# Characterization of Peroxidase Changes in Tolerant and Susceptible Soybeans Challenged by Soybean Aphid (Hemiptera: Aphididae)

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**ABSTRACT** Changes in protein content, peroxidase activity, and isozyme profiles in response to soybean aphid feeding were documented at V1 (fully developed leaves at unifoliate node, first trifoliate leaf unrolled) and V3 (fully developed leaf at second trifoliate node, third trifoliate leaf unrolled) stages of soybean aphid-tolerant (KS4202) and -susceptible (SD76R) soybeans. Protein content was similar between infested and control V1 and V3 stage plants for both KS4202 and SD76R at 6, 16, and 22 d after aphid introduction. Enzyme kinetics studies documented that control and aphid-infested KS4202 V1 stage and SD76R V1 and V3 stages had similar levels of peroxidase activity at the three time points evaluated. In contrast, KS4202 aphid-infested plants at the V3 stage had significantly higher peroxidase activity levels than control plants at 6 and 22 d after aphid introduction. The differences in peroxidase activity observed between infested and control V3 stage KS4202 plants at these two time points suggest that peroxidases may be playing multiple roles in the tolerant plant. Native gels stained for peroxidase were able to detect differences in the isozyme profiles of aphid-infested and control plants for both KS4202 and SD76R.

**KEY WORDS** peroxidase, host plant resistance, tolerance, soybean aphid

Since its detection in July of 2000, soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), has become the primary pest of soybeans (*Glycine max* (L.) Merrill) in the north-central region of the United States (Ragsdale et al. 2011, Hodgson et al. 2012). Soybean aphids are piercing–sucking insects that feed on soybean leaves, stems, and pods, withdrawing the phloem contents, which can result in the transmission of viral pathogens (Clark and Perry 2002), poor canopy development and significant reduction in photosynthesis (Macedo et al. 2003, Pierson et al. 2011). An indirect effect of heavy soybean aphid infestation is the development of dark sooty mold (*Capnodium* spp.) on the sugary honeydew (Tilmon et al. 2011). The injury caused by soybean aphid can reduce plant height, pod development, a lower number of seeds at maturity, and yield loss (Ragsdale et al. 2007). Furthermore, soybean aphids can also decrease the amount of seed oil, which can affect the market value of soybean seeds (Beckendorf et al. 2008).

Although alternative management strategies have been proposed (e.g., plant resistance and biological control), chemical control still remains the main method for suppressing soybean aphid in the United States (Hodgson et al. 2010). In general, most of the studies conducted in host plant resistance have focused on soybean cultivars possessing antibiotic or antixenotic traits (Hill et al. 2004, 2006a,b; Mensah et al. 2005; Diaz-Montano et al. 2006, 2007; Kim et al. 2008; Mian et al. 2008; Crompton and Ode 2010). Only a few studies have investigated soybeans that are tolerant to soybean aphid (Pierson et al. 2010, 2011; Marchi 2012; Prochaska et al. 2013). Despite the progress achieved in identifying soybean aphid resistant sources, limited research has been done to understand the mechanisms that are underlying plant resistance, specifically tolerance in soybeans.

The identification of mechanisms underlying plant tolerance is a crucial step to understanding how plants defend themselves from herbivores and identifying breeding strategies for incorporating tolerance traits into high-yielding plants (Panda and Khush 1995). Tolerance is the plant's ability to withstand arthropod feeding or injury and is conferred by a number of compensatory mechanisms (Strauss and Agrawal 1999, Smith 2005). Photosynthetic compensation, plant morphology and architecture, plant hormones, and oxidative enzymes have all been shown to be involved in the plant's defense response to insect herbivory (Gawronska and Kielkiewicz 1999; Haile et al. 1999; Heng-Moss et al. 2004, 2006; Boyko et al. 2006; Franzen et al. 2007; Gutsche et al. 2009; Pierson et al. 2011).

Biotic stressors (e.g., arthropods and pathogens) are known to trigger the production of reactive oxygen

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species (ROS), such as hydrogen peroxide ($H_2O_2$; Apel and Hirt 2004, Almagro et al. 2009). $H_2O_2$ acts as signaling molecule to activate plant defense; however, excessive accumulation of ROS can result in toxicity and cellular damage (Klessig et al. 2000, Delledonne et al. 2001). Therefore, the presence of ROS-scavenging enzymes, such as peroxidases and other oxidative enzymes, functions to degrade the $H_2O_2$ synthesized in response to stress, maintaining the ROS content to levels not harmful to plants (Apel and Hirt 2004). Plant peroxidases are frequently found in a broad range of isoforms (Siegel 1993, Passardi et al. 2004), and are known to participate in distinct physiological process, such as lignification, suberization, wound healing, and other forms of defense to arthropods and pathogens (Brisson et al. 1994, Kawano 2003, Almagro et al. 2009).

A limited number of studies have examined the role of oxidative enzymes in the defense response of soybean to arthropod feeding (Hildebrand et al. 1986, Pierson et al. 2011). Pierson et al. (2011) reported reproductive stage KS4202 (tolerant) soybeans to have higher peroxidase activity in response to aphid feeding, whereas the susceptible cultivar had similar levels of peroxidase activity between aphid-infested and control soybean plants. Additional studies are needed to further assess the role of peroxidases in the defense response of soybeans to the soybean aphid. Therefore, the purpose of this study was to investigate the effect of soybean aphid feeding on peroxidase activity in early vegetative stage tolerant and susceptible soybean plants.

## Materials and Methods

**Soybeans and Soybean Aphids.** Seeds of a susceptible genotype SD76R (Chiozza et al. 2010) and tolerant genotype KS4202 (Pierson et al. 2010, Prochaska et al. 2013) were selected for this study. The genotypes selected were not isolines. Four seeds of each genotype were planted in potting media (34% peat, 31% perlite, 31% vermiculite, and 4% soil mix) in 15-cm-diameter round plastic pots at a depth of ≈3 cm. Planting dates were staggered to ensure that plants would reach the designated plant stage at the same time. Upon germination, plants were thinned to one plant per pot and pots were placed in a plastic tray (35 by 50 cm) filled with water. The plants were maintained in a greenhouse under 400-W high-intensity lamps with a photoperiod of 16:8 (L: D) h at a temperature of 23 ± 3°C.

The experimental design was a completely randomized design, with a 2 × 2 × 2 × 3 factorial treatment arrangement including 2 soybean genotypes, 2 aphid infestation levels (control and 20 soybean aphids per plant), 2 vegetative stages (V1, fully developed leaves at unifoliate node, first trifoliate leaf unrolled; and V3, fully developed leaf at second trifoliate node, third trifoliate leaf unrolled; Fehr and Caviness 1977), and 3 harvest dates (6, 16, and 22 d after aphid introduction).

Each treatment combination was replicated five times. Adult apterous soybean aphid females used in this study were progeny of a Nebraska isolate (Biotype 1) collected from commercial soybeans near the University of Nebraska Northeast Research and Extension Center Haskell Agricultural Laboratory in Concord, NE (42° 23′3″ N, 96° 59′21″ W). The soybean aphid colony was maintained on KS4202 plants in a growth chamber at 21 ± 2°C and a photoperiod of 16:8 (L: D) h. Once soybeans were at the desired vegetative stage, 20 soybean aphids (fourth instars and adults) were placed on the youngest fully expanded trifoliate leaf using a small paintbrush. After aphid introduction, both infested and noninfested plants were individually caged using a tubular 0.05-cm clear polycarbonate plastic cage (Eplastics, San Diego, CA; 15 cm in diameter by 61 cm in height) covered with organdy fabric at the top. Plants were evaluated within 48 h of aphid infestation to assess aphid survival and reinfested if <20 aphids. At each harvest day, damage ratings were performed using a 1–5 scale, where 1 = ≤10% of leaf area with yellowish discoloration; 2 = 11–30% of leaf area with yellowish discoloration; 3 = 31–50% of leaf area with yellowish discoloration; 4 = 51–75% of leaf area with yellowish discoloration; and 5 = ≥75% of leaf area with yellowing discoloration or dead tissue (Heng-Moss et al. 2002, Pierson et al. 2010). At the time of harvest, the total number of soybean aphids on infested plants was determined, cumulative aphid–days (CAD) were calculated, and plant stage was recorded. Aphids were carefully removed with a paintbrush after aphid counts were performed. The youngest fully developed trifoliate was harvested, flash frozen with liquid nitrogen, and stored at −80°C for later processing.

**Preparation of Soybean Samples.** Soybean tissue was prepared for protein analysis through modified protocols from Hildebrand et al. (1986) and Pierson et al. (2011). Using a mortar and pestle, soluble proteins were extracted by grinding soybean tissue with 3.0 ml of 20 mM HEPES buffer (pH 7.2), 1% polyvinylpolypyrrolidone, and a plant protease inhibitor cocktail (0.3 ml per 1 g of tissue). The extracted homogenate was centrifuged at 10,000 rpm for 10 min at a temperature of 4°C. The supernatants were collected and stored at 4°C (<2 h) for protein and peroxidase analysis.

**Protein and Peroxidase Assays.** Total protein content was measured using a commercially available bicinchoninic acid protein assay (Pierce, Rockford, IL). Bovine serum albumin was used as the standard for protein concentration. Procedures were carried out according to Pierce's protein assay instructions. Five replications of each treatment combination were analyzed in triplicate.

Peroxidase activity was analyzed using a modified protocol from Hildebrand et al. (1986) and Pierson et al. (2011). The activity was determined by monitoring the increase in absorbance at 470 nm for 2 min. In each microplate well, 5 μl of plant extract, 75 μl of 18 mM guaiacol, 25 μl of 200 mM HEPES buffer pH 6.0, and 71.3 μl of distilled water were combined. The enzymatic reaction was initiated by adding 2.5 μl of 30% $H_2O_2$. Peroxidase specific activity was calculated using

**Table 1. Peroxidase activity for V1 and V3 KS4202 (tolerant) and SD76R (susceptible) soybeans at 6, 16, and 22 d after soybean aphid introduction**

| Genotype | Peroxidase activity ($\mu$mol/min × mg) | | | |
|---|---|---|---|---|
| | Stage of infestation | Control | Infested | *P* value[a] |
| Day 6 | | | | |
| SD76R | V1 | 1.43 ± 0.44 | 1.25 ± 0.35 | 0.79 |
| KS4202 | V1 | 1.37 ± 0.18 | 1.16 ± 0.27 | 0.75 |
| SD76R | V3 | 1.64 ± 0.39 | 2.53 ± 0.47 | 0.19 |
| KS4202 | V3 | 2.67 ± 0.62 | 4.44 ± 0.74 | 0.01 |
| Day 16 | | | | |
| SD76R | V1 | 1.84 ± 0.33 | 1.69 ± 0.36 | 0.86 |
| KS4202 | V1 | 2.34 ± 0.60 | 2.60 ± 0.64 | 0.76 |
| SD76R | V3 | 2.27 ± 0.24 | 3.19 ± 0.71 | 0.16 |
| KS4202 | V3 | 3.70 ± 0.97 | 4.35 ± 0.75 | 0.47 |
| Day 22 | | | | |
| SD76R | V1 | 2.65 ± 0.32 | 4.04 ± 1.18 | 0.13 |
| KS4202 | V1 | 2.68 ± 0.19 | 2.43 ± 0.30 | 0.78 |
| SD76R | V3 | 3.83 ± 0.83 | 4.39 ± 0.60 | 0.54 |
| KS4202 | V3 | 5.10 ± 0.39 | 7.33 ± 0.69 | 0.02 |

[a] Means significantly different at $P \leq 0.05$ by least significant difference.

the molar absorptivity of guaiacol at 470 nm ($26.6 \times 10^3$ $M^{-1}$ $cm^{-1}$). Five replications of each treatment combination were analyzed in triplicate.

**Peroxidase Profiles.** Native gel electrophoresis was performed to profile peroxidase patterns from extracted soybean proteins. Equal amounts of protein (60 $\mu$g) were diluted 1:1 with a gel-loading buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 40% glycerol, and 0.01% bromphenol blue before loading. The samples were loaded in precast 12-well 10–20% polyacrylamide gels (Bio-Rad Criterion gel, Bio-Rad, Richmond, CA) and electrophoresed at 120 V for 90–100 min at 4°C. Gels were stained for peroxidase activity using a modified protocol from Vallejos (1983) and Pierson et al. (2011). Gels were soaked for 10 min in a 50 mM sodium acetate solution at room temperature. Band development was initiated by adding 0.01 g of 4-chloronaphthol dissolved in 0.5 ml of methanol with 20 $\mu$l of 30% $H_2O_2$ to the buffer solution.

**Statistical Analysis.** Peroxidase activity, protein content, and CAD were analyzed using generalized mixed model (PROC GLIMMIX, SAS Institute 2008, Cary, NC) to detect differences between infested and

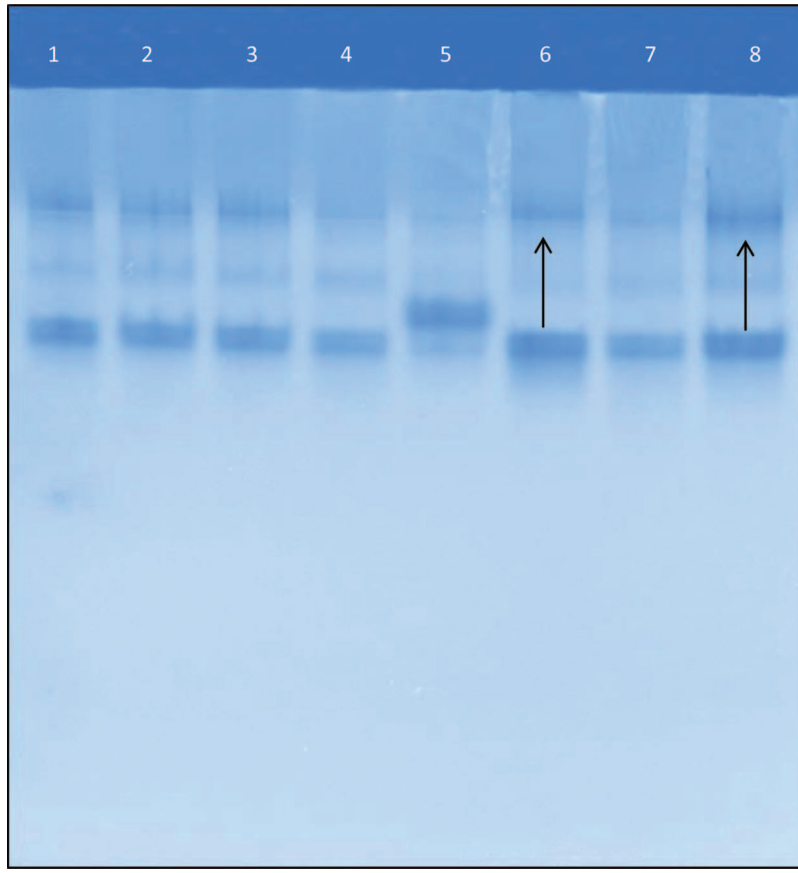


**Fig. 1.** Native gel (10–20%) of soybean leaf protein extract stained for peroxidase activity 6 d after soybean aphid introduction. Lane 1, V1 stage SD76R control; Lane 2, V1 stage SD76R infested; Lane 3, V1 stage KS4202 control; Lane 4, V1 stage KS4202 infested; Lane 5, V3 stage SD76R control; Lane 6, V3 stage SD76R infested; Lane 7, V3 stage KS4202 control; Lane 8, V3 stage KS4202 infested.

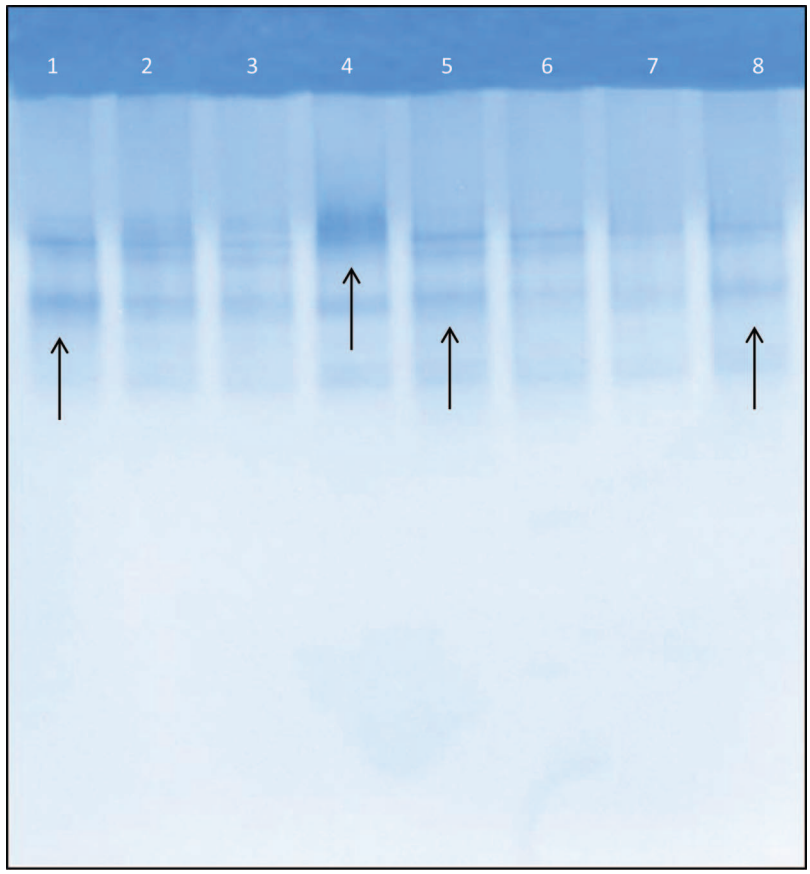


**Fig. 2.** Native gel (10–20%) of soybean leaf protein extract stained for peroxidase activity 16 d after soybean aphid introduction. Lane 1, V1 stage SD76R control; Lane 2, V1 stage SD76R infested; Lane 3, V1 stage KS4202 control; Lane 4, V1 stage KS4202 infested; Lane 5, V3 stage SD76R control; Lane 6, V3 stage SD76R infested; Lane 7, V3 stage KS4202 control; Lane 8, V3 stage KS4202 infested.

control KS4202 (tolerant) and SD76R (susceptible) soybeans at the V1 and V3 stages. When appropriate, means were separated using Fisher's least significant differences procedures ($\alpha = 0.05$).

## Results

**Aphid Numbers.** At day 6, CAD values were similar between KS4202 and SD76R at both V1 and V3, with 441.5 ± 39.7 and 425.5 ± 67.1 for V1 KS4202 and SDR76R plants, respectively. In contrast, aphid densities were lower for V3 with KS4202 and SD76R plants having 321 ± 31 and 234.5 ± 36.5 CAD, respectively.

By 16 d after aphid introduction, V1 KS4202 and SD76R had accumulated 5,080 ± 1,018 and 3,235.8 ± 662.8 CAD, respectively ($t = 1.40$; df = 20; $P = 0.17$). In a similar pattern, KS4202 and SD76R plants infested at V3 accumulated 3,633 ± 790 and 3,796.6 ± 1,174.9 CAD ($t = -0.12$; df = 20; $P = 0.90$).

Both SD76R and KS4202 V1 plants exceeded 10,000 CAD by day 22, a level at which 6.88% yield loss is expected for soybean aphid susceptible reproductive stage soybean (Ragsdale et al. 2007). KS4202 had 21,342 ± 2,122 CAD, which was significantly higher than SD76R plants (15,440 ± 2,360 CAD; $t = 2.2$; df = 20; $P = 0.04$). For treatments that had aphids introduced at V3, no differences in CAD were detected between the two genotypes at 22 d after aphid introduction ($t = 0.29$; df = 20; $P = 0.77$). KS4202 and SD76R plants had CAD values of 9,998 ± 968 and 9,196 ± 1,950, respectively.

No visible aphid injury was observed on the two genotypes at days 6 and 16. The two genotypes showed varying degrees of aphid injury by day 22. Mean damage ratings ± SE were as follows: KS4202 V1: 2.0 ± 0.29, SD76R V1: 2.6 ± 0.17; KS4202 V3: 1.33 ± 0.21, SD76R V3: 1.75 ± 0.25. These results are consistent with previous findings by Marchi (2012).

**Protein and Peroxidase Assays.** Protein content was not significantly different between infested and control V1 and V3 plants for both KS4202 and SD76R (data not shown). At 6 d after soybean aphid introduction, similar levels of peroxidase activity were detected between control and infested plants for both KS4202 and SD76R V1 stage plants (Table 1). For V3 plants, infested KS4202 had significantly higher peroxidase activity than KS4202 control plants ($t = -2.7$; df = 24; $P = 0.01$; Table 1). Although not significantly

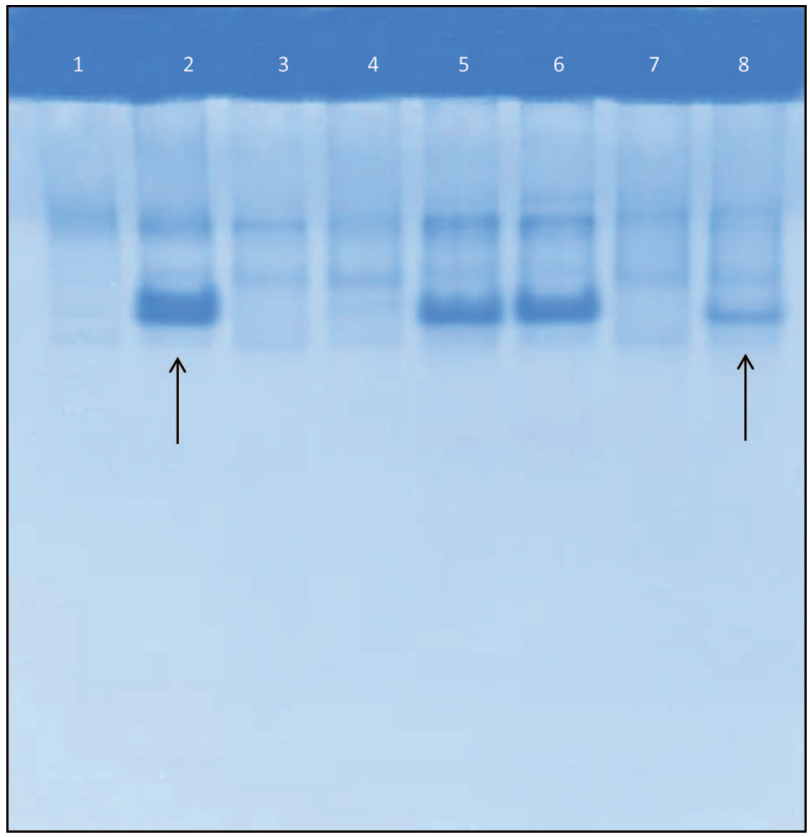


**Fig. 3.** Native gel (10–20%) of soybean leaf protein extract stained for peroxidase activity 22 d after soybean aphid introduction. Lane 1, V1 stage SD76R control; Lane 2, V1 stage SD76R infested; Lane 3, V1 stage KS4202 control; Lane 4, V1 stage KS4202 infested; Lane 5, V3 stage SD76R control; Lane 6, V3 stage SD76R infested; Lane 7, V3 stage KS4202 control; Lane 8, V3 stage KS4202 infested.

different ($t = -1.34$; df = 24; $P = 0.19$), SD76R infested plants had slightly higher peroxidase activity than control plants at 6 d after aphid introduction.

At 16 d after aphid introduction, KS4202 and SD76R V1 and V3 plants had similar levels of peroxidase activity when compared with their respective control plants (Table 1). By day 22, KS4202-infested V3 plants had significantly higher peroxidase activity levels than control plants ($t = -2.4$; df = 32; $P = 0.03$), whereas V1-infested and control KS4202 had similar activity levels (Table 1). Although not statistically different, V1 SD76R infested plants had slightly higher levels of peroxidase activity when compared with their control plants ($t = -1.54$; df = 32; $P = 0.13$). Peroxidase activity levels were similar between SD76R infested and control V3 plants.

**Peroxidase Profiles.** At day 6, no specific banding patterns were observed between control and infested plant for both genotypes at V1 stage (Fig. 1). However, differences in band intensity (indicated by arrows) between control and infested plants were evident in the isozyme profiles for KS4202 and SD76R plants at V3, which is consistent with the higher peroxidase activity detected in the kinetics study. SD76R control plants had slightly darker banding patterns (indicated by arrows) than their respective infested plants for both V1 and V3 at 16 d after aphid introduction (Fig. 2). KS4202 infested plants, however, had darker banding patterns than KS4202 control plants at day 16 (indicated by arrows). By day 22, visual differences in the banding intensity (indicated by arrows) were observed between control and infested SD76R V1 and KS4202 V3 plants (Fig. 3).

## Discussion

Peroxidases are a large class of oxidative enzymes involved in a broad range of physiological responses in plants. These molecules play a key role in the oxidation of ROS, such as $H_2O_2$. Peroxidases can also serve as generators of $H_2O_2$ through the oxidation of NADH (Apel and Hirt 2004, Koutaniemi et al. 2005), which can serve as signaling molecules to trigger defense pathways (Apel and Hirt 2004, Sukalovic et al. 2005). Peroxidases can also be involved in a broad range of physiological process, such as cell wall lignification, suberization, wound healing, and defense against pathogen infection (Brisson et al. 1994, Kawano 2003, Almagro et al. 2009). Previous research has found that higher levels of peroxidase activity are likely associ-

ated with resistance to a variety of arthropods in wheat (Ni et al. 2001, Boyko et al. 2006, Franzen et al. 2007, Han et al. 2009), barley (Gutsche et al. 2009), and buffalo grass (Heng-Moss et al. 2004). A recent study performed by Pierson et al. (2011) was the first report on the effects of soybean aphid feeding on peroxidase activity in soybeans. Their results indicated that peroxidase activity increased in the tolerant soybean KS4202, suggesting these enzymes might be involved in the tolerance response. In a subsequent study, Prochaska (2011) analyzed the transcriptional changes in infested and uninfested KS4202 and a susceptible genotype to gain insight into the genes involved in the tolerance response and mechanisms of the resistance. After 15 d of aphid feeding, five peroxidase genes were identified as being differentially expressed between KS4202-infested and uninfested plants. The same peroxidases genes were not differentially expressed in the susceptible soybean in response to aphid feeding. It was speculated that these peroxidases might be serving to detoxify the ROS accumulated and be involved in triggering signaling molecules for specific plant defense pathways.

In this study, KS4202 V1 and SD76R V1 and V3 control and aphid-infested plants had similar levels of peroxidase activity throughout the sampling periods. KS4202-infested plants at V3 had significantly higher peroxidase activity levels than control plants at 6 and 22 d after aphid introduction. The differences in peroxidase activity observed between infested and control V3 KS4202 plants throughout the course of the experiment suggest that peroxidases are playing multiple roles in the tolerant plant. The increase in peroxidase activity at day 6 may be resulting in the production of ROS, specifically $H_2O_2$, which can serve as a signaling molecule for triggering several plant defense pathways. However, the increases in peroxidase activity at day 22 are likely involved in the detoxification of ROS that accumulate as a result of aphid feeding. Although not significant, slight increases in peroxidase activity were observed between control and infested SD76R V1 and V3 plants. However, the level of activity was insufficient to prevent accumulation of ROS and as a result, these plants experienced visible plant damage.

The results presented here compare favorably to a study by Pierson et al. (2011), which also found higher levels of peroxidase activity in KS4202 exposed to soybean aphids during the reproductive stages. In addition, our results provide more evidence that oxidative enzymes, such as peroxidases, are likely involved in the tolerance response of KS4202 to soybean aphid.

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