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ARTICLE

Activation of Defense Mechanism in Wheat by Polyphenol Oxidase from Aphid Saliva

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The saliva of two cereal aphids, *Sitobion avenae* and *Schizaphis graminum* in third-instar nymphs, was collected after 24 h of feeding by 30 aphids, separately, on artificial diet sachets, and the salivary enzymes were determined. The result showed that polyphenol oxidase (PPO) existed in the saliva of both aphid species, and the enzymatic activities were 6.2×10^{-3} U/g for *S. avenae* and 2.37×10^{-1} U/g for *S. graminum*, revealing a 38-fold higher activity in the saliva of *S. graminum* than in the saliva of *S. avenae*. It was speculated that the higher PPO activity in *S. graminum* saliva was a contributing factor to the light yellow spot left on the feeding site of the wheat leaf by *S. graminum*; no such spot was left by *S. avenae*. After treatment of a wheat seedling with the saliva of *S. avenae* and *S. graminum* and PPO at the concentration of aphid saliva, transcript profiling data showed that aphid saliva and PPO significantly induced expression of the genes *aos* and *fps*. Because genes *aos* and *fps* encode the key enzymes in the defense signal pathways jasmonic acid and terpene signal pathways, respectively, it was deduced that PPO from aphid saliva, as the main elicitor, triggers an appropriate defense response in wheat through jasmonic acid and terpene signal pathways.

KEYWORDS: Aphid saliva; polyphenol oxidase (PPO); wheat; induced resistance mechanism

1. INTRODUCTION

Aphids, an important group of piercing−sucking agricultural pest insects, ingest nutrients from sieve tube in phloem, the photoassimilates transport conduit in plant. The insect has unique morphological adaptation features, host physiological sensation, digestion and excretory systems, and feeding behavior. It shares certain plant resources and, sometimes, can even exhaust all of the nutrients contained in the phloem (*1*). During the feeding process on host plants, aphid salivary stylets penetrate plant tissues accompanied with saliva secretion, which is very important for aphids to establish physical contact on the surface of plant tissues (*2*). On the other hand, the saliva components contain certain bioactive compounds that can induce defense responses in the host plant (*3*).

Prior to and during feeding, insects with plant-sucking mouthparts, such as aphids and white fly, secrete watery digestive saliva, which contains multiple enzymes. It was reported that the enzymes in aphid saliva have been found to be closely associated with plant defense (*4*). For instance, the aphid's salivary enzymes stimulate polyphenol oxidase (PPO) in the plant, which acts as the defensible antinutrient protein (*5*), causing bad digestion, or antifeedant activity, in the aphids and thus protecting plants from injury. The aphid saliva mainly contains pectinase, PPO, cellulose, etc. (*4*−*6*). These enzymes play important roles in feeding, digestion, metabolism, and self-detoxification processes of aphids (*7*,*8*).

Miles (*9*) first discovered that aphid saliva contained PPO in 1972. The probing of aphid on plants causes polyphenol accumulation (especially catechol) on the feeding spots. PPO catalyzes the conversion of polyphenol into benzoquinone, thereby alleviating harmful effects on aphids, thus overcoming the plant's defense system (*4*, *10*−*12*).

There are two types of responses in host plants infested by aphids. One is to activate the general defense genes, and the other one is to activate the specific anti-aphid genes upon the presence of the aphid-resistant trait, followed by the normal maintenance genes becoming the defense pattern in plant. To mobilize the defense response, plants produce various types of elicitors/activators, which activate expression of the genes in different defense signal pathways.

One is the salicylic acid (SA) defense signal pathway. Many publications have confirmed that genes in the SA defense signal pathway are induced in the plant tissues when aphids feed on the susceptible host plants, such as peach aphids, *Myzus persicae*, on the susceptible *Arabidopsis* and celery, and greenbugs, *Schizaphis graminum* on the susceptible sorghum cultivars (*1*, *13*−*15*). Genes encoding pathogenesis-related proteins (PRP/PRs), such as β-1,3-glucanase, lectin-like protein, and chitinase, are also activated. In contrast, the resistant wheat, upon feeding by *Diuraphis noxia*, had higher SA content (*16*). These results indicate that the SA mediated defense signal pathway plays an important role in the defense response of host plants to aphid.

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The second one is the jasmonic acid (JA) signal pathway. Tobacco leaves infested by tobacco aphid, *M. persicae* (*17*), and resistant wheat cultivars infested by *D. noxia* and *S. graminum* (*18*, *19*) increased significantly the JA synthesis and expression of JA-mediated defense associated genes, such as 12-oxo- PDA, 12-oxo-PDA reductase, lipoxygenase (LOX), and cytochrome P450. Aphid feeding obviously induced the transcriptional level of LOX gene on the leaves of tomato (*20*), *Arabidopsis* (*13*), tobacco (*17*), and wheat (*21*).

The third one is the terpene-mediated defense signal pathway. The major kinds of terpenes induced by insects are monoterpenes, diterpenes, sesquiterpenes, and their derivatives, mainly consisting of C10, C11, C15, and C16 terpenes. Farnesyl-pyrophosphate synthase (FPS) is the key enzyme in the process of manipulating the synthesis of isoprenoid compounds, and the expression level of FPS regulates the biological synthesis of terpenes.

On the basis of the *S. avenae*−wheat interactive system, Zhao et al. (*21*,*22*) have compared the induced effects by aphid feeding, mechanical wounding, and induced volatile substances from aphid infested wheat, as well as the active compounds, on the signal transduction pathway, namely, the activity of key enzymes, such as LOX, PPO, PAL, and β-1,3-glucanase, and the transcript level of key defense genes, such as *fps* (encoding for farnesyl-pyrophosphate synthase), *pal* (encoding for phenylalanine ammonia-lyase), and *aos* (encoding for allene oxide synthase). They found that aphid feeding stimulated the key enzymes from two defense signal transduction pathways, that is, JA and SA signal transduction pathways, at both enzymatic activity and gene expression levels, but the induced responses resulting from mechanical wounding (imitating aphid stylet without saliva solution) and aphid feeding are not equivalent. It was postulated that the components of saliva may be a specific elicitor to elicit herbivore-specific defense responses.

There are only few papers on the enzyme components in the watery digestive saliva of wheat aphid. Guo et al. (*23*) detected pectase in *S. avenae* saliva, which was responsible for the induction of indirect defense response in wheat seedling. It is unclear whether the presence of PPO in wheat aphid saliva functions in triggering the defense gene expression during feeding in the host plant.

In this study, saliva was collected by aphid feeding on artificial diet sachets. The PPO enzymatic activity in the aphid saliva was measured, and then the PPO was applied on susceptible wheat to detect the induced response on genes expression (*fps*, *pal*, and *aos*) using fluorescent quantitative PCR. In addition, PPO activity was compared between the saliva from *S. avenae* and *S. graminum*. The objective of this study was to explore the role of aphid saliva in the interaction between host and aphids during the process of inducing the host defense mechanism. The findings will be useful in understanding the elicitors of aphid saliva in elicited wheat defense signal pathways and of potential benefit for bioactive compound development for new aphid control techniques and also for the early assessment of food safety of aphid-infested wheat.

2. MATERIALS AND METHODS

2.1. Materials. *2.1.1. Wheat Cultivar and Growing Conditions in Laboratory*. Aphid-susceptible wheat seeds (cv. Beijing 837) were sterilized in 1% NaClO and germinated in plastic pots. The seedling was grown in a cultured room at 22 °C, 16 h light/8 h dark regimen, with 40−60% relative humidity. The experiment was conducted on seedlings at two-leaf stage.

2.1.2. Aphids. The routine culture of two aphid species, *S. avenae* and *S. graminum*, the Beijing clone, were maintained on wheat seedling (cv. Beijing 837) at 22 °C, 16 h light/8 h dark regimen, 40−60% relative humidity.

2.1.3. Chemicals and Reagents. Serine, methionine, and aspartate were manufactured by Shanghai Dongfeng Biochemical Technology Co. (Shanghai, China). Agarose and dopamine were from Sigma. Ruthenium Red was purchased from Fluka. Other common reagents and chemicals were from Beijing Guang Da HengYi Industrial Co., Ltd. (Beijing, China). Trizol reagent and reverse transcriptase kit were from Invitrogen. The SYBR Premix Ex Taq was from TaKaRa, China. The primers were synthesized at Beijing Sunbiotech Co., Ltd. (Beijing, China). All other chemicals were of analytical grade. Water was double-distilled sterile water.

2.2. Experimental Methodology. *2.2.1. Artificial Diet Preparation and Wheat Aphid Saliva Collection.*

2.2.1.1. Artificial Diet Preparation. The chemically defined diet of Miles et al. and Tjallingii et al. (*24*, *25*) was slightly modified to be 20% sucrose, 100 mmol/L serine, 100 mmol/L methione, and 100 mmol/L aspartic acid. The diet was held in Parafilm sachets; the diet mix was prepared under aseptic condition, stored at 4 °C in a refrigerator, and used within 7 days.

2.2.1.2. Aphid Saliva Collection for Enzyme Solution. The aphids, *S. avenae* and *S. graminum*, were reared on the underside of the sachets with 100 μL of diet solution, respectively, under a yellow light incubator at 22 °C and 16 h light/8 h dark, where they settled and initiated feeding. Each collection of saliva used 30 third-instar nymphs feeding for 24 h. To obtain the saliva from the aphid-fed diet sachet, a hole was pierced in the upper membrane of the diet sachet and the liquid was collected. After centrifugation at 16000*g* at 4 °C for 30 min, the supernatant was transferred to enzyme solution and stored at −20 °C.

2.2.1.3. Mass Collection of Aphid Saliva Solution for Application on Wheat. Each collection of saliva used ~3000 aphids feeding for 24 h as described in section 2.2.1.2. The saliva solution was collected from 100 aphid-fed diet sachets, pooled in final volumes of 8.9−9.0 mL, and stored at −20 °C. The two aphid saliva solutions applied to wheat leaves.

2.2.2. Identification of PPO in Wheat Aphid Saliva. PPO activity was assayed according to the method of Urbanska et al. (*7*). An agarose gel piece (125 mg) was mixed with 1 mL of diet solution, added to 1 mL of phosphate buffer (0.1 mol/L, pH 7.4) and dopamine to a final concentration of 0.1%, and then mixed thoroughly to form the diet gel for aphids. Thirty aphids of *S. avenae* and *S. graminum* in third-instar nymphs, respectively, were administered to the underside of the diet gel, feeding for 24 h. To observe the color changes under a microscope (NanoDrop ND-1000, Nanodrop Co.), the upper layer of the Parafilm membrane was removed. The presence of black spots validates PPO existence.

2.2.3. PPO Enzymatic Activity Assay. PPO enzymatic activity was assayed by ultraviolet spectrophotometer (*26*). The reaction mixture was prepared by adding 1.5 mL of *o*-dihydroxybenzene (100 mmol/L) into 1.4 mL of Na_2HPO_4−NaH_2PO_4 buffer (0.2 mol/L, pH 6.5), incubated in a water bath at 30 °C for 30 min. The enzymatic reaction was initiated by the addition of 0.1 mL of enzyme solution, the OD value at 410 nm was measured immediately and four more times at 30 s intervals during 2 min, for a total of five measurements. One unit of PPO activity (U) was defined as that which increases the absorbance at 410 nm by 0.001 per minute in the assay conditions, and the ratio of enzyme activity is expressed by the enzyme activity unit per milligram of protein. Assays were determined in three replicated samples.

2.2.4. Detection of PPO and Aphid Saliva Solution Induced the Transcript Level of Genes pal, aos, and fps in Wheat.

2.2.4.1. Induction Procedure of PPO and Aphid Saliva Solution. PPO (10 μg) was dissolved in 1 mL of ddH_2O. Thirty holes were punched with needles (2 mm diameter) on each leaf lamina of wheat seedlings at two-leaf stage, followed by spraying the enzymatic solution on both surfaces of leaf lamina. Leaf samples were collected at 0.5, 1, 2, 3, 4, 5, 6, 12, and 24 h after treatment. The untreated control plants were sprayed with ddH_2O. The aphid saliva solutions of *S. avenae* and *S. graminum* prepared as in section 2.2.1.3 were applied on wheat leaves, respectively, and the treated and sampled methods were similar to PPO solution treatment. The untreated control plants were sprayed with the artificial diet.

2.2.4.2. Total RNA Extraction and cDNA synthesis from Wheat Plants. Total RNA was extracted from leaves using the

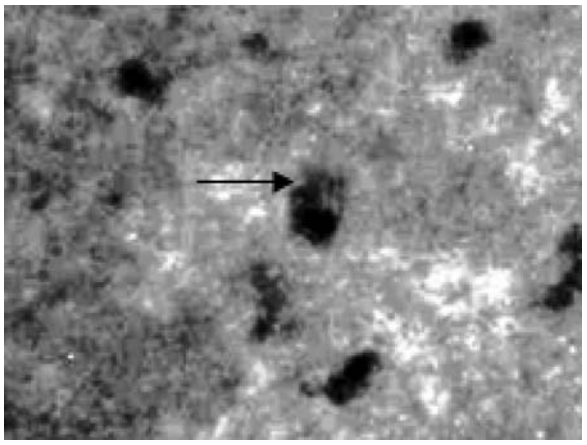

Figure 1. Identification of PPO in saliva of *S. avenae*. The arrow points to the black spots that resulted from PPO existence in aphid saliva.

TRIZOL kit (Invitrogen). First-strand cDNA was synthesized by a reverse-transcription reaction. Second-strand cDNA was amplified and quantified by adding forward and reverse primers, following the kit's protocol. Four genes, the genes encoding farnesyl pyrophosphate synthetase (*fps*), allene oxide synthase (*aos*), phenylalanine ammonia-lyase (*pal*), and a housekeeping gene *18SrRNA* referred to by Zhao et al., and the DNA sequences were retrieved from the GenBank database [*fps*, accession no. D85317, *aos*, accession no. AY196004, *pal*, accession no. AY005474, and the endogenous reference gene 18SrRNA, accession no. AY357914). Gene primers were designed as follows: *pal* F, 5′-GAGCACATCTTG-GAGGGAA-3′, R, 5′-TGAGCGGGTTGTCGTTG-3′; *aos* F, 5′-GTTC-TTCCCGGTACGACA-3′, R, 5′-ACGATTGACGGCTGCTATGA-3′; *fps* F, 5′-TCAAGACGGCTTCAGGG-3′, R, 5′-TCGCCAAAGTTAT-CCAAAT-3′; *18SrRNA*, F 5′-CCATCGCTCTGGATACATTAG-3′, R, 5′-CTTTCGCAGTTGTTCGTCTTT-3′.

2.2.4.3. Optimization of RT-PCR Reaction Condition. The target genes, *pal*, *aos*, and *fps*, and the endogenous reference gene, *18SrRNA*, with the expected fragments size of 196, 191, 184, and 165 bp, respectively, were amplified under the same PCR thermocycler (Bio-RAD iCycler) by iQ SYBR Green Supermix kit (Bio-Rad). To optimize the RT-PCR condition, the PCR program of amplification was designed for *pal*, *aos*, *fps*, and *18SrRNA* genes, respectively. The similar parts of the procedure were as follows: 95 °C for 10 s, followed by 45 cycles of denaturing at 95 °C for 10 s of annealing−extension, and final hold at 4 °C. However, there were changes in annealing temperature cascades and extension time among gene amplifications. The different parts of the procedure for the genes were as follows: for *18S rRNA*, annealing at 51−61 °C for 16 s and extension at 72 °C for 10 s; for *fps*, annealing at 51−61 °C for 18 s and extension at 72 °C for 12 s; for *pal*, annealing at 54−65 °C for 20 s and extension at 72 °C for 12 s; for *aos*, annealing at 52−62 °C for 20 s and extension at 72 °C for 12 s.

2.2.4.4. Gene Transcript Quantitative Analysis. Gene transcript levels were measured by SYBR fluorescent real-time quantitative PCR method. Amplifications of three genes (*fps*, *aos*, *pal*) and *18SrRNA* were assayed with the gene-specific primers, all under the optimum PCR conditions determined in section 2.2.4.3. Three replicates of assay reaction were conducted on each cDNA sample. The gene transcript level was calculated according to the following formula:

$$\text{relative transcript} = 2^{-\Delta\Delta Ct}, \Delta\Delta Ct = (Ct_{\text{target}} - Ct_{18SrRNA})_{\text{sample}} - (Ct_{\text{target}} - Ct_{18SrRNA})_{\text{control}}$$

3. RESULTS

3.1. PPO Enzymatic Activity in Wheat Aphid Saliva. From the aphid-fed diet gel by *S. avenae* and *S. graminum,* black spots were seen on the top surface of two aphid species diet gel (**Figures 1** and **3**), which showed that PPO existed in the saliva of *S. avenae*

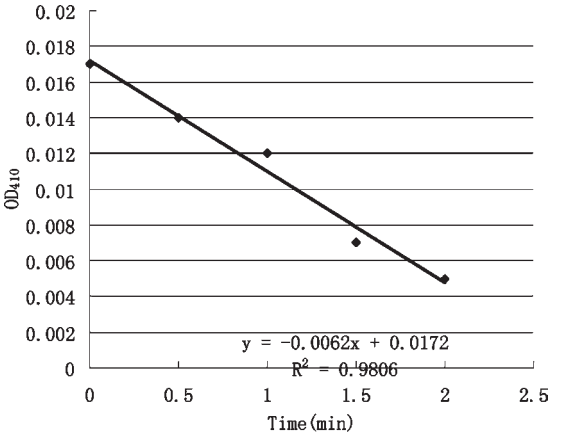

Figure 2. Time course of PPO activity in saliva of *S. avenae* within 2 min.

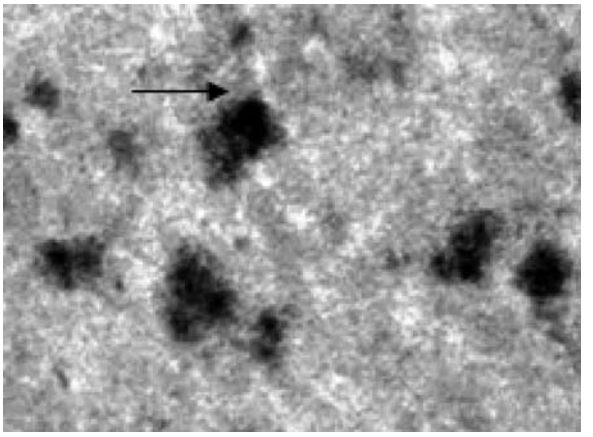

Figure 3. Identification of PPO in saliva of *S. graminum*. The arrow points to the black spots resulting from PPO existence in aphid saliva.

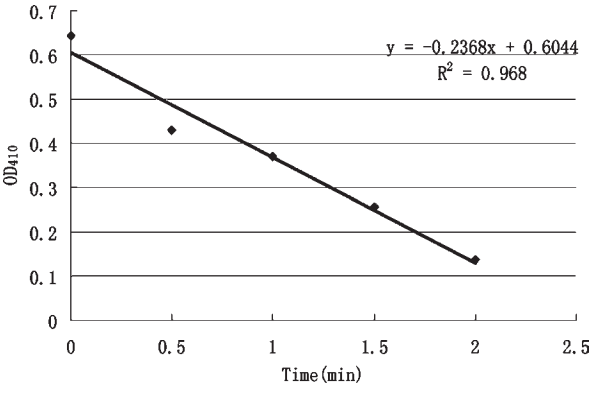

Figure 4. Time course of PPO activity in saliva of *S. graminum* within 2 min.

and *S. graminum*. PPO enzymatic activity of the saliva of two aphid species was determined from the aphid-fed diet sachets used by 30 aphids after 24 h. A linear relationship was found between the OD values and time within a 2 min assay (**Figures 2**and **4**). For *S. avenae*, the line's slope was 6.2×10^{-3} (**Figure 2**), which means the PPO activity was 6.2×10^{-3} U/g in the saliva. For *S. graminum*, the line's slope was 2.37×10^{-1} U/g (**Figure 4**) and the PPO activity was 2.37×10^{-1} U/g in the saliva. The result of PPO activity in saliva of two aphid species was 38-fold higher in *S. graminum* than in *S. avenae*. It was speculated that the higher PPO activity in *S. graminum* saliva was a contributing factor to

the light yellow spot left on the feeding site of wheat leaf by *S. graminum*; no such symptom was seen with *S. avenae* feeding.

3.2. RNA Quality Determination. The total RNA of wheat leaves had a 260/280 ratio at 1.8−2.1, with clear bands on the 2% agarose gel without obvious degradation (**Figure 5**). High RNA quality for reverse transcription was thus obtained.

3.3. Optimization of RT-PCR Reaction Conditions. *3.3.1. PCR Product from 18S rRNA Gene.* The result of PCR amplification of *18S rRNA* showed that one specific band was produced under annealing at 51−61 °C (**Figure 6**). Therefore, for *18S rRNA*, the optimum annealing regimen was 51−61 °C.

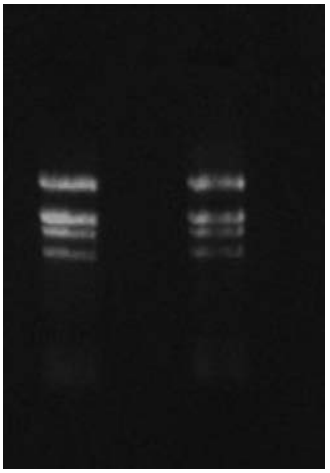

Figure 5. Detection of total RNA from wheat seedling.

3.3.2. PCR Product from fps Gene. The result of PCR amplification of *fps* showed that a single clean band was produced under annealing at 51−57.8 °C (**Figure 7**). Therefore, for *18S rRNA*, the optimum annealing regimen was 51−61 °C.

3.3.3. PCR Product from pal Gene. The result of PCR amplification of *pal* showed a single clean band appearing under the annealing at 54−65 °C regimen (**Figure 8**), but dimer was detected under the tested annealing temperatures except at 64.4 °C. Therefore, for *pal* PCR, the optimum annealing was at 64.4 °C.

3.3.4. PCR Product from aos Gene. The result of PCR amplification of *aos* gene showed that a single band was observed under annealing at 52−58.6 °C (**Figure 9**), but optimum annealing was at 52−52.7 or 56.8−58.6 °C.

3.4. Effects of Aphid Saliva and PPO on the Transcript of Key Genes in the Defense Pathways. In the aphid saliva and PPO treated leaves of wheat seedling, the transcripts of defense genes were detected as follows (**Table 1**−**3**; **Figures 10** and **11**): farnesyl pyrophosphate synthetase (*fps*) related to isoprene biosynthesis; allene oxide synthase (*aos*) in the JA-mediated signaling transduction pathway; and phenylalanine ammonia-lyase (*pal*) in the phenylpropanoid (SA-mediated) pathway.

3.4.1. Effects of S. avenae Saliva on the Transcript of Key Genes. The transcript profiling data (Table**1**) showed that treatment with saliva of *S. avenae* significantly induces the expression of genes *pal*, *fps*, and *aos*. The transcript of *pal* reached peak level at 4 h, 12.46-fold higher than the control. The transcript of *fps* reached peak level at 4 h, 8.43-fold higher than the control. *aos* was elicited rapidly at 0.5 h and reached peak level at 1 h, 15.66-fold higher than the control. Aphid saliva from *S. avenae* induces defense gene expression

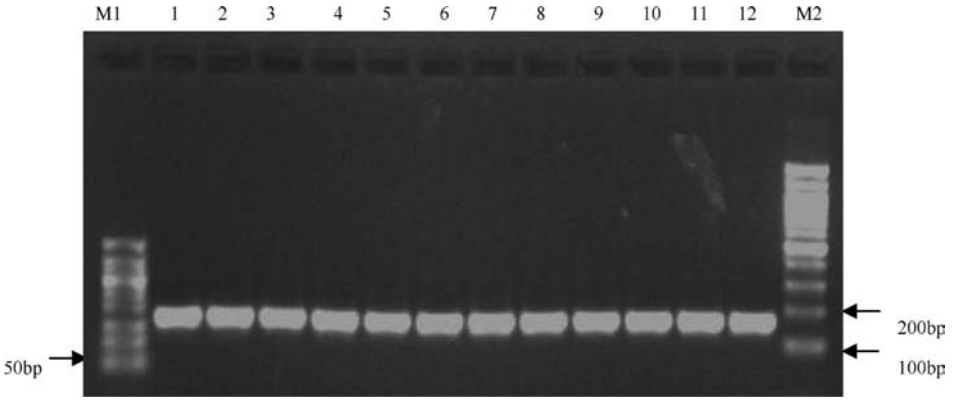

Figure 6. Detection of PCR product of *18S rRNA* gene on agarose gel. M1 and M2 were loaded with 50bp and 100bp marker ladders. Lanes 1-12 were loaded with the PCR products at annealing temperature of 51.5°C, 52°C, 53°C, 54.2°C 55.4°C, 56.6°C, 57.8°C, 59°C, 60°C, 60.5°C and 61°C, respectively.

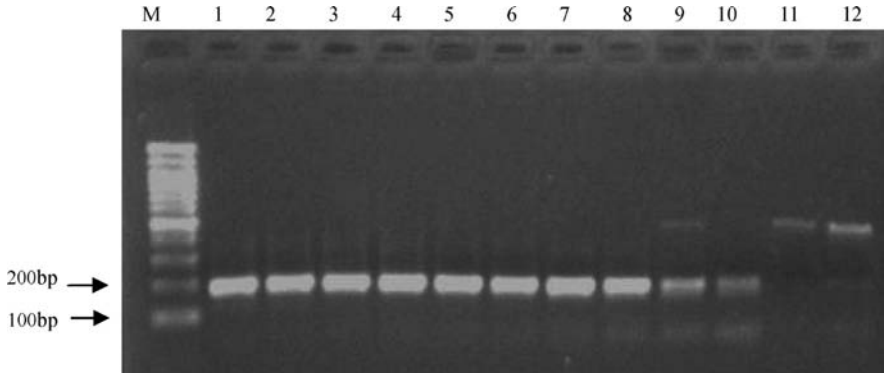

Figure 7. Detection of PCR product of *fps* gene on agarose gel. M1 and M2 were loaded with 50bp and 100bp marker ladders. Lanes 1-12 were loaded with the PCR products using annealing temperature of 51°C,51.5°C, 52°C,53°C,54.2°C, 55.4°C, 56.6°C,57.8°C,59°C,60°C,60.5°C,61°C, respectively.

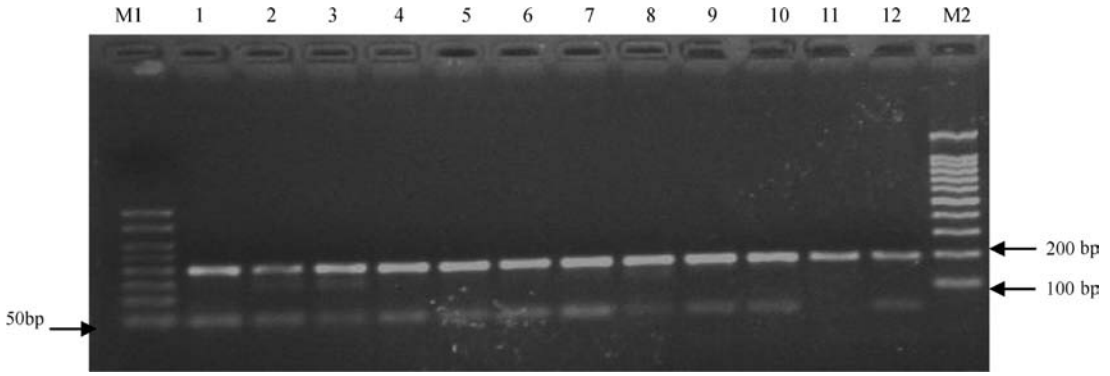

Figure 8. Detection of PCR product of *pal* gene on agarose gel. M1 and M2 were loaded with 50bp and 100bp marker ladders. Lanes 1-12 were loaded with the PCR products using annealing temperature of 54°C, 54.5°C, 55.1°C, 56.2°C, 57.5°C, 58.8°C, 60.1°C, 61.4°C, 62.8°C, 63.9°C, 64.4°C, 65°C, respectively.

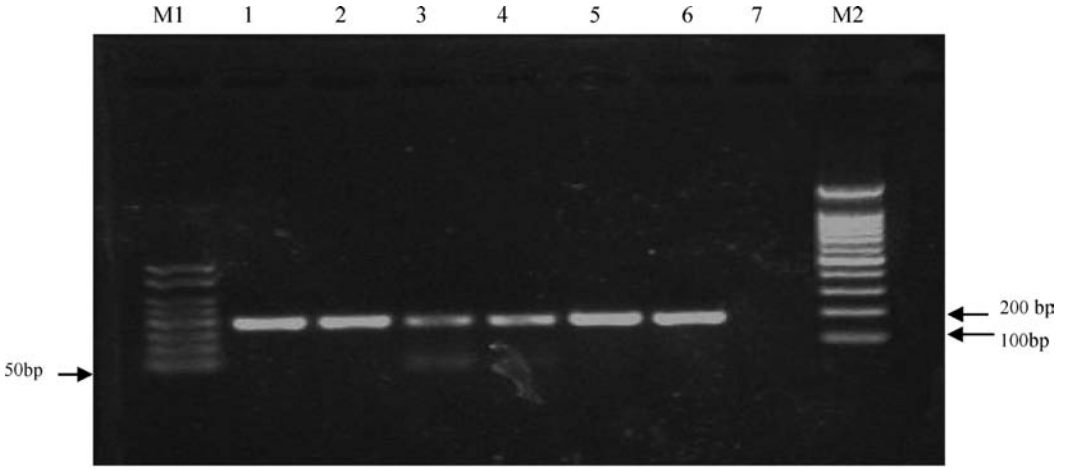

Figure 9. Detection of PCR product of *aos* on agarose gel. M1 and M2 were loaded with 50bp and 100bp marker ladders. Lanes 1-12 were loaded with the PCR products using annealing temperature of 54°C, 54.5°C, 55.1°C, 56.2°C, 57.5°C, 58.8°C, 60.1°C, 61.4°C, 62.8°C, 63.9°C, 64.4°C, 65°C, respectively.

in wheat through JA, SA, and terpene signal transduction pathways. The earliest induced response occurred in the JA-mediated pathway, by activation of the *aos* gene within 0.5−1 h of treatment, then promoted the expression of other defense genes such as *pal* and *fps* in the SA and terpene pathways.

3.4.2. Effects of S. graminum Saliva on the Transcript of Key Genes. Similar to the leaves treated with saliva of *S. avenae*, transcript levels of *pal*, *fps*, and *aos* increased significantly (by 3.13-, 8.39-, and 8.35-fold, respectively) after treatment with *S. graminum* saliva and reached peak transcript levels at 1−2 h after treatment. Compared with *S. avenae* saliva, *S. graminum* simultaneously induced wheat defense response through JA, SA, and terpene signal transduction pathways, but the latter two were the major pathways induced by the saliva of *S.graminum*.

3.4.3. Effects of PPO on the Transcript of Key Genes. For the wheat leaves treated with PPO, the fluorescent real-time quantitative PCR reaction and melting curve are shown in **Figure 10**. All of the PCR products had single-peak melting curve, indicating specific amplification of the genes isolated from the PPO-treated wheat leaves.

The transcription level of the three genes, *pal*, *fps*, and *aos*, increased to different extents (2.31-, 18.69-, 263.31-fold, respectively) (**Table 3**) and reached peak transcript levels at 1, 2, and 3 h after treatment, respectively. The result of PPO increased the transcripts of *aos* and *fps* significantly, which indicated that PPO induced wheat defense response through JA and terpene defense signal pathways.

The results of the effect of saliva from two aphid species and PPO on transcripts of *pal*, *fps*, and *aso* are shown in **Figure 11**. The salivary components from *S. avenae* induced the transcripts of *pal*, *fps*, and *pal* significantly, which may exist as elicitors for SA, JA, and terpene defense signal pathways, and the salivary components from *S. graminum* may exist as elicitors for SA and terpene defense signal pathways; PPO solution was the major elicitor for JA and terpene defense signal pathways.

4. DISCUSSION

Grain aphid, *S. avenae*, and greenbug, *S. graminum*, are two common cereal aphids on wheat in subtropical areas. The two species own different ecological niches on plants and also produce different symptoms. *S. avenae* damages the upper leaf and ear, whereas *S. graminum* infests the abaxial surface of the lower leaves. The wheat leaves fed on by *S. graminum* form light yellow spots produced by necrotic hypersensitive reaction, caused by a toxin injected by the aphid (*27*). The symptom could be hardly observed from *S. avenae* aphid feeding sites.

The qualitative and quantitative tests of salivary enzymes found that both species existed PPO, and the PPO enzyme activity in *S. graminum* saliva was almost 38-fold higher than that of the *S. graminum*. It is very likely that PPO in aphid saliva could be responsible for the light yellow leaf spots upon infestation by *S. graminum*. Oxidases can induce oxidative reaction and lead to chlorotic wheat leaves, but it needs to be proved by further investigation of the observable symptom by PPO application on wheat. This enzyme is reported to be closely associated with plant

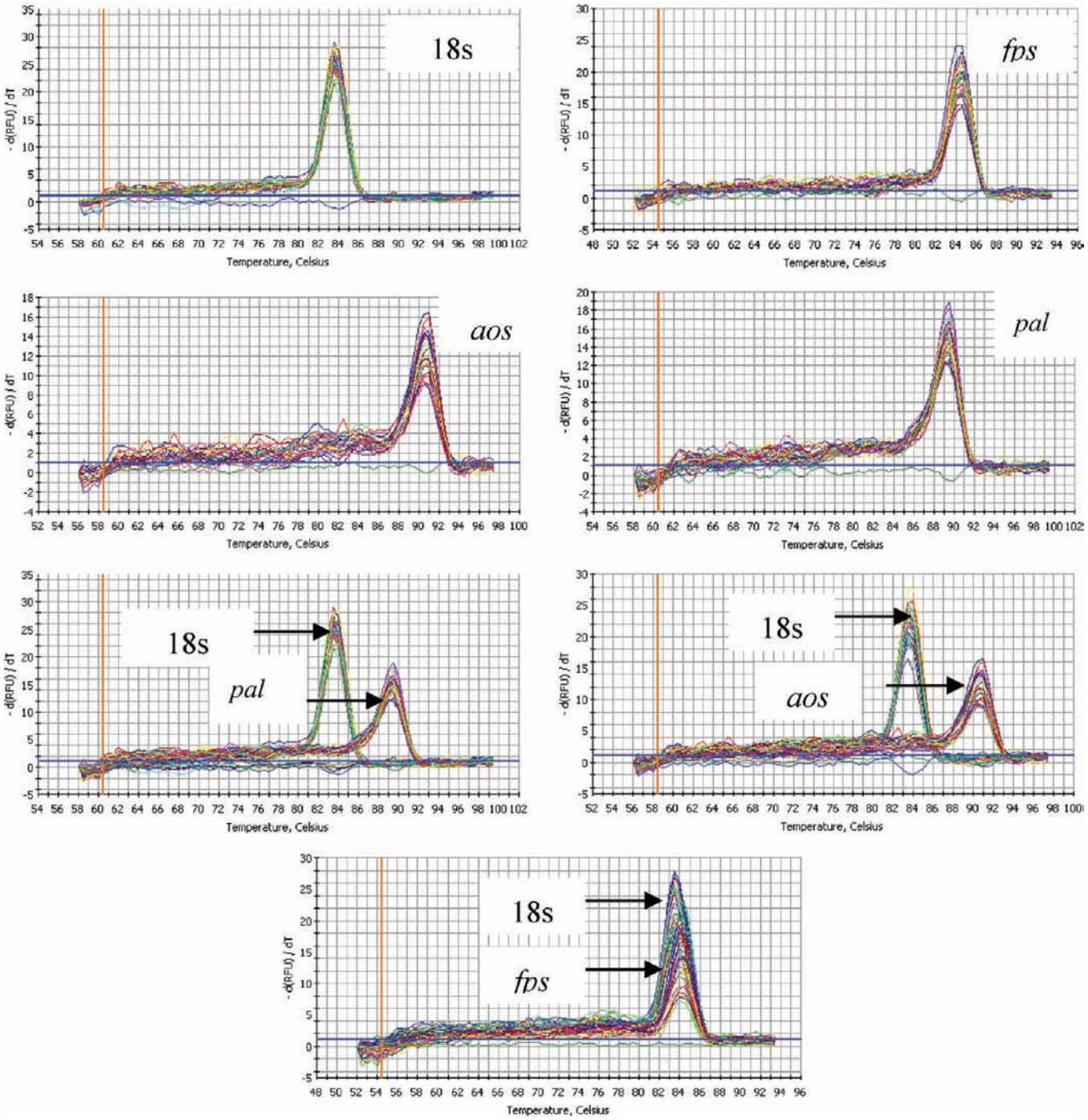

Figure 10. Melting curves of endogenous reference gene (*18S rRNA*) and the target genes (*aos*, *fps*, *pal*) in real-time PCR.

disease resistance, mainly by participating in oxidation of phenol into quinones. It is also involved in polymerization of lignin precursors to form lignin, thus affecting lignifications of cell wall and tissues. The necrotic spots formed on the leaf surface could result from localized tissue necrosis. The role of PPO from aphid saliva in causing necrotic hypersensitive reaction and the impact on the PPO and peroxidase activities in the host plants are awaiting clarification in future study.

PPO in aphid saliva was validated to be an elicitor in the defense response of the host plants at the biochemical and molecular level. Real-time fluorescent quantitative PCR was performed to measure the expression of the relevant genes. After PPO treatment, the transcript level of key defense genes, such as *fps* and *pal*, were induced to 18- and 2.3-fold higher levels after treatment for 1 h, and that for *aos* increased to 263-fold after treatment for 3 h. The results indicate that PPO was the major elicitor to activate efficiently to JA and terpene defense signal pathways. Therefore, PPO in aphid saliva served as the elicitor for plant defense reaction.

From the saliva of both aphid species, except for PPO, other kinds of enzymes have been detected (data are not shown), which may contribute to affect aphid host identification and acceptance and the interaction of aphid and plant defense systems.

Smith et al. (*28*) summarized the progress of the molecular basis of plant resistance and defenses to aphid feeding and concluded that aphid feeding may trigger multiple signaling pathways dependent on both salicylate and jasmonate signaling

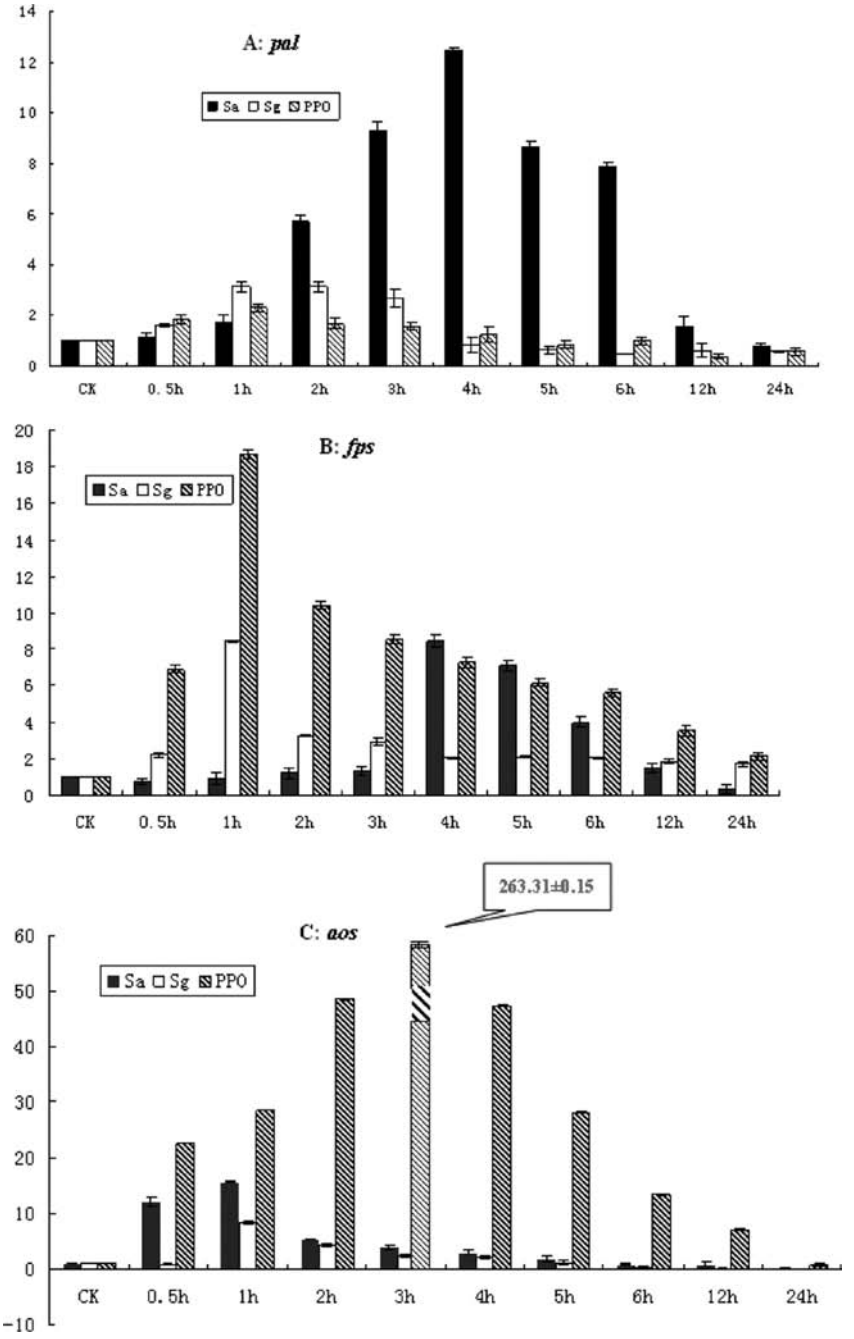

Figure 11. Effects of saliva from two cereal aphids and PPO treatments on (**A**) *pal*, (**B**) *fps*, and (**C**) *aso* expression. Sa, *S. avenae*; Sg, *S. graminum*.

Table 1. Relative Expression of *pal*, *fps*, and *aos* in Leaves Exposed to *S. avenae* Saliva

	relative expression of three genes		
time after treatment with *S. avenae* saliva	*pal*	*fps*	*aos*
CK	1	1	1
0.5 h	1.11 ± 0.22	0.78 ± 0.17	11.98 ± 0.81
1 h	1.73 ± 0.26	0.91 ± 0.30	15.66 ± 0.09
2 h	5.72 ± 0.21	1.23 ± 0.29	5.28 ± 0.06
3 h	9.30 ± 0.38	1.31 ± 0.23	3.88 ± 0.40
4 h	12.46 ± 0.13	8.43 ± 0.35	2.97 ± 0.45
5 h	8.66 ± 0.21	7.10 ± 0.29	1.74 ± 0.53
6 h	7.89 ± 0.20	3.99 ± 0.29	0.89 ± 0.12
12 h	1.52 ± 0.45	1.50 ± 0.23	0.86 ± 0.38
24 h	0.76 ± 0.15	0.33 ± 0.23	0.11 ± 0.00

molecules in host plants. There is evidence that aphids can produce various types of elicitors to activate gene expression in the respective defense signal pathways, including the SA and JA signal pathways. Our research group has proved grain aphid, *S. avenae*, feeding could induce SA and JA signal pathways and propose that the major elicitor may exist in the aphid saliva (*21*). From this study, the saliva from two cereal aphids could induce the expression of the genes *aos* and *fps* to different extents. The data showed that the saliva of *S. avenae* induced the transcript of *pal*,*fps*, and *aos* by 12.46-, 8.43-, and,5.66-fold, respectively, which may exist as major elicitors to JA, SA, and terpene defense signal pathways. The saliva of *S. graminum* induced the transcript levels of *pal*, *fps*, and *aos* by 3.13-, 8.39-, and 8.35-fold, respectively, which may exist as major elicitors to SA and terpene defense signal pathways.

Table 2. Relative Expression of *pal*, *fps*, and *aos* in Leaves Exposed to *S. graminum* Saliva

time after treatment with *S. graminum* saliva	relative expression of three genes		
	pal	*fps*	*aos*
CK	1	1	1
0.5 h	1.63 ± 0.06	2.20 ± 0.12	0.83 ± 0.12
1 h	3.13 ± 0.20	8.39 ± 0.07	8.35 ± 0.29
2 h	3.13 ± 0.20	3.25 ± 0.06	4.26 ± 0.26
3 h	2.66 ± 0.37	2.93 ± 0.23	2.29 ± 0.30
4 h	0.85 ± 0.29	2.05 ± 0.03	2.13 ± 0.26
5 h	0.63 ± 0.15	2.10 ± 0.06	1.17 ± 0.31
6 h	0.50 ± 0.00	2.05 ± 0.03	0.25 ± 0.15
12 h	0.61 ± 0.26	1.83 ± 0.12	0.08 ± 0.03
24 h	0.59 ± 0.03	1.71 ± 0.13	0.02 ± 0.03

Table 3. Relative Expression of *pal*, *fps*, and *aos* in Leaves Exposed to PPO

time after treatment with PPO	relative expression of three genes		
	pal	*fps*	*aos*
CK	1	1	1
0.5 h	1.84 ± 0.20	6.87 ± 0.20	22.65 ± 0.06
1 h	2.31 ± 0.15	18.69 ± 0.26	28.52 ± 0.03
2 h	1.68 ± 0.19	10.42 ± 0.20	48.62 ± 0.10
3 h	1.56 ± 0.15	8.49 ± 0.23	263.31 ± 0.15
4 h	1.26 ± 0.31	7.24 ± 0.26	47.48 ± 0.09
5 h	0.86 ± 0.12	6.13 ± 0.21	28.01 ± 0.15
6 h	0.98 ± 0.17	5.58 ± 0.20	13.32 ± 0.09
12 h	0.38 ± 0.09	3.55 ± 0.29	6.99 ± 0.12
24 h	0.59 ± 0.15	2.11 ± 0.19	0.73 ± 0.18

From a Smith et al. (*28*) hypothesis, two different processes are involved in elicitation of plant response to aphid feeding. One process involves a gene-for-gene recognition of aphid-derived elicitors by plant resistance genes followed by the activation of aphid-specific resistance and defense responses. The second process involves plant recognition of aphid-inflicted plant tissue damage, which leads to changes in plant chemistry, followed by the production of plant signaling molecules that trigger a general stress response, similar to the basal plant defense to phytopathogen. Both processes include the chemical change in plant triggered by aphid feeding and toxin from aphid saliva. There are very few reports of aphid feeding kernel protein and quality traits of wheat flour (*29*), and no report on the impact of aphid saliva. It will be necessary to be aware if aphid infestation, followed by sap ingested and saliva injected in plant phloem and other tissue, may pose any risk to wheat food safety, so early emerging risk analysis deserves attention from scientists.

ABBREVIATIONS USED

PPO, polyphenol oxidase; JA, jasmonic acid; SA, salicylic acid; LOX, lipoxygenase; FPS, farnesyl-pyrophosphate synthase; PAL, phenylalanine ammonia-lyase; PRP/PRs, pathogenesis-related proteins; *fps*, gene for farnesyl-pyrophosphate synthase; *pal*, gene for phenylalanine ammonia-lyase; *aos*, gene for allene oxide synthase; CK, control; Sa, *Sitobion avenae*; Sg, *Schizaphis graminum*.

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