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ORIGINAL PAPER

The involvement of peroxidases in soybean seedlings' defense against infestation of cowpea aphid

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Abstract Changes in the level of hydrogen peroxide (H_2O_2) and activity of peroxidases towards phenolic substrates (EC 1.11.1.7) such as pyrogallol (PPX), syringaldazine (SPX) and guaiacol (GPX), and cytosolic ascorbate peroxidase (cAPX, EC 1.11.1.11) in response to infestation of cowpea aphid (*Aphis craccivora* Koch) were analyzed in soybean (*Glycine max* (L.) Merr. cv. "Nam Dan") at the V3 stage (first two trifoliate leaves fully developed, third trifoliate leaf unrolled) for 96 h post-infestation (hpi). Influence of *A. craccivora* at a varied population size (10, 20 and 30 individuals per each soybean plant) caused a burst of H_2O_2 generation in the aphid-infested leaves at 12 hpi. Paralleling the H_2O_2 accumulation, peroxidase activity in all the infested plants remarkably increased and was significantly higher than that observed in controls (uninfested plants). The cascade of enzymes induced was continuously overlapped by the early enhancement of SPX within 6–24 hpi, an expression of cAPX (12–48 hpi) followed by an accumulation of GPX (24–72 hpi) and PPX (24–96 hpi). The differential induction of SPX, GPX, PPX and cAPX resulted in a rapid reduction of H_2O_2 content in aphid-infested leaves, and the activity of peroxidase was closely correlated with the intensity of *A. craccivora* infestation around the defined points of time at which the activity of each enzyme reached the maximum level. The increase in activity of peroxidases matched their function as controlling accumulation of H_2O_2 and detoxifying this reactive oxygen product when soybean plants were challenged with cowpea aphid. Furthermore, peroxidases could directly deter cowpea aphid feeding through other functions such as the anti-nutritive and/or toxicological defenses and/or limiting the penetration of aphid stylets into plant tissues via participating to strengthen and reinforce the cell wall barrier. These results indicated that peroxidases may be some elements of the defense system that increased the resistance of *G. max* cv. "Nam Dan" to infestation of *A. craccivora*.

Keywords *Aphis craccivora* · Cowpea aphid · *Glycine max* · Hydrogen peroxide · Peroxidases · Soybean

Abbreviations

cAPX	Cytosolic ascorbate peroxidase
FW	Fresh weight
GPX	Guaiacol peroxidase
H_2O_2	Hydrogen peroxide
hpi	Hours post infestation
nkat	Nanokatal
PPX	Pyrogallol peroxidase
SPX	Syringaldazine peroxidase

Introduction

The III class plant peroxidases (EC 1.11.1.7) are members of a large multigenic heme-containing enzyme family that control reactive oxygen species (ROS) generation when plants have been challenged with various stressors (Schenk et al. 2000). Catalyzing the reduction of peroxides, e.g., hydrogen peroxide (H_2O_2), to water in the presence of electron acceptors (Koksal 2011), peroxidases play as a

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major system for the enzymatic removal of peroxidative damage in plant cells (Velikova et al. 2000). In addition to detoxifying H_2O_2, peroxidases also oxidize a variety of phenolic compounds, and they were initially characterized following their substrates as guaiacol peroxidase (GPX), pyrogallol peroxidase (PPX) and syringaldazine peroxidase (SPX). These enzymes have been known to be multifunctional, participating in a broad range of physiological processes, e.g., somatic embryogenesis, auxin catabolism, lignification and degradation of the cell wall, as well as defense against biotic and abiotic factors (Hiraga et al. 2001; Allison and Schultz 2004; Passardi et al. 2005).

Accumulation of peroxidase activity was evaluated as part of plant defense responses, and the increased peroxidase levels may enhance the plant's ability to tolerate insect feeding (Ni et al. 2001; Heng-Moss et al. 2006; Gulsen et al. 2010). Infestation of Russian wheat aphid (*Diuraphis noxia*) enhanced the activity of peroxidases in wheat leaves from *D. noxia*-resistant plants, whereas wheat leaves from the *D. noxia*-susceptible variant did not exhibit a similar increase (Ni et al. 2001). The resistant buffalograss (*Bouteloua dactyloides*) had higher levels of peroxidase activity compared with the control, whereas enzyme activities for control and susceptible plants remained at similar levels or were slightly lower for chinch bug-infested plants (Heng-Moss et al. 2004). These findings suggested that the increased activity of peroxidases may serve to enhance the plant's resistance to aphids.

The accumulation of H_2O_2 in plant cells is controlled not only by GPX, PPX and SPX, but also by cytosolic ascorbate peroxidase (cAPX, EC 1.11.1.11), a member of the class I heme-peroxidases in the cytosol (Takeda et al. 2000; Almagro et al. 2009). cAPX is widely known to play a role as a protective element against adverse environmental conditions such as drought and salt stress, high light, high and low temperatures, and pathogens (Agrawal et al. 2003; Fryer et al. 2003; Menezes-Benavente et al. 2004; Bonifacio et al. 2011; Caverzan et al. 2012). However, the knowledge regarding the expression of cAPX in plants challenged by insect pests is limited. Additional studies to further assess the role of cAPX in plant defense responses to aphids are currently essential.

Soybean (*Glycine max* (L.) Merr.), called the miracle crop, has been extensively grown in the world for its edible seed as the foremost material of vegetable protein and oil (Lee et al. 2007). The interactions between soybean and aphids have been mentioned in various reports, of which a few studies have revealed the change in activity of peroxidases and expression of genes encoding these enzymes related to the aphid tolerance (Hildebrand et al. 1986; Felton et al. 1994; Prochaska 2011, Pierson et al. 2011; Prochaska et al. 2013; Marchi-Werle et al. 2014). The tolerant plants had higher peroxidase activity in response to aphid infestation, whereas the susceptible cultivars had similar levels of peroxidases between aphid-infested variants and control. The aphid-tolerant plants, based on the high expression of peroxidases, were able to effectively detoxify the excessive accumulation of ROS, while the susceptible soybeans were not able to reduce the effect of ROS (Pierson et al. 2011). Analyzing the transcriptional changes in aphid-tolerant and aphid-susceptible soybean plants, Prochaska (2011) discovered the expression of two genes encoding peroxidases involved in the tolerance responses after aphid feedingfor 15 days. The same genes were not differentially expressed in the susceptible soybean in response to aphids. Despite the progress achieved in identifying soybean aphid-resistant/-tolerant sources, limited information has been provided toward understanding the mechanisms underlying soybean resistance or tolerance to aphids (Pierson et al. 2011; Prochaska et al. 2013; Marchi-Werle et al. 2014).

Cowpea aphid (*Aphis craccivora* Koch) is one of the most destructive aphid species of soybean [*Glycine max* (L.) Merr. cv "Nam Dan"]—a valuable crop in the agricultural production in Nghe An province (Vietnam). When feeding, *A. craccivora* directly injects saliva containing toxins into the plant tissues, produces honeydew on the surfaces of leaves that reduces photosynthesis and transmits a number of viral and bacterial phytopathogens (Sorensen 2003); therefore, it causes serious damages to plant growth and significantly reduces the yield of soybean. To date, information in the available literature regarding the interaction between *G. max* cv. "Nam Dan" and its dangerous pest, *A. craccivora*, is still missing. The aim of present study was to investigate the changes in activity of peroxidases such as GPX, PPX, SPX and cAPX in the response of soybean "Nam Dan" to infestation from different numbers of cowpea aphid individuals at the different time points evaluated. It should be stressed that the aforementioned aspects of *G. max* cv. "Nam Dan"-*A. craccivora* interaction are novel problems whose investigation would contribute to the knowledge about the anti-herbivore defense mechanism of this soybean cultivar.

Materials and methods

Materials

Experiments in this study were carried on soybean (*Glycine max* (L.) Merr. cv. "Nam Dan") plants at the V3 stage (first two trifoliate leaves fully developed, third trifoliate leaf unrolled). Soybean seeds were exclusively provided by Nghe An seed center (Vietnam). Seeds were surface-sterilized for 10 min in 0.01 % $HgCl_2$ solution and held for imbibition in the incubator at 22–23 °C for 48 h.

The germinated seeds were cultured in 20-cm-diameter plastic pots containing Hoagland medium placed in a phytotron with temperature of 23–25 °C, related humidity of 70–75 %, light intensity of 110–130 μM photons m^{-2} s^{-1} and light period of 14 light/10 dark h.

Materials for analyses were leaves of soybean seedlings. The following variants were applied as (1) control seedlings without aphid infestation and (2) soybean seedlings infested by *A. craccivora* populations of various size, e.g., 10, 20 and 30 individuals per plant. Leaves in the control and infested plants were carefully collected after 6, 12, 24, 48, 72 and 96 h post-infestation (hpi) of cowpea aphid. After all aphid individuals had been carefully removed, leaves were weighed, frozen in nitrogen liquid and kept at −70 °C for subsequent analyses of peroxidases. Content of hydrogen peroxide (H_2O_2) was determined in fresh materials at particular time points for all variants.

Aphid infestation

The aphid species used in this study was cowpea aphid (*Aphis craccivora* Koch), originally cultured and supplied by the Department of Applied Entomology (Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology). Virus-free individuals of *A. craccivora* were reared on the host, *G. max*, in the phytotron with controlled environmental factors.

Each soybean seedling at the V3 stage was treated by 10, 20 or 30 wingless adults of *A. craccivora*. Generally, cowpea aphid infests heavily on developmentally young plants with high turgor rather than developmentally mature plants. We used soybean seedlings at the V3 stage, because cowpea aphid daily fertility on soybean seedlings was high in this stage of development.

Aphid individuals were carefully transferred to soybean leaves with a fine paintbrush. Nymphs and winged aphid adults were monitored through all experiments; therefore, the number of wingless adults of *A. craccivora* was constant. The control plants in experiments were soybean seedlings without aphid infestation. All control and aphid-infested variants were separately put in glass boxes (50 cm × 50 cm × 50 cm) covered by nylon gauze and placed in a phytotron, in which the environmental factors such as temperature, relative humidity, light intensity and light period were controlled.

Analysis

Chemicals

All analytical chemicals were purchased from Sigma-Aldrich (USA).

Determination of hydrogen peroxide content

Content of hydrogen peroxide (H_2O_2) was determined following the spectrophotometric method described by Becana et al. (1986). Leaves of soybean (500 mg fresh weight, FW) were homogenized with 3 ml 5 % trichloroacetic acid (TCA) and 0.10 g activated charcoal. The homogenate was filtered through four layers of cheesecloth and centrifuged at 12,000×*g* for 30 min at 4 °C. The reaction mixture contained extracted solution, 100 mM potassium phosphate buffer (pH 8.4) and reagent containing 0.6 mM 4-(-2 pyridylazo)resorcinol and 0.6 mM potassium-titanium oxalate in a 1:1 proportion. The decrease of absorbance was measured at a wavelength of 508 nm (A_{508}) in the UV-VIS Double PC 8 Auto Cell Scanning Spectrophotometer (UV-VIS UVD-3200, LABOMED-USA), connected to a computer; the spectral data on the PC monitor were analyzed by using the UV-Win 6.0 UV-VIS application software. Blanks were obtained by using 5 % TCA to replace extracted solution in the mixture. Content of H_2O_2 was determined from the difference of A_{508} between each sample and blank. The amount of hydrogen peroxide in soybean leaves was expressed as μm g^{-1} FW.

Extraction and assay of peroxidase activity towards phenolic substrates

Frozen leaves (500 mg) were homogenized at 4 °C with a mortar and pestle in 3 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 10 % (w/v) polyvinylopyrrolidone (PVP). The mixes were centrifuged at 15,000×*g* for 30 min at 4 °C, and the supernatants were selected for enzyme assays.

Peroxidases (EC 1.11.1.7) activity was spectrophotometrically measured using phenolic substrates such as pyrogallol, syringaldazine and guaiacol and was expressed as nanokatal per 1 mg of protein (nkat mg^{-1} protein).

Activity of peroxidase towards pyrogallol (PPX) was estimated by measurement of the purpurogallin-a product of pyrogallol oxidation (Nakano and Asada 1981). The assay mixture contained 50 mM sodium phosphate buffer (pH 7.0), 40 μl enzyme extract, 180 mM pyrogallol and 2 mM H_2O_2. The absorbance was measured at a wavelength of 430 nm in the UV-VIS UVD-3200 spectrophotometer.

Activity of peroxidase towards syringaldazine (SPX) was assayed by measurement of the content of the colored product of syringaldazine oxidation according to method of Imberty et al. (1985) with a minor modification. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 60 μl enzyme extract, 41.6 mM syringaldazine and 4 mM H_2O_2. Absorbance was measured at a wavelength of 530 nm in the UV-VIS UVD-3200 spectrophotometer.

Activity of peroxidase towards guaiacol (GPX) was assayed according to the modified method of Maehly and Chance (1954) via determination of the tetraguaiacol-a colored product of guaiacol oxidation. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 100 µl enzyme extract, 20 mM guaiacol and 6 mM H_2O_2. Absorbance was measured at a wavelength of 470 nm in the UV-VIS UVD-3200 spectrophotometer.

Extraction and assay of cytosolic ascorbate peroxidase (cAPX) activity

For extraction of cAPX (EC 1.11.1.11), 500 mg of frozen leaves was ground in a mortar at 4 °C by using 3 ml of 50 mM potassium phosphate buffer at pH 7.0, 1 mM EDTA, 1 mM sodium ascorbate and 1 % PVP. Mixes were centrifuged at 15,000×*g* for 30 min. Supernatants were selected for enzyme assay.

Activity of cAPX was determined by the spectrophotometric method of Nakano and Asada (1981). The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 60 µl enzyme extract, 10 mM ascorbic acid and 2 mM H_2O_2. Absorbance was recorded at a wavelength of 265 nm and analyzed in the UV-VIS UVD-3200 Spectro system. Activity of cAPX was expressed as nkat mg^{-1} protein.

Determination of protein

Protein content in soybean leaves was determined following the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

All analyses were performed in three replicates in three independent experiments. Analysis of variance (ANOVA) was applied to verify whether means from independent experiments within given experimental variants were significant with the level of significance $\alpha = 0.05$. Data shown in the figures are means of triplicates for each variant and standard errors (SE).

Results

Accumulation of hydrogen peroxide in soybean leaves after aphid infestation

Cowpea aphid infestation caused a big fluctuation in the content of endogenous hydrogen peroxide (H_2O_2) in leaves of soybean "Nam Dan" during experimental time (Fig. 1). Resulting from an early remarkable increase of H_2O_2 generation, a burst of this ROS product was obtained at 12 hpi in all aphid-infested plants. The highest content of H_2O_2 was recorded in 20 aphid-infested soybean leaves, having values 3.88- and 4.23-fold higher than in the beginning and in the control (the uninfested plants) at the same time, respectively. ANOVA results at the level of $P < 0.05$ concerning the burst of H_2O_2 in soybean leaves through P values such as $P_{10\ \text{aphids-control}}$, $P_{20\ \text{aphids-control}}$ and $P_{30\ \text{aphids-control}}$, obtained respectively as 0.000669, 0.006377 and 0.002748, showed highly significant differences between the infested and uninfested plants. However, statistical significance was not seen between H_2O_2 contents

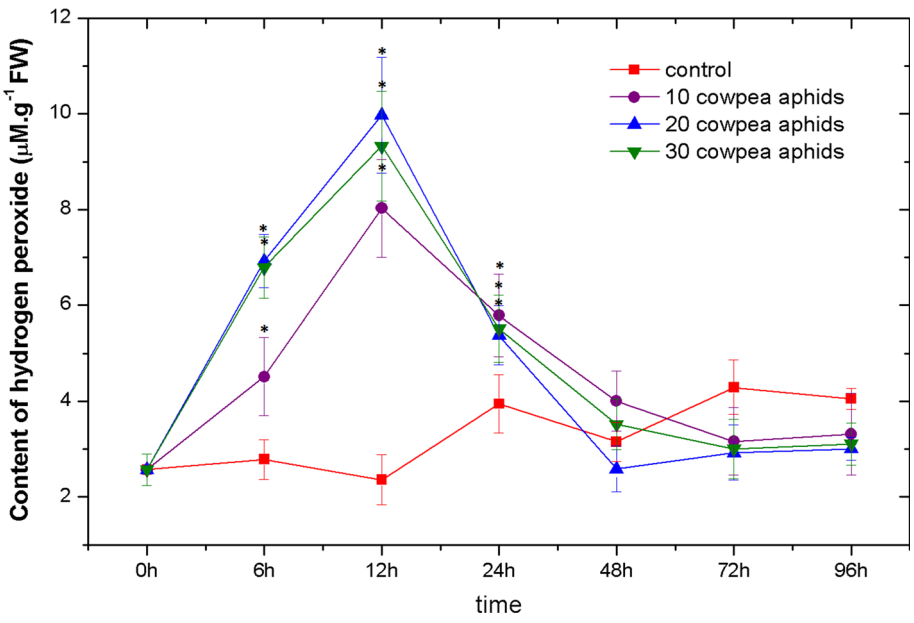

Fig. 1 Accumulation of hydrogen peroxide (H_2O_2) in leaves of *G. max* "Nam Dan" control and *Aphis craccivora*-infested seedlings. Values represent means and SE from three independent experiments. *Significant difference between aphid-infested variant and control ($P < 0.05$)

in the infested variants. After reaching a peak, this signaling molecule strongly decreased to low levels; particularly within 72–96 hpi, the content of H_2O_2 in aphid-infested leaves was less than in controls. Without aphid infestation, the content of H_2O_2 in control plants remained at a low level and changed little throughout the experiment.

Accumulation of peroxidase activity

Activity of peroxidase-oxidized phenolic substrates such as pyrogallol (PPX), syringaldazine (SPX) and guaiacol (GPX) in leaves of *G. max* "Nam Dan" were differently enhanced following the evaluated time points and intensity of aphid infestation when this soybean cultivar was challenged with *A. craccivora* (Fig. 2).

As the first enzyme accumulated in the aphid-infested plants, activity of SPX increased slightly from the beginning, reaching a peak at 12 hpi. At the specific points of time evaluated, such as 0, 12 and 24 hpi, SPX activity in 30 aphid-infested plants was significantly higher than in others; e.g., the maximum activity of SPX in this infested variant at 12 hpi obtained 14.597 nkat mg^{-1} protein, which was 1.59- and 1.75-fold higher than that in 0 h and in controls at the same time point, respectively. SPX activity then reduced to a comparatively stable level, and the infested and uninfested soybean plants had similar levels of peroxidase activity within 48–96 hpi (Fig. 2a).

Following the accumulation of SPX was an enhancement in the activity of both PPX (Fig. 2b) and GPX (Fig. 2c) in the aphid-infested plants, which strongly increased from 12 hpi, reached the highest levels at 48 hpi and then slightly reduced until the end of the experiment. Activities of these two enzymes in aphid-infested variants were significantly higher than those observed in controls within 24–96 hpi. For example, activities of PPX in ten aphid-infested leaves (the least aphid-induced variant) were 6.708, 12.871, 14.029 and 12.203 nkat mg^{-1} protein, being 1.35-, 2.09-, 2.13- and 2.32-fold higher than in controls at the respective time points of 24, 48, 72 and 96 hpi.

Furthermore, activities of both PPX and GPX in aphid-infested soybean leaves were expressed to closely match with the intensity of *A. craccivora* infestation around 48 hpi. The highest activity of PPX in 30 aphid-infested plants was 16.438 nkat mg^{-1} protein, which was 8.70 % and 27.71 % higher than that in soybean plants infested by 20 aphids (15.122 nkat mg^{-1} protein) and 10 aphids (12.871 nkat mg^{-1} protein), respectively. Similar to PPX, the maximum level of GPX activity in 30 aphid-infested variants having 9.584 nkat mg^{-1} protein was much higher than that in 20 aphid-infested plants (8.025 nkat mg^{-1} protein) and 10 aphid-infested plants (5.703 nkat mg^{-1} protein). Interestingly, in all the aphid-infested variants and control plants, the activity of PPX was remarkably higher than that of GPX at every evaluated time point.

Accumulation of cytosolic ascorbate peroxidase (cAPX) activity in soybean leaves after aphid infestation

cAPX enzyme showed different activity levels in the infested and uninfested soybean plants at different times after cowpea aphid infestation. Whereas activity of cAPX in controls was little changed at a low level during the experimental time, a considerable induction of this enzyme was observed in aphid-infested variants, which rapidly increased to maximum levels at 12 hpi (Fig. 3). The highest activity of cAPX recorded in 20 aphid-infested leaves was 22.73 nkat mg^{-1} protein, being 2.20- and 1.98-fold higher compared with controls and in the beginning, respectively. After reaching the peak, cAPX slightly reduced until the end of the experiment. Additionally, cAPX activity in aphid-infested variants was significantly different from that in controls within 6–48 hpi. For the example of the least induced variant of 10 aphids per seedling, activities of cAPX in aphid-infested leaves at 6, 12, 24 and 48 hpi obtained 11.50, 18.68, 19.32 and 18.27 nkat mg^{-1} protein, which were higher than in controls (10.235 nkat mg^{-1} protein) by 12.36, 82.25, 88.76 and 78.51 %, respectively. However, a lack of correlation between enzyme activity and intensity of infestation from different numbers of cowpea aphids was recorded.

Discussion

The enhancement of hydrogen peroxide (H_2O_2) together with induction of cell death in herbivore-damaged areas is known as the oxidative response in plant defense mechanisms (Radville et al. 2011) and might be a central component mediating cross tolerance in plants (Maffei et al. 2007). H_2O_2 also function as a deterrent that prevents insects from feeding (Apel and Hirt 2004). Generation of H_2O_2 has been known to be induced as a plant defense response in numerous plant-aphid interactions (Argandona et al. 2001; Moran et al. 2002; Zhu-Salzman et al. 2004; Divol et al. 2005; Moloi and van der Westhuizen 2006; Boyko et al. 2006; Kusnierczyk et al. 2008). In a recent study of Mai et al. (2013), an early remarkable enhancement of H_2O_2 was observed to activate the defense mechanisms of pea (*Pisum sativum* cv. Cysterski) against pea aphid (*Acyrthosiphon pisum*) attack. Similar to the above results, an increase in the level of H_2O_2 content in aphid-infested leaves of soybean (*G. max* cv. "Nam Dan") within 6–24 h after cowpea aphid (*A. craccivora*) infestation was evaluated as closely related to the defensive aspects of H_2O_2.

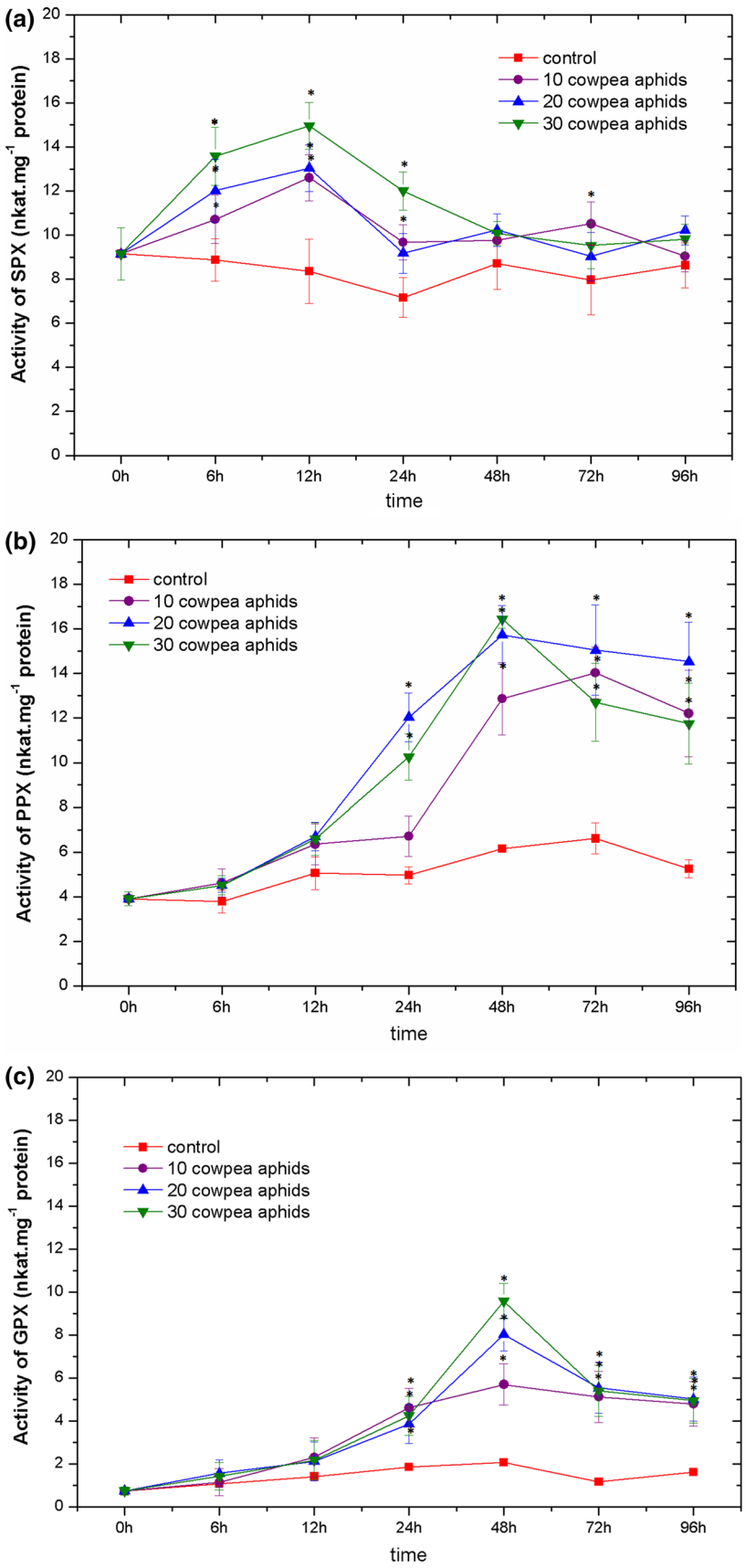

Fig. 2 Accumulation of peroxidase activity towards syringaldazine-SPX (**a**), pyrogallol-PPX (**b**) and guaiacol-GPX (**c**) in leaves of *G. max* "Nam Dan" control and *Aphis craccivora*-infested seedlings. Values represent means and SE from three independent experiments. *Significant difference between aphid-infested variant and control ($P < 0.05$)

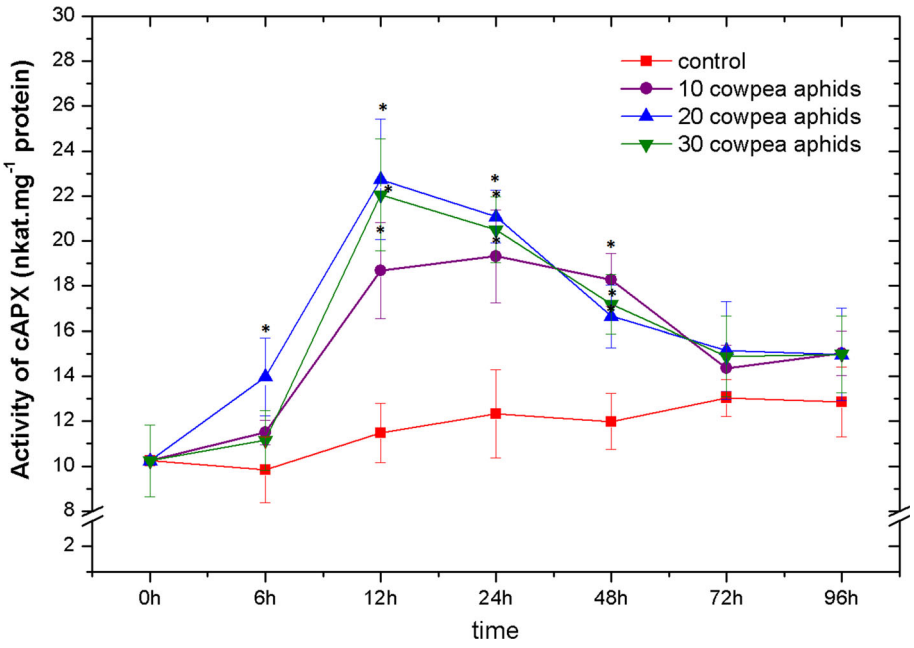

Fig. 3 Accumulation of cytosolic ascorbate peroxidase (cAPX) activity in leaves of *G. max* "Nam Dan" control and *Aphis craccivora*-infested seedlings. Values represent means and SE from three independent experiments. *Significant difference between aphid-infested variant and control ($P < 0.05$)

H_2O_2 is a relatively stable, partially reduced form of ROS, and its ability to diffuse freely allows H_2O_2 to play as a control role in plant defense responses (Bóka et al. 2007). H_2O_2 causes the release of Ca^{2+} flux into the cellular matrix, which is a very important ubiquitous intracellular second messenger molecule involved in many signal transduction pathways in plants (Tuteja and Mahajan 2007). H_2O_2 also activates several members of the mitogen-activated protein kinase (MAPK) family, one of centrals for mediating plant cellular responses to stresses, e.g., AtMPK3 and AtMPK6 in *Arabidopsis* (Desikan et al. 2005). As a signaling molecule in plant defense mechanisms, the accumulation of H_2O_2 could be the beginning of a cascade of events that trigger physiological and molecular responses to reduce aphid attacks (Argandona et al. 2001).

On the contrary, the high content of H_2O_2 engaged in oxidative stress can exert toxic effects, and the uncontrolled generation often causes damage to cellular components such as proteins, lipids and nucleic acids (Ahmad et al. 2008). However, plant cells have a number of ROS-scavenging systems that are able to maintain a relatively low H_2O_2 content by the relevant protective mechanisms using antioxidative enzymes such as peroxidases. In the presence of electron acceptors, peroxidases serve to catalyze the reduction of H_2O_2 toxicity to water (Koksal 2011), which involves the role of peroxidases as a major enzymatic system to control cellular damage (Velikova et al. 2000).

A cascade of remarkable enhancement and overlap of class III peroxidases (SPX, GPX and PPX) in aphid-infested leaves of soybean "Nam Dan" was also observed in parallel with the accumulation of H_2O_2 after *A. craccivora* infestation. The first was SPX induction (6–24 hpi) and then an increase of cAPX activity (12–48 hpi) followed by an enhancement of GPX (24–72 hpi) and PPX (24–96 hpi). Activities of PPX, GPX and SPX in aphid-infested variants were always significantly higher than those observed in controls at each sampling time point. The enhancement of these peroxidases is able to control the balance between accumulating H_2O_2 generation as a defense response and decreasing the H_2O_2 level to reduce oxidative damage in plant cells. Genes coding peroxidases participating in the transduction of the oxidative signal controlling the concentration of H_2O_2 were discovered to exhibit diverse expressions in plants (Divol et al. 2005; Boyko et al. 2006). It is interesting that the stimulation of peroxidase activity and accumulation of H_2O_2 in plant responses to the infestation of aphids were correlated with the generation of ethylene, a phytohormonal signaling molecule functioning in plant defense systems (Argandona et al., 2001).

The enhancement of peroxidases is crucially known to play directly in plants' defenses against insects. Levels of peroxidase activity were enhanced in plants resulting from herbivore attacks, and the increases of peroxidases were recorded to reduce feeding of insects effectively (Ni et al. 2001; Allison and Schultz 2004; Heng-Moss et al. 2004, 2006; Boyko et al. 2006; Franzen et al. 2007; Gutsche et al. 2009; Gulsen et al. 2010). First, peroxidases function as anti-nutritive and/or toxicological defenses against herbivores (Felton et al. 1989; Duffey and Stout 1996). Generally, plant tissues generate phenol compounds in their cells in stress conditions. Peroxidases and other oxidative enzymes serve to oxidize phenols or their derivatives in the damaged tissues to form reactive quinones, which in turn polymerize or form electrophiles that impair the plant

phloem nutrient uptake by insects (Felton et al. 1989; Maffei et al. 2007), leading to a decrease in their appetite or deterring them from feeding. Quinones additionally pose a remarkable oxidative stress to herbivores by their direct toxicity to insects (Felton et al. 1994; Duffey and Stout 1996). Second, modification of the cell wall structure can play an important function in the plant's aphid resistance capability (Thompson and Goggin 2006). Peroxidase is involved in the strengthening of plant cell wall by early initiation of the lignification process. The synthesis of lignin can be beneficial to plants because lignification serves to reinforce the cell wall barrier (Fincher and Stone 1986), which would locally and systematically limit the penetration of aphid stylets into plant cells (Chaman et al. 2001), thereby increasing the plant's resistance to herbivores.

In soybean plants, Hildebrand et al. (1986) and Felton et al. (1994) found an increase of peroxidase activity in defenses against herbivory such as mites, bean leaf beetles and three-corned alfalfa leafhoppers in resistant variants. The resistant plants had higher peroxidase activity in aphid-infested leaves, whereas the susceptible cultivar had a similar level of enzyme activity compared with controls. In the transcriptional changes, expression of two genes encoding peroxidases involved in the resistance response of soybean was identified, whereas, the same genes were not differentially expressed in the susceptible soybean in response to aphids (Prochaska 2011). Once identified, the enhancement of peroxidases may be useful as markers for aphid resistance of soybean.

Interestingly, peroxidases were also found to induce in responses of the aphid-infested plants to pathogens (Stout et al. 1999) and mechanical wounding (Allison and Schultz 2004). Aphids not only ingest phloem sap but also inject their saliva and transmit virus pathogens to plants (Pirone and Blanc 1996). When feeding, aphid stylets penetrate through cells by symplast punctures and cause wounding to tissues (Tjallingii and Hogen 1993). The expression of resistance to aphid infestation may compromise plant defenses against other stressors. These are additional evidence for reciprocal induced resistance involving the combination of the plant-induced response to complex stimuli such as aphids, pathogens and wounding. Although information to understand the simultaneous mechanisms to protect plants from multiple of stressors is still limited, it is suggested that peroxidases may be a multifunctional component of the soybean defense system in which the host plant could select the effective response(s) to pathogens and/or wounding resulting from aphid infestation. Further experiments will be required to understand fully the soybean mechanism in the integrative responses to multiple stimuli.

Belonging to class I within the superfamily of plant peroxidases, cAPX plays a protective role in plant responses to adverse environmental conditions (Agrawal et al. 2003; Fryer et al. 2003; Menezes-Benavente et al. 2004; Bonifacio et al. 2011; Caverzan et al. 2012). The potential defense mechanism in plants against herbivores regarding cAPX is the prominent control of generation of H_2O_2 and other ROS products (Apel and Hirt 2004). As one of the most widely distributed enzymatic antioxidants, cAPX is the main enzyme responsible for H_2O_2 removal in the cytosol of plant cells (Takeda et al. 2000; Almagro et al. 2009). cAPX had a much higher affinity for H_2O_2 than other antioxidants, making it an efficient scavenger of H_2O_2 (Wang et al. 1999). A considerable increase in the activity of cAPX observed in *G. max* "Nam Dan" leaves after *A. craccivora* infestation resulted in a remarkable decrease of the accumulated H_2O_2, and a relatively low content of H_2O_2 remained in the infested leaves within 12–96 hpi (Fig. 1). The close relation between an early enhancement of cAPX activity and reduction of H_2O_2 content in aphid-infested soybean plants supported the evidence for a protective role of this enzymatic antioxidant in the soybean defense mechanisms.

In conclusion, it should be emphasized that peroxidases are the vital element in the defense system involved in soybean "Nam Dan" responses to cowpea aphid infestation. The different induction of SPX, GPX and PPX was likely a cascade of events in soybean plants to directly deter cowpea aphid infestation via functioning as the anti-nutritive and/or toxicological defense and trigger other signaling pathways to protect themselves from cowpea aphid attack. These peroxidases, together with the enhancement of cAPX, additionally serve to detoxify the accumulated H_2O_2 and reduce 'oxidative damage' to cellular components. Prominently, the induction of the activity of peroxidases has been known to enhance plant's ability to tolerate insect feeding (Ni et al. 2001; Heng-Moss et al. 2006; Gulsen et al. 2010). Therefore, from the results obtained in the soybean-aphid interactions, we suggested that *G. max* cv. "Nam Dan" may be an aphid-tolerant variety of soybean plant. However, it is necessary to clarify this aspect in prospective studies to obtain adequate evidence for a convincing conclusion.

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