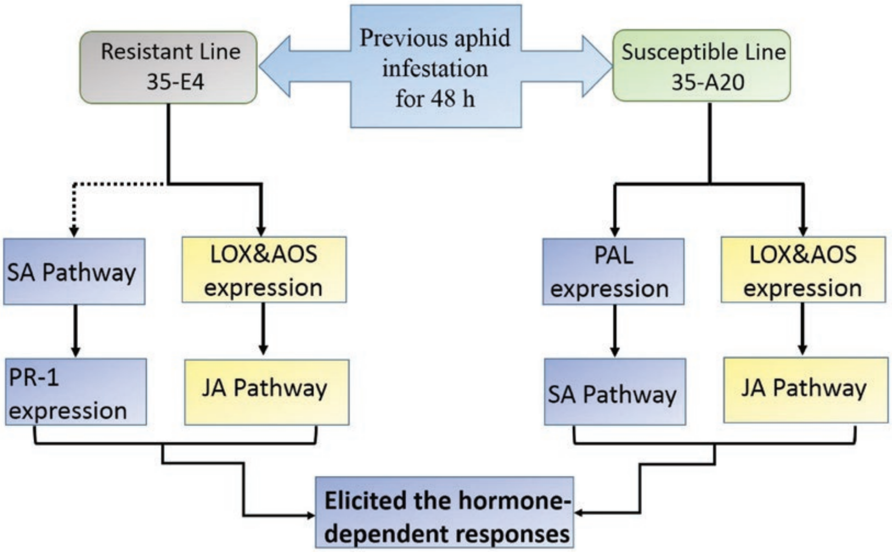

**Fig. 8.** Proposed hormone-dependent responses pathways induced by aphid infestations in near-isogenic winter wheat lines with different resistant to *R. padi*. Filled arrows represent conversions observed in or deduced from this study; and dotted arrows represent steps predicted from the literature.

Moran and Thompson 2001, Rayapuram and Baldwin 2007, Zhao et al. 2009, Alvarez et al. 2013). The extensive accumulation of SA in plant tissues could be a critical step in triggering downstream defense responses (Mohase and van der Westhuizen 2002). Previous studies have suggested that the PAL gene is crucial for the conversion of chorismate into cinnamate, which is associated with SA formation in plants. Interestingly, the PAL gene was not expressed in resistant line 35-E4 after the *R. padi* treatment. In addition, the SA concentration in resistant wheat cultivar Tugela DN steadily increased with time and reached a peak after 96 h of *D. noxia* infestation, and then it rapidly decreased to normal levels at later time points (Mohase and van der Westhuizen 2002). Taken together, we speculated that *R. padi* infestation strongly upregulates the expression of the PAL gene during aphid feeding, which leads to a large increase in SA accumulation. This speculation was supported by the expression profiling results for the PAL gene in susceptible line 35-A20 after *R. padi* infestation. Moreover, in *Arabidopsis*, at least two possible routes involved in SA biosynthesis are derived from chorismate in plastids, and these two pathways are frequently activated during the response to pathogens (Mauch-Mani and Slusarenko 1996; Wildermuth et al. 2001). Thus, additional experiments are required to clarify the biosynthesis pathways of SA in wheat.

After aphid-induced SA is distributed within plant tissues, competitive binding of SA via SA-binding proteins, such as catalase (CAT), results in the accumulation of hydrogen peroxide ($H_2O_2$) and dissociation of oligomeric nonexpressor of PR genes1 (NPR1) into its monomeric form. Then monomeric NPR1 translocates into the nucleus and activates the expression of pathogenesis-related proteins (PRs) to induce plant resistance against intruders (Wu et al. 2012). The wheat PR-1 gene encodes a PR protein causing protease resistance, which is induced or upregulated after pathogen or insect attack (Ibeas et al. 2001, Lu et al. 2013). In this study, the PR-1 gene was highly expressed in resistant line 35-E4 after *R. padi* infestation. To the best of our knowledge, the PR-1 gene is expressed in the resistant wheat cultivar only after an initial aphid infestation (van der Westhuizen et al. 1998, Mohase and van der Westhuizen 2002, Zhu and Zhao 2006). In addition, the expression profiles of SA-dependent responses, including PR genes, were upregulated in different aphid-susceptible plant systems (Moran and Thompson 2001, Moran et al. 2002, Zhu-Salzman et al. 2004, Divol et al. 2005). However, the PR-1 gene was not expressed in susceptible line 35-A20 after *R. padi* or *S. avenae* infestation, which was likely because the process of SA accumulation in the susceptible line employed a distinct pathway compared with that in the resistant wheat line, which resulted in SA production at a relatively small level. These results are consistent with the previous studies in which the accumulation of SA in susceptible Tugela was not pronounced (Mohase and van der Westhuizen 2002). Thus, we cannot exclude the possibility that susceptible line 35-A20 could employ the SA signaling pathway as a defense against subsequent herbivore attacks.

In addition, we observed that *R. padi* infestation caused different expression patterns between lines 35-E4 and 35-A20 in the LOX and AOS genes, which are involved in JA biosynthesis, which is supported by an experiment conducted on different aphid–plant systems that found that genes associated with JA synthesis and JA-mediated defense responses were significantly induced by aphid infestation in resistant plants (Gao et al. 2007). In the octadecanoid pathway of JA biosynthesis, linoleic acid undergoes a dioxygenation reaction that depends on LOX to form 13-hydroperoxide. This intermediate is then converted into 12-oxophytodienoic acid by AOS (Schaller 2001, Shivaji et al. 2010). Accordingly, the timing of elevation of increased LOX expression occurs earlier than that of AOS. This finding was supported by the results of the experiment in which lines 35-A20 and 35-E4 were exposed to hormone spraying, such as direct MeJA or SA spraying, and the expression of the LOX gene was significantly upregulated at 3 h. Based on these findings, we can infer that the aphid-induced expression of LOX was activated within 48 h of aphid preinfestation. Additional work is required to unravel the timing of elevated LOX gene expression during aphid infestation. Furthermore, the protein encoded by the LOX gene can adversely affect the growth and development of insects (Constabel 1999, Bruinsma et al. 2009). Therefore, the JA-dependent resistance response induced by *R. padi* damage was stronger in line 35-E4 than in 35-A20. The difference may also be because line 35-E4 is not a suitable host for *R. padi*. *R. padi* may produce multiple punctures when seeking a colonial site and that the salivary components of *R. padi* retained during this wounding stimulate the host to express genes associated with the JA-dependent pathway (War et al. 2012). Previous studies have revealed that wounding punctures can lead to the extensive accumulation of JA in plant tissues via the increased expression of enzymes in the JA biosynthesis pathway (Shivaji et al. 2010). However, mechanical wounding under our experimental conditions did not significantly affect the expression levels of the genes in the tested winter wheat lines, potentially because the distinct defense pathways were activated between aphid feeding and mechanical wounding (Wu and Baldwin 2009). Moreover, the relative expression levels of the LOX and AOS genes were higher in line 35-A20 than those in 35-E4 under exogenous application of MeJA and SA. Thus, the JA-dependent response pathway plays a vital role in the defense of line 35-A20 against subsequent herbivore attacks.

Compared with *R. padi* or *S. avenae* infestation alone, sequential aphid infestation significantly upregulated the expression of the AOS gene. It is possible that sequential aphid infestation introduces additional wounding of the host because these aphids prefer to colonize different parts of wheat plants (Ni and Quisenberry 2006). However, the expression level of the AOS gene increased more in secondary *S. avenae* infestation than in secondary *R. padi* infestation. Because this wheat line is susceptible to *R. padi*, the initial infestation with *R. padi* exhibited fewer wounded leaves and induced less PAL gene expression in host seedlings; thus, the effect on the feeding of subsequent colonizers was reduced. Subsequent infestation with *S. avenae* might lead to an increased response and inflict greater damage because of a reduction in the nutritional quality of the host plant (Luo et al. 2016). However, in secondary *R. padi* infestation treatment, an initial *S. avenae* infestation had a larger effect on the expression level of the PAL gene, which causes toxic effects and a defense response against subsequent *R. padi* infestation. The above reasons might account for the greater expression level of the AOS gene to secondary *S. avenae* infestation. Moreover, our results showed that sequential aphid attacks antagonistically affected or reduced the expression level of the PAL gene in line 35-A20. This effect may be related to a difference in the expression level of the AOS gene during sequential aphid attacks in susceptible line 35-A20 and the subsequent accumulation of JA. The JA defense responses act antagonistically with the SA signaling pathway (Shigenaga and Argueso 2016). Therefore, either *R. padi* or/and *S. avenae* preinfestation induced strong upregulation of the AOS and PAL genes, which are involved in JA- and SA-dependent responses in line 35-A20.

Although terpenes are important compounds produced in plants that adversely affect herbivore performance (War et al. 2012), the relative expression of the FPS gene, which is associated with terpene biosynthesis (Arimura et al. 2000), was seldom significantly affected by the aphid, spraying, or mechanical treatments applied to the two wheat lines. Thus, aphid attack did not induce lines 35-A20 and 35-E4 to produce terpenes defenses to reduce damage from late-season herbivore infestations.

In summary, herbivore-induced responses in host plants can affect the preference and performance of late-season conspecific or heterospecific herbivores by upregulating the expression of genes associated with the JA- and SA-dependent pathways, the defense profiles in resistant and susceptible winter wheat plants were significantly different.

## Supplementary Material

Supplementary data are available at *Journal of Economic Entomology* online.

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## Author Contributions

K.L., H.Z., and G.Z. conceived and designed the research. K.L., X.Y., and X.H. performed the research and wrote the manuscript. K.L., C.L., X.Y., and Z.H. analyzed the data and prepared the figures. All authors have read and approved the manuscript.

## Competing Interests

The authors declare that they have no competing interests.

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