Preliminary studies on differential defense responses induced during plant communication

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ABSTRACT

We compared the expression patterns of three representative genes in undamaged tomato and tobacco plants in response to exposure to either tomato or tobacco fed on by *Helicoverpa armigera* (cotton bollworm). When tomato and tobacco, two species of one family, were incubated in the chambers with the tomato plants damaged by the cotton bollworm, the expression of the *PR1, BGL2,* and *PAL* genes was up-regulated in leaves of both plants. However, the levels of gene expression were significantly higher in the tomato than that in the tobacco. In addition, the activities of enzymes, peroxidase, polyphenol oxidase, and lipoxygenase were found to be higher in the tomato than those in the tobacco. Similar results were obtained when the damaged plants were replaced by the tobacco.

***Keywords*:** plant–plant communication, tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), cotton bollworm (*Helicoverpa armigera*), inducible defenses.

INTRODUCTION

Plants are frequently damaged by herbivorous insects. Many types of defensive strategies have evolved in response to herbivore attack [1, 2], including physical, mechanical and chemical defenses that deter herbivore activity [3, 4]. Furthermore, some plants defend themselves indirectly by emitting volatile chemicals, which in turn attract the natural enemies of the herbivores [5, 6].

Several studies have shown that volatile chemicals are released when plants are damaged [7]. It is demonstrated that the plant defense response is not limited to the individual being damaged [8]. Damage to a plant can result in induction of chemical defenses and defense related gene expressions in neighboring plants. Plants that are exposed to volatile compounds released by damaged neighbors may increase the production of defensive chemicals even though they themselves have not been damaged [8, 9].

Interestingly, in interplant communication, the receiver and emitter can be of different species [10-12]. This phenomenon suggests that healthy plants near to the damaged plants may use volatile chemicals released from damaged neighbors as cues to induce anti-herbivore defenses as a deterrent of future attack by herbivores.

Such inter-plant signal transfer has been hypothesized for over 20 years and has been a controversial topic [13, 14]. Although some studies have found no evidence for the transfer of signals between damaged and undamaged plants [15], others supported the hypothesis that chemical information was conveyed between damaged and undamaged plants [8, 9, 16]. In recent years, inter-plant communication has appeared to be better supported by more experimental evidences [10, 17].

Previous researches mainly concentrated on intra-specific information transfer through the volatile chemicals between plants. Oudejans and Bruin [18] extended the scope to inter-specific information transfer between cucumber and Lima bean. Since then, additional experiments were done on information transfer between inter-specific plants [10-12]. However, no reports are available about compara-

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tive study related to intra- and inter-species signaling to aerial signal emitted from damaged plants. In this study, we selected the tomato and tobacco, both of which belong to the same family, to investigate whether there exists response sensitivity difference between inter-species and intra-species information transfer.

MATERIALS AND METHODS

Plant materials and insect culture

Tomato (*Lycopersicon esculentum cv Castlemart*) and tobacco *(Nicotiana tabacum cv Xanthinc*) seeds were germinated in pots and grown for 2 w in a growth chamber. Experiments were initiated when plants were in the late rosette stage of growth.

Cotton bollworm (*Helicoverpa armigera*) larvae were collected from Jiangxi province in 2003 and reared in the laboratory at 24 ± 1°C, and 80 ± 5% R.H. under a 14 h light/10 h dark photoperiod. The larvae were fed on a modified semi-artificial diet as described by Li *et al* [19].

Experimental design and sampling

For feeding experiments, eight 4th instar larvae that had been starved for 12 h were placed on each of the plant's primary leaf and allowed to feed continuously during incubation (t = 6, 12 h). Experiments were carried out in a clean, sealed container (diameter = 33 cm, depth = 21 cm). In one series of experiments, larvae were placed on two tomato plants; while in the second series of experiments, larvae were placed on two tobacco plants. In each replicate, two plants being fed by the cotton bollworms were positioned on one side of the container and two healthy, potted tomato and tobacco plants were placed on the other side of the container (n = 4 uninfested and 2 infested plants per container). To prevent invasion of the uninfested plants by the cotton bollworm, we used wet cotton wool to isolate the container into an infested- and an uninfested-plant area. We used a cleaned container for each experimental replicate in order to exclude effects from the previous experiment or experimental replicate. Experiments were maintained at 25 ± 2°C, 50-70% RH, and 16L-8D (2,150 lx fluorescent light). Uninfested tomato and tobacco plants that were kept in the same chamber with damaged tomato or tobacco plants were sampled at 6 and 12 h after the initiation of the experiments (t = 6, 12 h). Two uninfested potted tomato and tobacco seedlings maintained with uninfested tomato or tobacco seedlings in a container for the equal time were used as control. Cotton bollworm wounded tomato and tobacco were also sampled as the positive control. These experiments of a time course were repeated four times. Undamaged healthy plants were used in each repeat.

RT-PCR analysis

Total RNA was extracted using the cooled phenol method [28]. Total RNA pellets were dissolved in 30 μl of RNase-free water and quantified by spectrophotography. RNA quality was determined by gel fractionation in agarose followed by ethidium bromide staining and UV light visualization before analysis for specific mRNAs. Reverse transcription reactions were carried out as recommended by the manufacturer (Takara, R019A, Japan). The products of reverse transcription were used as templates for PCR analysis. The primers used for PCR were designed according to the sequence reported in Genbank (Tab. 1). And the primers for β-actin gene were designed according to the conserved sequence of tomato and tobacco. To compare expression levels and minimize PCR artifacts, the number of PCR cycles was kept low [17 cycles for β-actin, 29 cycles for *LOX* (lipoxygenase), 27 cycles for *BGL* (β-1, 3-glucanase), 22 cycles for *PAL* (phenylalanine ammonia-lyase)], and PCR products were detected by agarose gel electrophoresis. Each experiment was repeated four times using independent samples. The PCR primers used were listed in Tab. 1.

PPO, POD and LOX activity measurement

Extracts for assays of foliar polyphenol oxidase (PPO), peroxidase (POD) and lipoxygenase (LOX) [20] were prepared by homogenizing individual, weighed leaflets in 1.25 ml of pH 7 K Phos (0.1 M) buffer containing 7% polyvinylpolypyrrolidine. An aliquot (0.4 ml) of a 10% solution of Triton X100 was added and the homogenate was centrifuged at 6,000 g for 15 min. The resulting supernatant was used directly as an enzyme source. The activity of polyphenol oxidase and peroxidase was measured as the rate of formation of melanin-like material from phenolic substrates [20]. For the assay of polyphenol oxidase, 10–30 μl of enzyme extract were added to 1 ml of 2.92 mM caffeic acid in pH 8 K Phos buffer (0.1 M) and the change in absorbance of the mixture at 470 nm was measured for 30 s. For assaying peroxidase activities, the procedure was identical, except that the substrate was 5 mM guaiacol with 0.02 mM H_2O_2 added as a cofactor. Polyphenol oxidase and peroxidase activities were reported as ΔOD/min/mg protein. The activity of lipoxygenase

Tab. 1 Primers used in RT-PCR analysis

Gene	Gene product	Forward primer (5'→3')	Reverse primer (5'→3')
Tomato *PR-1*	Pathogenesis-related protein	ATCTCATTGTTACTCACTTGTC	AACGAGCCCGACCA
Tomato *BGL2*	β-1,3-glucanase	CACCAACATTCACATAACAGAGGC	CAGGGCTGATTTCATTACCAAC
Tomato *PAL*	Phe-ammonia lyase	TTCAAGGCTACTCTGGC	CAAGCCATTGTGGAGAT
Tomato *LOX*	Lipoxygenase	TTTCTG CGACTTGAGGTTCGG	ATTAGTCTTTACCTTCTTGTCCAGT
Tobacco *PR-1*	Pathogenesis-related protein	CTCGGTTCGTGTTGGATGT	TCGCAAGTAGCTAGACCATCA
Tobacco *BGL2*	β-1,3-glucanase	GCACAGTCTATTGGAGTATGCTATG	GGT ATT CGC TAA GAC CCC TGA
Tobacco *PAL*	Phe-ammonia lyase	CCTACAAGGCTACTCTGG	AGCCGCCTTCACATA
Tobacco *LOX*	Lipoxygenase	GAGCCATTCGTGATCGCAAC	GCGATTAGGGAGATAACCAGCA
Actin	House-keeping gene	GGGGAGGTAGTGACAATAAATAACAA	GACTGTGAAACTGCGAATGGC

was measured using the formation of conjugated dienes from linoleic acid [21]. The reaction mixture consisted of 15–20 μl enzyme extract and 2.9 ml of 0.4 mM linoleic acid dispersed in pH 7 K Phos buffer containing 0.1% Tween 20. Rate of change in absorbance of this mixture was measured at 234 nm for 10 min. Each experiment was repeated four times using independent samples. Protein concentrations were determined using Bradford method [22].

Statistics

Statistical analysis was performed by the Student's *t* test. The data shown here was represented as mean ± SD. Values of $P < 0.05$ were considered as significance.

RESULTS

Expression of defense-related genes induced by plants incubated with caterpillar-damaged plants

In leaves of tomato and tobacco plants exposed to *H. armigera*-infested plants, we detected transcripts of *BGL* (β-1, 3-glucanase, pathogenesis-related (PR) proteins) and phenylalanine ammonia-lyase (PAL, an enzyme involved in the phenylpropanoid pathway) genes. And transcript of *LOX* (a key enzyme of the octadecanoid pathway) gene was undetectable or weak in both treatments (Fig. 1). In the control experiments where uninfested tomato and tobacco plants were incubated with other uninfested tomato or tobacco plants, we did not detect transcripts of these three genes (Fig. 1). Consistent with a previous study [23], transcription of *BGL* and *PAL* in undamaged plants was induced by exposure to the infested plants and any volatile compounds they released. As a positive control, in the infested tomato or tobacco plants, the transcripts of these three genes were all strongly up-regulated (Fig. 1 "infested").

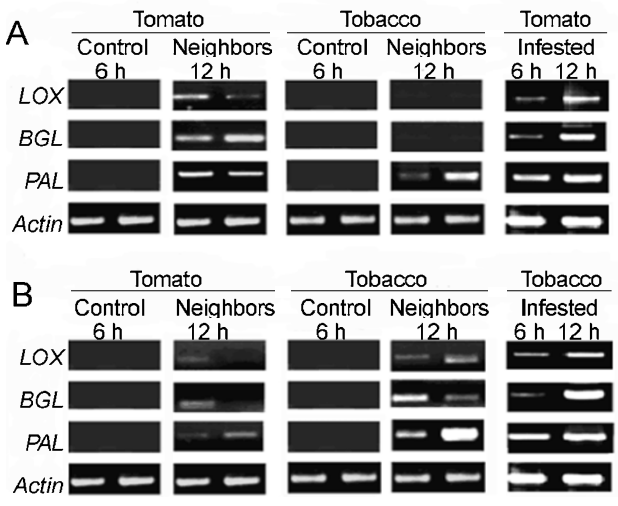

Fig. 1 Intensity of gene expression in tomato and tobacco plants exposed to grazed plants of the same and different species fed on by *H. armigera*. Data from intact plants exposed to grazed (**A**) tomato or (**B**) tobacco plants for 6 and 12 h. Total RNA was isolated from the leaves of the plants and the expression of *LOX*, *BGL* (basic PR-2), and *PAL* genes was analyzed using RT-PCR. Control experiments were carried out following the same procedure but without insect feeding. Infested tomato or tobacco was shown as a positive control. These experiments of a time course were repeated four times on independent samples.

Gene-expression patterns were compared between the two species. In the damaged tomato treatment, adjacent undamaged tomato seedlings showed a significantly stronger expression of *BGL* and *PAL* than undamaged tobacco (Fig. 1A). In contrast, in the infested-tobacco treatment, neighboring tomato seedlings showed a significantly weaker expression of *BGL* and *PAL* than the tobacco seedlings (Fig. 1B). These experiments of a time course were repeated four times on independent samples, with similar results each time, and only representative result was shown here.

Plant enzyme activity assays

After an exposure to grazed plants for 12 h, PPO, LOX, and POD activities of leaves were measured. As shown in Fig. 2 and Tab. 2, after incubation with infested tomato, PPO activity of adjacent, undamaged tomato increased by 2.0-fold compared with the control tomato while PPO activity of the undamaged tobacco increased by 1.5-fold compared with the control tobacco (two-sample *t* test, $t_4 = 2.447$, $P < 0.05$). When incubated with damaged tomato plants, the activity of POD in undamaged tomato seedlings increased by 2.7-fold and of LOX in undamaged tomato seedlings by 1.8-fold, whereas the activity of POD and LOX in the tobacco seedlings was 1.7- and 1.5-fold, respectively (two-sample *t*-test, for POD, $t_4 = 2.318$, $P < 0.05$; for LOX, $t_4 = 2.777$, $P > 0.05$).

In contrast, incubation with damaged tobacco seedlings significantly increased the activity of PPO and POD in the undamaged tobacco plants as compared with that in undamaged tomato plants (Fig. 2, Tab. 2). Activity increased by 2.2- and 2.4-fold in the tobacco whereas it was 1.3- and 1.8-fold in the tomato plants (two-sample *t* test, PPO, $t_4 = 2.777$, $P < 0.01$; POD $t_4 = 2.776$, $P < 0.05$). However, LOX activity in the neighboring tobacco plants did not differ significantly from that of tomato (two-sample *t* test, $P > 0.05$, non-significant).

DISCUSSION

The three oxidative enzymes, PPO, POD and LOX, investigated in this study were demonstrated to play an important role in anti-herbivore defense [24]. PPO is an oxidative enzyme that is induced by actual herbivory or by exogenous application of methyl jasmonate. It has been found to be a reliable indicator of other systemic induced responses in solanaceous plants, including tobacco [24-

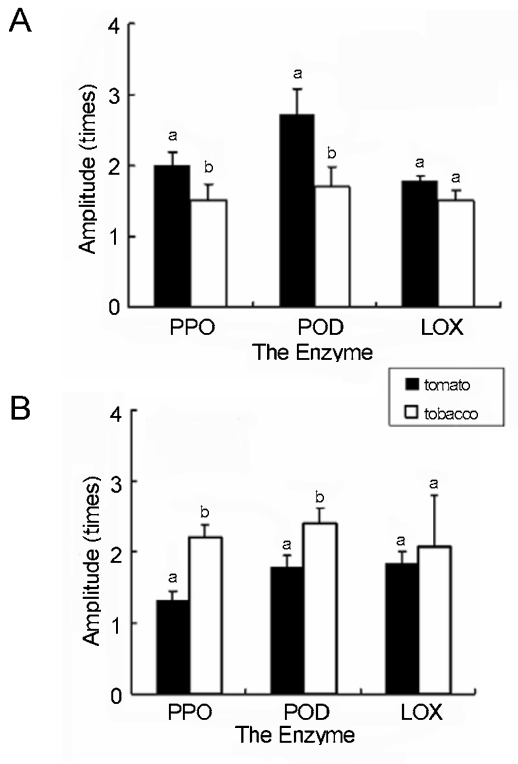

Fig. 2 Enzyme activity in neighboring tomato and tobacco seedlings after 12 h incubation with grazed (**A**) tomato or (**B**) tobacco plants. Amplitude = $EA_{treatment}/EA_{control}$, EA: the enzyme activity. The data are shown as mean ± standard deviation, n = 4. Bars with the different letters are significantly different within treatment (*t* test, $P<0.05$). PPO = polyphenol oxidase; POD = peroxygenase; LOX = lipoxygenase.

25]. LOX is the key enzyme for the synthesis of JA and it catalyzes the production of JA from linolenic acid, which stimulates the expression of defense-related genes serving as secondary signals activating a subset of defense genes [26]. Increasing activities of these enzymes is considered as one measure of the activation of defense response.

We found that intact plants responded to some volatile cue in the presence of *H. armigera* -infested intra- and inter-specific plants. Transcripts of *BGL* and *PAL* were detected in both tomato and tobacco, suggesting that the neighboring tomato or tobacco can respond to grazing-induced volatiles through the activation of defensive genes. However, the intensity of the expression of three defense genes, *LOX*, *BGL* and *PAL* varied, with weak increase in the expression of the LOX pathway in either neighboring tomato or tobacco. Weak response in *LOX* is surprising in terms of its role in jasmonic acid pathway. Probably it responds only to direct damage as opposed to volatiles, and may also be related to weak gene expression at the gene level (Fig. 1).

The sensitivity of the responses differed between species. The response to damaged plants of the same species was significantly higher than the response to damaged plants of the second species tested (Fig. 1). After incubation with infested tomato, PPO activity in neighboring undamaged tomato plants increased significantly than adjacent undamaged tobacco plants ($P < 0.05$). Similar results were observed in tobacco plants ($P < 0.05$). Different intra- and inter-specific responses may be related to different reception and succedent signal transduction mechanisms in the two species tested. The same expression pattern was found in the activity of the prooxidant enzyme POD. It was reported that POD activity increases with insect attack or leaf tissue damage [27]. Such an increase is possibly due to a direct role of POD in plant resistance mechanisms.

Since the whole plants were confined in the sealed container for 6-12 h, available CO_2 may have been depleted,

Tab. 2 Enzyme activities[a] (mean ± SD, $n = 4$) in seedlings exposed to the infested plants for 12 h.

Treatment	PPO[b]		POD[b]		LOX[c]	
	Tomato	Tobacco	Tomato	Tobacco	Tomato	Tobacco
Control[1]	17.9 ± 2.7	17.2 ± 0.7	16.5 ± 1.9	18.9 ± 2.7	6.3 ± 0.3	6.4 ± 1.1
Treated[1]	35.6 ± 2.6	26.1 ± 4.3	44.9 ± 2.7	32.9 ± 0.6	11.2 ± 0.2	9.7 ± 0.9
Control[2]	15.5 ± 0.3	15.3 ± 3.5	14.2 ± 2.3	15.3 ± 3.5	6.2 ± 0.5	7.0 ± 0.9
Treated[2]	20.4 ± 2.3	33.8 ± 3.5	25.5 ± 5.9	36.8 ± 4.8	11.5 ± 1.7	14.5 ± 3.2

[a]: Enzyme activities in units of ΔOD/min/mg (protein concentration). PPO (ΔE490 min^{-1} mg^{-1}protein), POD (ΔE470 min^{-1} mg^{-1}protein), LOX (ΔE234 min^{-1} mg^{-1}protein).
[b]: Assays for PPO and POD measured the rate of formation of melanin-like material from substrates.
[c]: Assays for LOX assay measured the formation of conjugated dienes from linoleic acid.
[1]: exposure to grazed (treated) and ungrazed (control) tomato seedlings
[2]: exposure to grazed (treated) and ungrazed (control) tobacco seedlings

thus stressing the plants during incubation. However, Arimura and his coworkers reported that photosynthetic activity of plants in a similar sealed environment was not affected [23], suggesting that the plants were not stressed. Therefore, it seems likely that the transcriptional responses in the tomato and tobacco shown in the present study were not due to CO_2 depletion.

Our results suggest that plant defensive responses are species specific. In an analysis of the effects of volatile compounds on the induction of plant defensive systems, Arimura *et al* [16] reported that three volatile compounds released by green leaves of lima bean species ((*Z*)-3-hexenol, (*E*)-2-hexenal, and (Z)-3-hexenyl acetate) elicited the expression of defensive genes, but the patterns of gene expression differed for each compound. This indicated that the lima bean could distinguish among different signals. In different plant species receiving the same chemical signals, different signaling pathway may be activated, resulting in the transcription of different genes and in differing levels of enzyme activity. Evolutionarily, every plant may develop a specific response system for defense. Different species of plants have probably also evolved differing levels of alarm/defense responses. However, details of these mechanisms, and their ecological importance, remain elusive.

In conclusion, our study on the defensive responses of tomato and tobacco plants has revealed differences in the sensitivity of the response in the levels of gene expression and enzyme activity in plants exposed to damaged conspecific as compared with damaged plants of a different species in the same plant family. However, whether this phenomenon has an ecological role in the field or it extends to more distantly related plants remains an open question.

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