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ORIGINAL ARTICLE

Larval feeding induced defensive responses in tobacco: comparison of two sibling species of *Helicoverpa* with different diet breadths

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Abstract Plants respond differently to damage by different herbivorous insects. We speculated that sibling herbivorous species with different host ranges might also influence plant responses differently. Such differences may be associated with the diet breadth (specialization) of herbivores within a feeding guild, and the specialist may cause less intensive plant responses than the generalist. The tobacco *Nicotinana tabacum* L. is the common host plant of a generalist *Helicoverpa armigera* (Hübner) and a specialist *H. assulta* Guenée (Lepidoptera, Noctuidae). The induced responses of tobacco to feeding of these two noctuid herbivores and mechanical wounding were compared. The results showed that the feeding of the specialist *H. assulta* and the generalist *H. armigera* resulted in the same inducible defensive system, but response intensity of plants was different to these two species. Inductions of jasmonic acid (JA), lipoxygenase (LOX), and proteinase inhibitors (PIs) were not significantly different concerning these two species, but *H. assulta* caused the less intensive foliar polyphenol oxidase (PPO) increase, more intensive nicotine and peroxidase (POD) increases in tobacco than *H. armigera*. The defensive response of plant to herbivores with different diet breadth seems to be more complicated than we expected, and the specialist does not necessarily cause less intensive plant responses than the generalist.

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Abbreviations

JA	Jasmonic acid
LOX	Lipoxygenase
PI	Proteinase inhibitor
POD	Peroxidase
PPO	Polyphenol oxidase

Introduction

Chemical defense is important for plants. The phenomenon of induced resistance to arthropods is nearly ubiquitous in plants, having been reported in over 100 plant species from approximately 30 families (Karban and Baldwin 1997). Herbivorous insect damage is one of the most important threats that plants meet in nature (Marquis 1992). Plants can produce a variety of chemical defenses to protect themselves against herbivores (Karban and Baldwin 1997). The defenses include the production of chemicals from small organics to large proteins, and enzymes that have various deterrent effects on attacking herbivores. In tomato, wounding causes a systemic reprogramming of leaf cells that result in the synthesis of over 20 defense-related proteins (Ryan 2000). Ryan divided most of the newly synthesized protein after damage into three functional groups: (1) antinutritional proteins including proteinase inhibitors (PIs) and polyphenol oxidase (PPO), (2) signal pathway components such as lipoxygenase (LOX) and (3) proteinases (Ryan 2000).

In many situations, plants respond differently to herbivore feeding and mechanical wounding (Baldwin

1988). Damage by mechanical wounding and feeding by the cabbage butterfly (*Pieris rapae*) caterpillar resulted in different gene transcript profiles in Arabidopsis (Reymond et al. 2000). Plants also respond differently to damage by different herbivorous insects. Responses of tomato (*Lycopersicon esculentum*) to the damage by the aphid (*Macrosiphum euphorbiae*) and *Helicoverpa zea* were distinct: aphid's feeding could induce peroxidase (POD) and LOX activities but not PPO and PI activities, whereas *H. zea* feeding induces PPO and PIs but not POD (Stout et al. 1998). We speculated that sibling herbivorous species with different host ranges might also influence plant responses differentially. Such differences may be associated with the diet breadth (specialization) of herbivores within a feeding guild, and the specialist may cause less intensive plant responses than the generalist. Very limited studies about the relationship of plant chemical defense and diet breadth of caterpillar species were reported (Hartley and Lawton 1987; Bowers and Stamp 1993; Agrawal 2000), and they found either there was no difference in plant-induced defense due to herbivory by the specialists compared to the generalists, or there was no clear relationship of the specificity of induction and effects of induced plant resistance with diet specialization in the herbivores. However, these studies deal with herbivores, which are not sibling species.

Tobacco (*Nicotiana tabacum* L.) is an important commercial plant. The toxic secondary metabolite nicotine is an important defense weapon of this plant. Both secondary metabolites and protein-based defenses have been described in tobacco. Different genotypes of cultivated tobacco (*N. tabacum*) are known to vary in their ability to accumulate and store nicotine, and in the native tobacco species, *N. sylvestris* and *N. attenuata*, wounding and herbivore attack are known to dramatically induce a rapid increase in jasmonic acid (JA) in wounded leaves and a slightly delayed systemic increase in the roots, which result in a systemic, whole plant nicotine increase (Baldwin 1988, 1999; Baldwin and Ohnmeiss 1994a, b; McCloud and Baldwin 1997). Nicotine is produced in roots and its de novo synthesis is regulated by the endogenous signal JA (Baldwin and Ohnmeiss 1994a; Baldwin et al. 1997). In the JA-related defense-induction- signaling pathway, LOX plays an important role. LOX catalyzes the production of JA from linolenic acid, which stimulates the expression of defense-related genes (Farmer et al. 1992). Severa1 species of the families Solanaceae and Fabaceae accumulate PIs in their storage organs and in leaves, in response to wounding (Richardson 1979; Brown and Ryan 1984). Trypsin inhibitors were also found in *N. attenuate* (Zavala et al. 2004). The ornamental tobacco (*N. alata*) produces one 6 kDa chymotrypsin inhibitor and four 6 kDa trypsin inhibitors from a single 40.3 kDa precursor protein, which enhances plant resistance to *H. punctigera* and *Telogryllus commodus* (Anderson et al. 1997). PPO is an oxidative enzyme that can be induced by herbivory or by exogenous application of methyl jasmonate, and has been found to be a reliable indicator of other systemic-induced responses to herbivory in solanaceaous plants including tobacco (Stout et al. 1998; Constabel and Ryan 1998). PODs are heme-containing enzymes, which use H_2O_2 to oxidize a wide variety of biological substrates including phenolics, indole acetic acid, and ascorbate (Butt 1980), and have been studied in various stress-related and developmental processes. Stout et al. (1998) found that in tomato leaves aphid feeding can induce POD activities, but *H. zea* feeding cannot induce POD activities. *Leptinotarsa decemlineata* regurgitant treating on wound of *Solanum tuberosum* L. and *Phaseolus vulgaris* L. can also stimulate POD production (Kruzmane et al. 2002).

In this study, we focused on the induced defensive responses of tobacco to the feeding of two sibling noctuid species with different diet breadth, the cotton bollworm *H. armigera* (Hübner) and the oriental tobacco budworm *H. assulta* Guenée. The former is a typical generalist, which can feed on several-hundred plant species in at least 30 families, whereas the latter is a specialist, which only feed on several plants in Solanaceae. They share tobacco as their host plant. The aim of this study is to determine if there are any differences in chemical defense of tobacco plant to feeding of these two caterpillar sibling species and mechanical wounding, especially in induction of JA, nicotine, LOX, PPO, PIs, and POD activities in tobacco leaves.

Materials and methods

Plant and insects

Seeds of a common cultivar of tobacco (*Nicotiana tabacum* L.) "Putongyan" were provided by Institute of Crop Germplasm Resources of Chinese Academy of Agricultural Science and germinated in potting soil containing the appropriate nutrients in a growth chamber of 24 ± 1°C, with a photoperiod of 16-h light and 8-h dark.

Two moth species, *H. armigera* and *H. assulta* were collected in Zhengzhou, Henan province of China. The larvae were reared on an artificial diet for many generations in a laboratory of 26 ± 1°C with a photoperiod

of 15-h light and 9-h dark. The diet was prepared based on method of Wu (1985).

Treatments of plant

The healthy plants (weight 8.2 ± 1.06 g with five to six expanded leaves at the start of experiment) were selected and randomly divided into three groups for the following treatments: (1) Caterpillar feeding: two-day-old fifth instar *H. armigera* or *H. assulta* were individually caged on the second uppermost fully expanded leaf of tobacco plant; the caterpillars were limited to the space of the cages and could not move out during the experiment. After feeding for 5 h from the first bite, the caterpillars and the cages were removed from the plants. (2) Mechanical wounding: the counterpart leaves of another plant were punched with capillary (diameter 1.5 mm) one time each hour for 5 h, to spatially and temporally simulate caterpillar feeding. A preliminary experiment showed that after feeding for 5 h, each caterpillar consumed similarly, about 7 cm^2 leaf area and so we controlled the mechanically damaged area of the leaves about equal to that of caterpillar feeding in 5 h. (3) Undamaged control plants. The damaged leaves of treated plants and the undamaged counterpart leaves of control plants were harvested at 30 min and 3 h after the start of damage, to determine wound-induced changes of JA, at 30 min, 1, 3 and 5 days after the start of damage to determine wound-induced changes of nicotine. For JA and nicotine assays, the whole experiment was repeated separately for three times, and in each repeat five replications were run at each time point of each treatment. For LOX, POD, PPO and PIs induction experiments, we first assayed the time course of induction (30 min, 3 h, 6 h, 24 h, 3 days and 5 days) by *H. armigera* feeding and determined the time with maximal induction, then compared their activities in the above treated leaves at that time. Five replications were run for LOX, POD, PPO and PIs assays at each time point of each treatment. For each time point in all the treatments new plants were used.

Quantification of endogenous JA

The quantification of endogenous JA was done based on the protocol of Koch et al. (1999). Tobacco leaves (1.0 g fresh tissue) were frozen and ground under liquid nitrogen. The powder was suspended in a solution of acetone and 50 mm citric acid (70:30 v/v). [9, 10-$^2H_2$]Dihydro-JA (198 ng) was added as an internal standard. The organic solvent was allowed to evaporate overnight at room temperature. The aqueous solutions were filtered and extracted with 10 ml diethyl ether for three times. The pooled extracts were then loaded onto a solid-phase extraction cartridge (Argnoaut, Mid Glamorgan, UK) containing 500 mg of the aminopropyl sorbent. After loading, the cartridges were washed with 7 ml of a solvent mixture of trichloromethane:2-propanol (2:1 v/v). JA and the standard were eluted with 10 ml diethyl ether:acetic acid (98:2 v/v). After evaporation of solvents and esterification of the residue with excess diazomethane, the sample was adjusted to 50 µl with hexane. The solutions were analyzed by GC-MS without further purification. The methyl esters of JA and the standard [9, 10-$^2H_2$] JA, were separated by GC on a DB-wax column (60 m long, 0.2 mm i.d.), allowing quantification of the former. To enhance the sensitivity of the method, spectra were recorded in the selective ion mode, monitoring only the fragment ion at m/z = 83, 151, 156, 224, 228, and 83 is the base peak of both the methyl esters of JA and [9, 10-$^2H_2$] JA. The amount of endogenous JA was calculated from the peak areas of the methyl esters of JA and the [9, 10-$^2H_2$] JA standard.

Nicotine analysis

Leaf nicotine contents were determined by HPLC as described by Saunders and Blume (1981). One gram sample of fresh tobacco leaves was ground in 10 ml of 40% (v/v) methanol containing 0.1% (v/v) 1 N HCl with a homogenizer. The homogenate was centrifuged at 500*g* for 3 min and filtered through a 0.45 µm Millipore filter prior to HPLC injection. Nicotine standard was from Sigma (St. Louis, MO, USA). A Waters 600E HPLC system equipped with a Model 486 detector was used. Nicotine was separated on a Millipore µBondapak C_{18} reversed-phase column (7.8 × 300 mm) with an isocratic mobile phase of 40% methanol containing 0.2% (v/v) phosphoric acid buffered to pH 7.25 with triethylamine and detected at 254 nm.

Defense-protein assay

To assay for foliar enzymes, about 0.2 g fresh leaf with midribs removed, was homogenized in 1.25 ml 0.1 M ice-cold Tris–HCl buffer, pH 7.0, containing 7% (w/v) polyvinlypolypyrollidone (PVPP; Sigma, MO). Then, 0.4 ml 10% (v/v) Triton X-100 (Sigma) solution was added to the homogenate. It was centrifuged at 12,000*g* for 10 min at 4°C, and the supernatant was stored at −20°C until used for spectrophotometric assays of PPO and POD (Stout et al. 1996, 1998; Thaler et al. 1996) and LOX assay (Gökmen et al. 2002).

For PPO assay, an aliquot of 30 µl of leaf enzyme extract was added to 1 ml of 2.92 mM caffeic acid (Sigma) in phosphoric acid buffer (0.1 M, pH 8.0) and the change in absorbance of the mixture at 470 nm was measured for 5 min. The procedure for assaying of POD activities was identical, but the substrate for POD activities consisted of 2.92 mM guaiacol (Sigma) with 0.02 mM H_2O_2 added as a cofactor. PPO and POD both measured the rate of formation of melanin-like material from phenolic substrates (Stout et al. 1996). Activities of PPO and POD are reported as ΔOD per min per gram fresh weight.

The LOX assay was followed the method of Gökmen et al. (2002). The substrate solution was prepared by mixing 157.2 µl of pure linoleic acid (Sigma), 157.2 µl of Tween-20 (Sigma) and 10 ml of deionized water. The solution was clarified by adding 1 ml of 1 N NaOH and diluting to 200 ml with 0.05 M sodium phosphate buffer (pH 7.0). The reaction was started, by adding 1 ml of crude enzyme extract with 29 ml substrate solution at 30°C. Five minutes later, the aliquots of 1 ml from the reaction medium were transferred into glass tubes containing 4 ml of a 0.1 N NaOH solution. The absorbance at 234 nm was recorded. The blank solution was prepared by mixing 1 ml of substrate solution with 4 ml of a 0.1 N NaOH solution.

The assay for PIs measured the inhibition by plant extracts on the degradation of a peptide substrate Nα-p-tosyl-L-arginine methyl ester (TAME; Sigma) by the proteinase trypsin (Sigma; Stout et al. 1996). One g of fresh leaf was homogenized in Tris–HCl buffer (pH 7.0) containing polyvinylpolypyrrolidine (7%), phenylthiourea (1.67 mmol l^{-1}), KCl (0.3 mmol l^{-1}), and ascorbic acid (0.4 mmol l^{-1}). Extracts were then frozen for later use. For PIs assays, the frozen leaf extract was thawed and centrifuged and 10 µl of the clear supernatant was added to an identical volume of a 0.001 M solution of HCl containing 0.0015 mg trypsin. The mixture was allowed to incubate for 10 min in a quartz cuvette. Following incubation, 2.9 ml of 0.5 mM TAME in a methanol/ phosphate buffer mixture (12:13 v/v; pH 8.0) was added to the cuvette and the increase in absorbance at 247 nm was monitored for 5 min. For every sample run, a control was run with TAME and trypsin only and proteinase inhibitors activities were calculated relative to this control.

Bioassays

Two-day-old fifth instar caterpillars of *H. armigera* or *H. assulta* were individually caged on the second uppermost fully expended leaf of a *N. tabaccum* plant for 5 h. Three days later, the damaged leaves were removed from the caterpillar-exposed plants and used to feed neonates of *H. armigera*. Plants without damage were used as controls. Every ten neonates of *H. armigera* were reared in one glass-tube with the leaves damaged by *H. armigera* or *H. assulta*. The damaged leaves fed to *H. armigera* neonates were renewed with equally treated leaves every day to keep fresh, undamaged control leaves also renewed every day. Two hundred *H. armigera* larvae were used for testing their survival and growth on each kind of damaged plant. Five days later, weights and survival rates of the *H. armigera* larvae were recorded.

Statistical analysis

All data including JA, nicotine, LOX, POD, PPO, PIs, the larva weight, survival rate were analyzed by the one-way ANOVA for analysis of variance, and the least significant difference (LSD) test was performed for means multiple comparisons ($P = 0.01$). The survival rates were transferred to arcsin before ANOVA analysis. The SPSS 10.0 software package was used (SPSS 2001).

Results

Induction of JA in tobacco leaves

Undamaged control leaves consistently showed low level of JA (Fig. 1). At 30 min after the start of damage, the JA concentration in the leaves damaged by both insect species increased to high level at the same extent, which were two to threefold higher than that of mechanically wounded leaves ($df = 3, 56$; $F = 314.33$; $P < 0.0001$). At 3 h after the start of damage, the JA concentrations in the caterpillar damaged leaves increased further, and no difference was found concerning these two species. JA concentrations of caterpillar-damaged leaves were still over 2-fold higher than that of mechanically wounded leaves ($df = 3, 56$; $F = 324.99$; $P < 0.0001$; Fig. 1).

Induction of nicotine in tobacco leaves

Undamaged control leaves showed low levels of nicotine, two herbivore species damaged leaves showed higher levels of nicotine throughout the 5 days of the experimental time (Fig. 2). Larval feeding and mechanical wounding all induced the production of nicotine of the tobacco leaves but with significantly different magnitudes. At 30 min after the start of damage, nicotine concentration of *H. armigera* damaged

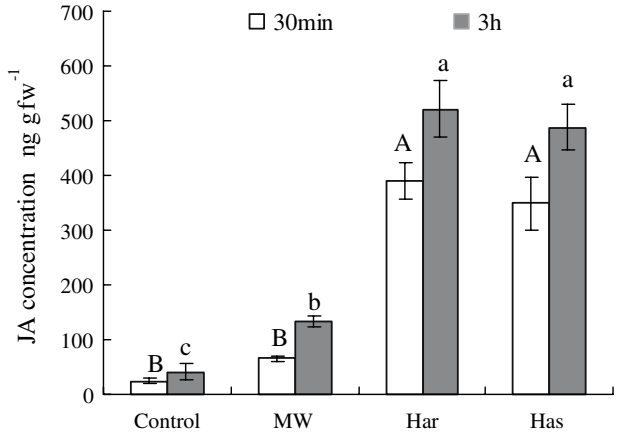

Fig. 1 Concentration of JA in undamaged plants and plants subjected to mechanical wounding and *H. armigera* or *H. assulta* larvae feeding. The whole experiment was repeated separately for three times, and in each repeat five replications were run at each time point of each treatment. Data are mean ± SE, and unique letters of the same series above symbols represent groups that differ significantly ($n = 15$; $P < 0.01$). *MW* mechanical wounded leaves, *Har* leave damaged by *H. armigera* larvae, and *Has* leave damaged by *H. assulta* larvae

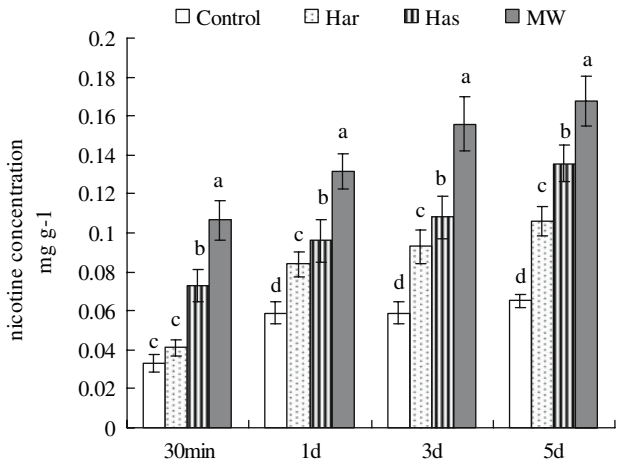

Fig. 2 Nicotine in undamaged plants and plants subjected to mechanical wounding and *H. armigera* or *H. assulta* larval feeding. *Control* undamaged leaves, *Har* leave damaged by *H. armigera* larvae, *Has* leave damaged by *H. assulta* larvae, and *MW* mechanical wounded leaves. The whole experiment was repeated separately for three times, and in each repeat five replications were run at each time point of each treatment. Data are mean ± SE, and unique letters in the same groups above symbols represent groups that differ significantly ($n = 15$; $P < 0.01$)

leaves were no different from control leaves, and both of them were lower than that of *H. assulta* damaged and mechanically damaged leaves; nicotine concentration of mechanically damaged leaves also were higher than that of *H. assulta* damaged leaves ($df = 3,56$; $F = 48.42$; $P < 0.0001$). At 1, 3 and 5 days after the start of damage the nicotine concentration among differently treated leaves all had significant difference. At 5 days after the start of damage, the nicotine concentration in damaged leaves was increased by 1.58-fold for the mechanical wounding, 1.07-fold for *H. assulta* larval damage, and 0.63-fold for *H. armigera* larval damage. *H. armigera* feeding resulted in significantly lower increase of nicotine concentration than *H. assulta* feeding, and both of them caused significantly less increase of nicotine concentration than mechanical wounding ($df = 3,56$; $F = 120.41$; $P < 0.0001$).

Induction of defense enzymes and PIs activities in tobacco leaves

The time courses of inductions of LOX, POD, PPO and PIs by *H. armigera* larval feeding within five days were shown in Fig. 3. The peak induction of POD, PPO and PIs all occurred at 6 h after the start of damage, but that of LOX appeared at 3 h (Fig. 3a–d).

Based on the above results, the foliar LOX activities of various treated plants at 3 h and the foliar POD, PIs and PPO activities at 6 h after the start of damage were compared. The two caterpillar feedings all induced significantly higher activities of foliar LOX, POD, PIs and PPO in damaged leaves than the mechanical wounding did (Fig. 4a–d). There was no significant difference on inductions of LOX and PIs by feeding of *H. armigera* and *H. assulta* larvae, but significant difference between inductions of POD and PPO by two caterpillars feeding were found. Feeding of *H. armigera* induced higher PPO and lower POD levels than feeding of *H. assulta* (Fig. 4a–d).

Survival and weight of *H. armigera* on the larvae damaged plants

Helicoverpa armigera larvae showed lower weight and survival rate when fed on the leaves formerly fed by *H. armigera* than fed on undamaged control leaves, but showed no difference from larvae fed on *H. assluta* damaged leaves (Fig. 5a, b). In five days, the larvae that fed on undamaged control leaves got the highest weight (19 ± 1.0 mg), and the larvae that fed on *H. armigera* damaged leaves got the lowest weight (9.3 ± 1.6 mg), and the weight of the larvae fed on *H. assulta* damaged leaves and undamaged control leaves had no significant difference ($df = 2, 57$; $F = 5.905$; $P < 0.05$; Fig. 5a). The survival rate of the *H. armigera* larvae fed on undamaged control leaves was the highest (0.53 ± 0.02), and that fed on *H. armigera* damaged leaves was the lowest (0.25 ± 0.03) ($df = 2, 57$; $F = 27.498$; $P < 0.01$), and also there was significant difference in the survival rate between caterpillars fed on *H. assulta* and *H. armigera* damaged leaves (Fig. 5b).

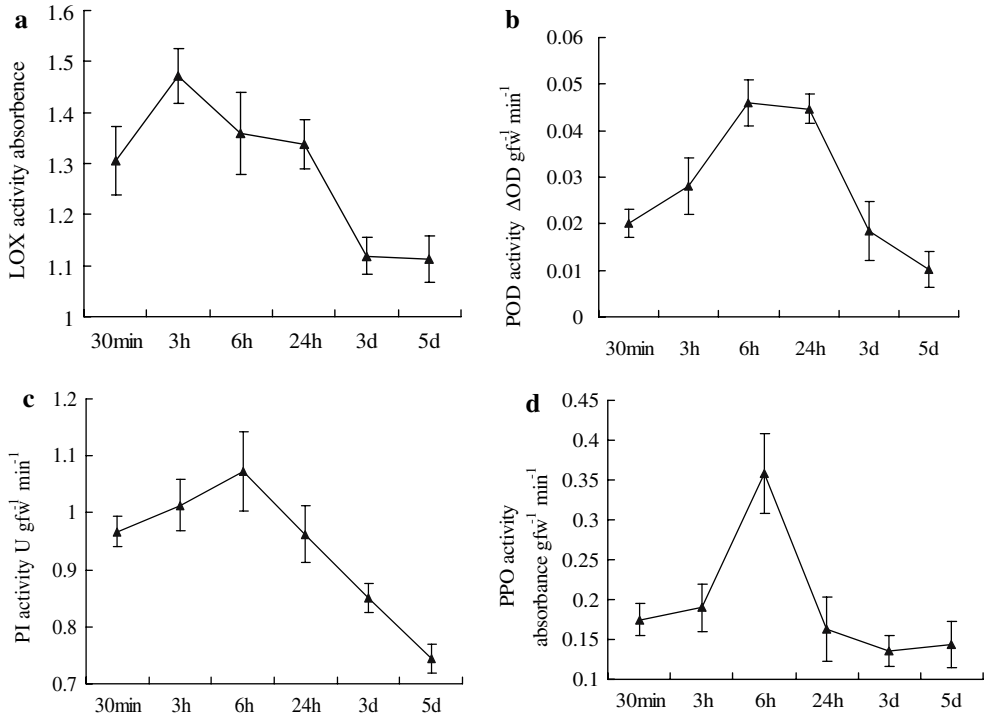

Fig. 3 Foliar defense enzymes and PIs activities in plants subjected to *H. armigera* larval feeding at 30 min, 3 h, 6 h, 24 h, 3 d and 5 d after the first bite of the larva. **a** LOX activity, **b** POD activity, **c** PI activity, and **d** PPO activity. Data are mean values ± SE of five replications ($n = 5$)

Discussion

Chemical defense of tobacco has been studied extensively (Baldwin 1988, 1999; Baldwin and Ohnmeiss 1994a, b; McCloud and Baldwin 1997; Voelckel and Baldwin 2004), but there have been few comparative studies of the chemical defense of tobacco to sibling herbivores with different host ranges. Plants may have the chemical defense specificity to herbivores with different diet breadth. The sibling species, *H. armigera* and *H. assulta* give us an ideal system to study chemical defense specificity of tobacco to sibling herbivores within a feeding guild.

Our results clearly demonstrated that the caterpillar feeding and mechanical wounding could induce the same defensive responses of the plant, but the plant response levels were different. We suggest that some elicitor agents in the caterpillar's oral cavity may be responsible for the induction of the plant responses since the caterpillar feeding and the mechanical wounding experiments were carefully operated to guarantee the modes and kept synchronous, and the two herbivores damaged leaf areas were similar at the 5-h-feeding time (data not show).

JA is a ubiquitous, damage-inducible compound, which elicits a diverse suite of plant defense responses (McCloud and Baldwin 1997). The JA concentrations increased about 12- to 13-fold when the leaf was damaged by caterpillar's feeding compared with the 2- to 3-fold increases when the leaf was damaged by mechanical wounding (Fig. 1). Many former studies also indicated that JA increases in mechanically wounded leaves from a variety of species range from 2- to 9-fold (reviewed in Farmer 1994; McCloud and Baldwin 1997), but herbivores and pathogens always induced JA increases much higher (13- to more than 100-fold increases; Farmer 1994; Mueller and Brodschelm 1994; McCloud and Baldwin 1997).

However, the similar amplification of foliar JA concentrations caused by these two herbivores does not result in the same nicotine amplification. It seems that relationship between JA induction and plant defense may be labile and some elicitor agents in *H. armigera* and *H. assulta* may be involved in suppressing defensive responses in tobacco. It is reported that *Manduca sexta* feed induced higher JA responses and lower nicotine responses in *Nicotiana attenuata* than mechanical wounding did, and it was speculated that the fatty acid-amino acid conjugates (FAC) in the oral secretions of *M. sexta* was responsible for the difference in induction of JA and nicotine (McCloud and Baldwin 1997; Halitschke et al. 2001). Recently,

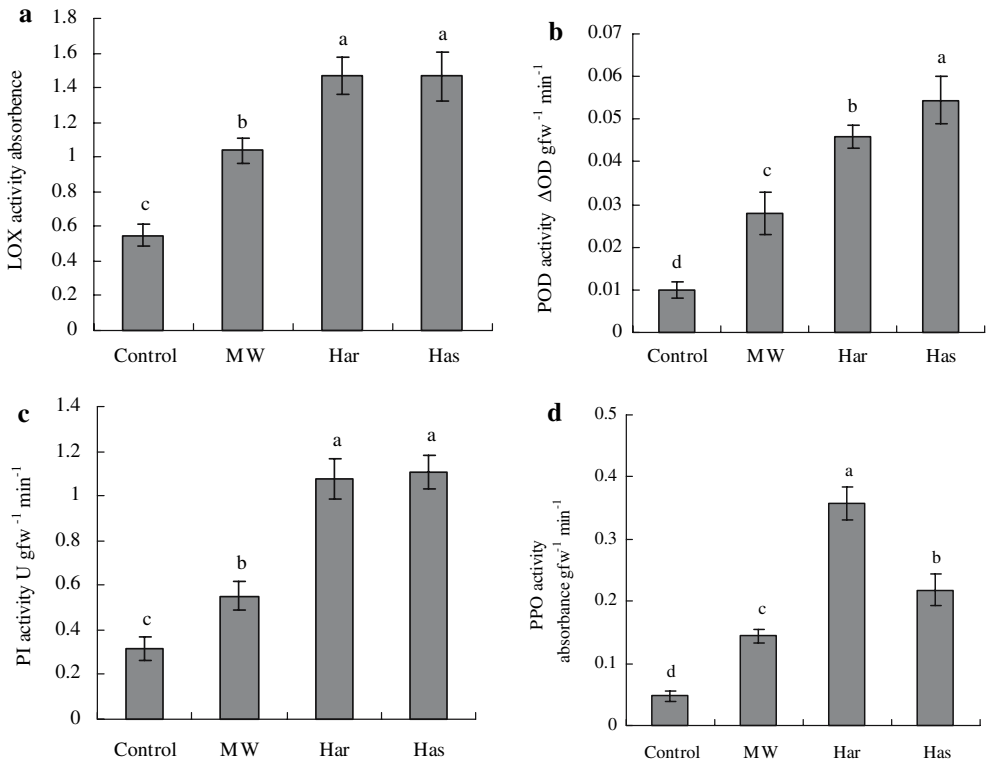

Fig. 4 Foliar defense enzymes and proteinase inhibitors activities in undamaged plants and plants subjected to mechanical wounding and *H. armigera* or *H. assulta* larvae feeding. **a** LOX activity (assayed at 3 h after the start of damage), **b** POD activity (assayed at 6 h after the start of damage), **c** PI activity (assayed at 6 h after the start of damage), and **d** PPO activity (assayed at 6 h after the start of damage). *MW* mechanical wounded leaves, *Har H. armigera* larva damaged leaves, and *Has H. assulta* larva damaged leaves. Data are means of five replications and unique letters above symbols represent groups that differ significantly ($n = 5$, $P < 0.01$)

it was reported that *N. tabacum* L. nicotine synthesis induced by mechanical wounding is regulated by auxin (Shi et al. 2006). But the authors did not discuss the relationship of the *N. tabacum* L. nicotine synthesis and herbivore bite. A glucose oxidase (GOX) found in saliva and labial gland of *H. zea* has been shown to suppress the tobacco nicotine induction through wounding (Musser et al. 2002). GOX catalyzes the oxidation of glucose to produce gluconic acid and H_2O_2, which could increase the level of ethylene and free salicylic acid (SA) in tobacco (Chamnongpol et al. 1998). Ethylene in *N. attenuate* induced by herbivory attack can suppress nicotine accumulation (Winz and Baldwin 2001). We have also found a GOX in the labial gland (salivary gland) of *H. armigera* and *H. assulta* larvae (Zong and Wang 2004). The nicotine response of tobacco to these two caterpillars' feeding was also significantly different. The GOX activity in *H. armigera's* labial gland was about ten times higher than that in *H. assulta's* labial gland. We speculate a quantitative relationship between caterpillar GOX and nicotine responses. However, further study is needed to validate this issue.

Just like the induction of foliar JA concentrations in tobacco by damage, induction of foliar LOX, POD, PIs and PPO activities were also observed. The results showed that *H. armigera* and *H. assulta* feeding not only could suppress nicotine responses, but also promote LOX, POD, PIs and PPO based chemical defenses of tobacco. It's well known that the foliar LOX is a key enzyme in JA synthesis, which can elicit a diverse suite of plant defense responses including PIs (Liechti and Farmer 2002). PPO and POD all participate in the oxidative responses in the foliar cell. PPO can lead to protein crosslinking, rendering them less digestible when it is ingested by herbivores together with phenolics (Constabel et al. 1996). POD is the key enzyme in the process of building the plant cell wall (Chittoor et al. 1999), and its activation also decreases the nutritive quality of the foliage (Felton et al. 1989). Our results showed that the foliar JA, LOX, and PIs of plants had no significant differences in response to the feeding of two caterpillar species, but *H. armigera* induced higher level of foliar PPO and lower level of POD than *H. assulta* did.

Different herbivores within a feeding guild may influence plant responses differentially, but based on

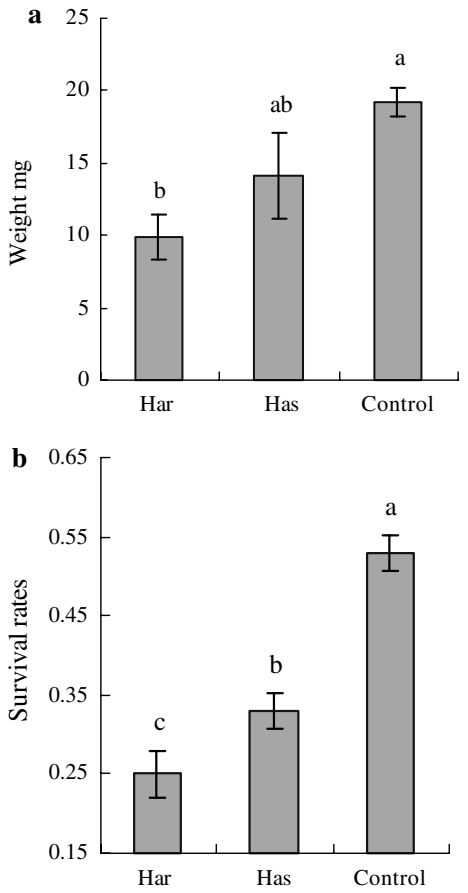

Fig. 5 *H. armigera* larva weight (**a**) and survival rates (**b**) 5 days after fed on undamaged leaves (Control), *H. armigera* damaged leaves (Har) or *H. assulta* damaged leaves (Has). Data are mean ± SE, and different letters above symbols represent groups that differ significantly ($n = 20$; $P < 0.01$)

our results for sibling species such as *H. armigera* and *H. assulta*, though two herbivores have distinctly different host ranges, the induced defense systems of plants against them seems to be the same; the only difference may be the response intensity. Similar results were observed in a global transcriptional response assay of Arabidopsis to damage of a specialist *P. rapae* and a generalist insect *Spodoptera littoralis*, almost identical transcript profiles were observed in the study (Reymond et al. 2004). Our results proved that, induced defense of tobacco plants was effective against *H. armigera* larvae even though it was induced by *H. assulta*. However, greater survival rates of *H. armigera* larvae on the leaves previously damaged by *H. assulta* than those on the leaves previously damaged by *H. armigera* indicated the specificity of induced plant defense concerning these two caterpillars exists to some extent. Agrawal (2002) tested specificity of induced plant resistance employing a different system; four lepidopteran herbivores with wild radish plants. He found that the specificity of induction and effects of induced plant resistance varied when plants were damaged by different herbivore species, but the variation was not associated with diet breadth of the herbivores (Agrawal 2000). These results were based on four lepidopteran herbivores that are not close phylogenetically. For testing the relationship of diet specialization in herbivores with the specificity of induced plant defense, we assume that using closely related herbivorous species with sufficient difference in their host ranges might give more convincing results.

In conclusion, we found that the feeding of tobacco by the specialist *H. assulta* and the generalist *H. armigera* resulted in the same inducible defensive system, but the responding intensities to these two species were different. Induction of JA, LOX, and PIs was not significantly different concerning the two species, but *H. assulta* caused a less intensive foliar PPO increase in tobacco, more intensive nicotine production, and increase in POD compared to *H. armigera*. Contrary to our expectations, the present results show that herbivores with a different diet breadth do not seem to induce specific plant defense responses, and the specialist does not necessarily cause less intensive plant responses than the generalist. We just focused on the performance of *H. armigra* on plants damaged by either herbivore in this study. It would be more informative to obtain complete results on the performance of the two herbivores on plants damaged by either herbivore, mechanically damaged plants, and undamaged controls. Further surveying of other plant signals such as salicylic acid and ethylene may provide more detailed knowledge about the physiological responses of tobacco, induced by feeding of herbivores.

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