Induction of phenylalanine ammonia-lyase and lipoxygenase in cotton seedlings by mechanical wounding and aphid infestation*

QIN Qiuju, SHI Xueyan, LIANG Pei and GAO Xiwu**

(Department of Entomology, China Agriculture University, Beijing 100094, China)

Received August 24, 2004; revised October 10, 2004

Abstract It has been suggested that infestation of plants causes increases in the activities of phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX), key enzymes in the phenolic compounds synthesis pathway and the octadecanoid pathway, respectively. The purpose of this work is to investigate whether the infestation of cotton aphid (*Aphis gossypii*) and mechanical wound can cause the induction of PAL and LOX activities in cotton seedlings, and whether the induction occurs in healthy seedlings growing nearby the attacked ones. The specific activities of PAL and LOX were measured using spectrophotometric method after aphid infestation and mechanical wounding. Result indicated that PAL activity and LOX activity were greatly induced by mechanical wounding and aphid infestation in cotton seedlings. The induction of PAL and LOX occurred not only in wounded and infested seedlings but also in intact healthy seedlings growing nearby. After exposed to the aphid infestation-induced volatiles, the specific activity of PAL in cotton seedlings increased by 6% at 24 h, 80% at 48 h, 235% at 72 h compared to the control, and the specific activity of LOX increased by 18% at 24 h, 34% at 48 h, 24% at 72 h, respectively. In comparison, the specific activity of PAL in unwounded seedlings exposed to wound-induced volatiles increased by 0.0 at 24 h, 200% at 48 h, 164% at 72 h, respectively and the specific activity of LOX increased by 28% at 24 h, 37% at 48 h, 8% at 72 h, respectively. It suggests that the induced volatiles are involved in plant-plant communication as airborne transferred signals.

Keywords: phenylalanine ammonia-lyase, lipoxygenase, cotton aphid, mechanical damage, cotton plants.

Plants have developed a multitude of inducible defense mechanisms against aggressive biotic and abiotic agents[1]. Wound and herbivory-induced plant responses can negatively affect herbivore's physiology directly by stimulating the synthesis of toxic metabolites[2—4]. In addition to such direct defense mechanisms, plants can also emit specific blends of volatiles that attract carnivorous enemies to defend themselves against herbivores[5—9].

Herbivory-induced plant volatiles can not only attract natural enemies, but also affect plants growing nearby the attacked plants. In the lima bean (*Phaseolus lunatus*)—spider mite (*Tetranychus urticae*)—predatory mite (*Phytoseiulus persimilis*) system, the neighboring lima bean plants become more attractive to predator mites and less susceptible to spider mites[10, 11]. Using detached lima bean leaves in a lidded glass container, Arimura et al. found that several defense genes could be induced to express in the infested and uninfested lima bean leaves by RT-PCR analysis[12, 13]. Many indoor and outdoor studies have demonstrated that herbivory-induced volatiles and wound-induced plant volatiles are involved in plant-plant communication as airborn transferred signals[14—18]. Field studies have demonstrated that the herbivore resistance and specific activity of polyphenyl oxidases (PPO) increased in native tobacco plants growing downwind of damaged sagebrush[19—21].

Phenylalanine ammonia-lyase (PAL) is a key enzyme in the synthesis of phenolic compounds and salicylic acid in plants. It plays a vital role in defense against fungi[22]. Studies on insect-plant interaction suggest that phenylpropanoid compounds are involved in plant defense against pests[23]. Lipoxygenase (LOX) is a key enzyme in the octadecanoid pathway and catalyzes the hydroperoxidation of linoleic acid and other polyunsaturated lipids. Terminal metabolites jasmonic acid is a bioactive product that has many physiological functions and mediates responses to mechanical trauma, pathogenesis and pests in plants as a long-distance transferred signal.

At present, most studies on herbivory-induced plant-plant communication have primarily focused on the tritrophic system consisting of lima bean plants,

* Supported by the Major State Basic Research Development Program of China (Grant No. G200016207) and the National Natural Science Foundation of China (Grant Nos. 30170621 and 39970496)

** To whom correspondence should be addressed. E-mail: gaoxiwu@263.net.cn

spider mites and predatory mites. Moreover, many researchers have used excised leaves or shoots rather than intact plants in their assays. The use of excised leaves may increase a response because they are more sensitive to herbivore-induced volatiles (HI-VOCs) than intact plants[24, 25]. Cotton aphid (*Aphis gossypii*) is an important phloem feeding insect on cotton plants. There is no report on cotton aphid-induced plant-plant communication up to now. In order to understand the regulatory mechanism of plant defense response, we measured the specific activities of PAL and LOX in the aphid infested, artificially damaged seedlings and intact healthy cotton seedlings growing nearby.

1 Materials and methods

1.1 Plant and insect

Cotton plants, cv. Zhongmian 35, was grown from seeds and germinated in sterile sand (28±2 ℃, 50% to 70% relative humidity). After full expansion of two cotyledons, plants were transplanted to plastic cups containing Hoagland nutrient solution and placed in an air-conditioned greenhouse for two to three weeks under natural light (25—35 ℃, 50% to 70% relative humidity).

Cotton aphids were reared on caged cotton seedlings in the greenhouse for several generations, and the temperature was maintained at 25—35 ℃ with a 50%—70% relative humidity under natural light.

1.2 Reagents

L-phenylalanine was purchased from Shanghai Chemical Company, linoleic acid from Sigma, β-mercaptoethanol from Merk, phenylmethylsulfonyl fluoride (PMSF) from Roche, bovine serum albumin (BSA) from North-Bio, and coomassie brilliant blue G-250 from Fluka.

1.3 Treatment

Cotton seedlings with the same growth potential were used for the experiment. Intact healthy cotton plants were kept as control (without wound and aphid infestation), artificially damaged plants were prepared by punching 35 holes (1mm in diameter) on two cotyledons, and aphid infestation was achieved by placing 50 apterous females on the cotyledons of a cotton plant. The treatments were repeated 6 times. Activities of PAL and LOX were measured 24, 48 and 72 hours after the treatments.

The experimental design was the following: four cotton plants (each with 100 apterous female aphids infestation) placed in two plastic cups as the emitter plants and four uninfested cotton plants as the plant volatile receivers were placed in the same desiccator. To prevent the escape of cotton aphids from the infested emitter plants, the infested plants were caged with fine nylon net and the outside wall of the two plastic cups containing uninfested plants sealed with glue. Artificially damaged cotton plants were placed in another desiccator together with four undamaged cotton seedlings as wound-induced volatiles receiver. Four healthy plants were placed in the third desiccator together with four healthy plants as control. Each assay was repeated three times. The experimental setup was maintained for 1 to 3 days, then the specific activities of PAL and LOX in each plants were examined.

1.4 Assay for enzyme activities

Phenylalanine ammonia-lyase (PAL) activity was measured by a modified spectrophotometric assay according to Koukol et al.[26] One activity unit is defined as a change in absorbance of 0.01 at 290 nm · h^{-1}(mg protein)$^{-1}$ at 30 ℃.

Lipoxygenase (LOX) activity was determined following the procedure of Axelord et al.[27] One unit of lipoxygenase is defined as the quantity of enzyme that generates 1 μmol conjugated diene per minute under the assay conditions.

1.5 Protein determination

Protein content was determined by the dye-binding method of Bradford with bovine serum albumin as a standard[28].

1.6 Statistical method

Data were analyzed by ANOVA, a statistical software package (Graphpad instat).

2 Results

2.1 Induction of PAL activity in cotton seedlings

There were significant induction of PAL activity as functions of artificial damage and aphid infestation after 48 hours (Fig. 1). Specific activity of PAL in artificially damaged cotton seedlings and aphid infested seedlings increased by 1.58-fold and 4.84-fold respectively compared to the activity in control seedlings. The values of PAL activity showed a significant difference among aphid infested, artificially damaged seedlings and control seedlings ($p<0.05$).

Table 1 summarizes the PAL induction in cotton seedlings with different treatments. The results indicated that the PAL activity increased not only in the artificially damaged and aphid-infested cotton seedlings, but also in the healthy seedlings exposed to wound-induced and aphid infestation-induced plant volatiles. The specific activities of PAL in the cotton seedlings receiving aphid infestation reached the maximum value after exposed to aphid infestation-induced volatiles for 72 hours, whereas the PAL activity in wounded seedlings reached the maximum value after exposed to wound-induced volatiles for 48 hours.

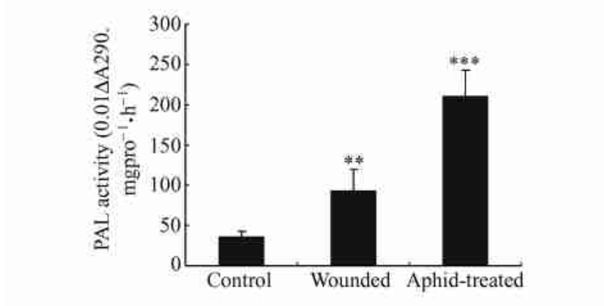

Fig. 1. The activity of phenylalanine ammonia-lyase in cotton seedlings after 48 hours of treatment. All the values are means ± standard deviation. ** $p<0.01$, *** $p<0.001$, when compared with control.

Table 1. Induction of phenylalanine ammonia-lyase by different treatments

Treatment	Specific activity (ΔA290·mgpro^{-1}·h^{-1})[a]		
	24 h	48 h	72 h
Control	29.04±2.82 (1.00)	29.42±1.33 (1.00)	23.70±4.28 (1.00)
Wounded	43.03±5.70 (1.48) *	54.05±14.21 (1.84)	58.27±15.29 (2.46)
Aphid	56.13±4.06 (1.93) ***	46.40±14.72 (1.58)	70.94±17.48 (2.99) *
Wound-receiver	29.04±7.51 (1.00)	88.33±8.67 (3.00) ***	62.67±26.68 (2.64)
Aphid-receiver	30.85±3.95 (1.06)	52.98±14.31 (1.80)	79.38±15.73 (3.35) *

a) All the values are means ± standard deviation of three experiments. * $p<0.05$, *** $p<0.001$, when compared with control.

2.2 Induction of LOX activity in cotton seedlings

The specific activity of LOX in cotton seedlings increased obviously after aphid infestation and artificial damage (Fig. 2). In cotton leaves with aphid infestation, the specific activity of LOX was enhanced by 0.92-fold at 24 h, 1.29-fold at 48 h and 1.16-fold at 72 h, respectively, when compared to that in control cotton leaves. In artificially damaged leaves LOX was enhanced by 1.27-fold at 24 h, 1.21-fold at 48 h and 1.16-fold at 72 h, respectively, compared to that in undamaged leaves. There was no obvious difference in activity of LOX between the above two treatments.

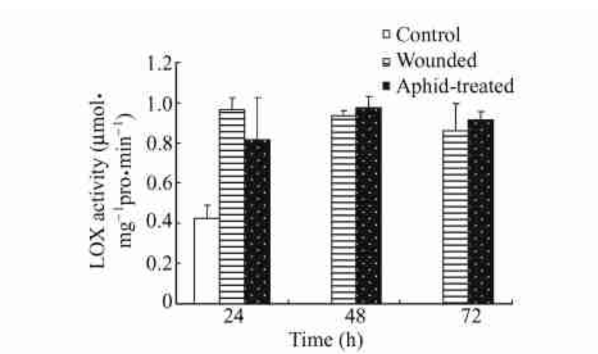

Fig. 2. The LOX activity in cotton leaves after aphid infestation and mechanical wounding.

Table 2 shows LOX activity in cotton seedlings with different treatments. The results indicated that after 24 hours of the treatments, either by artificial damage or by aphid infestation, the specific activity of LOX in cotton leaves increased by 17%—28% compared to that in control leaves. The activity of LOX enhanced further after 48 hours by 34%—47% and reached its maximum value. Later on, LOX activity in artificially damaged leaves and the leaves exposed to wound-induced volatiles declined to the control level, whereas the LOX activities in aphid infested leaves and the leaves exposed to aphid infestation-induced volatiles were enhanced by 21% and 24%, respectively, compared to the control.

3 Discussion

The biochemical mechanism of plant defense induced by pathogen has been extensively studied in plant-pathogen interactions in cotton, whereas the herbivore-induced biochemical changes in cotton is a relatively new research area[29, 30]. PAL catalyses the first step of reaction in the phenylpropanoid pathway and is thought to be the key reaction in the control of lignin, flavonoids and salicylic acid biosynthesis. PAL gene expression has been associated with abiotic stress, wounding and infection[31]. Studies of Mercedes

Table 2. LOX activity in cotton seedlings with different treatments

Treatment	Specific activity (μmol·mg^{-1}pro·min^{-1})[a]		
	24 h	48 h	72 h
Control	0.7435±0.03 (1.00)	0.5518±0.03 (1.00)	0.5163±0.04 (1.00)
Wounded	0.8814±0.02 (1.19) *	0.7851±0.06 (1.42) *	0.5474±0.05 (1.06)
Aphid-treated	0.8715±0.09 (1.17) *	0.8114±0.07 (1.47) **	0.6239±0.12 (1.21)
Wound-receiver	0.9519±0.01 (1.28) **	0.7540±0.08 (1.37) *	0.5569±0.02 (1.08)
Aphid-receiver	0.8794±0.03 (1.18) *	0.7383±0.09 (1.34) *	0.6386±0.03 (1.24)

a) All the values are means±standard deviation of three experiments; * $p < 0.05$, ** $p < 0.01$, compared with control.

et al. revealed that after infested with aphids there were much higher PAL activity and concentration of salicylic acid in the infested barley cultivar LM-109 than those in uninfested ones[23]. Our results of the elevated PAL in aphid infestation-treated cotton seedlings are consistent with conclusions of Mercedes.

PAL activity increased not only in aphid infested and artificially damaged cotton seedlings but also in seedlings growing nearby. Baldwin et al. reported that potted poplar ramets (*Populus euroamericana*) showed increased concentrations and rates of synthesis of phenolic compounds within 52 hours of having 7 percent of their leaf area removed by tearing, as did undamaged plants sharing the same enclosure. They also found that damaged sugar maple (*Acer saccharum* Marsh) seedlings and nearby undamaged maples had increased levels of phenolics and hydrolysable and condensed tannin within 36 hours[32]. Studies of Karban suggested that the mechanical clipping of sagebrush increased the polyphenyl oxidase activity in native tobacco plants growing downwind of the clipped sage[19]. Using cotton seedlings as materials, we proved that PAL activity increased in both wounded cotton seedlings and the seedlings growing nearby. All of these results imply that an increased PAL activity can lead to biosynthesis of phenylpropanoid and related secondary metabolites, which might be a defense strategy of plants, and signaling volatiles emitted from plants could be involved in plant-plant communication.

There are both similarity and difference in effect of herbivory and mechanical wounding on plant defense system. For example, the fatty acid-amino acid conjugate (FACs), volicitin, isolated from beet armyworm *Spodoptera exigua* oral secretions can induce volatiles emission from plants[33]. The results in our study demonstrated that both mechanical damage-induced and aphid infestation-induced plant volatiles could evoke plant-plant communication, although the duration and extent of the communication are different. The PAL activity in cotton seedlings exposed to damage-induced and aphid infestation-induced volatiles for 24 hours did not differ from that of the control, which suggests that the defense reaction of the volatile receiver lags behind the volatile emitter, therefore a high concentration or accumulation of plant volatiles is required to elicit defense responses of the healthy plants growing nearby.

Studies of Arimura revealed that LOX activity obviously increased in both spider mite-infested detached lima bean leaves and leaves exposed to the spider mite-induced volatiles for 1 or 3 days, whereas the detached leaves exposed to wound-induced volatiles showed no significant increase of the LOX activity compared to the control. However, the results in our study are different. The LOX activity increased in both intact cotton seedlings exposed to aphid infestation-induced volatiles and seedlings exposed to wound-induced volatiles, but at a lower level. The discrepancy between the results of the two studies might be due to the fact that we used different plant materials, different herbivore and different infestation.

Many indoor and outdoor studies have verified that plants can communicate through airborne transferred signals. In our study, it is also proved that volatiles induced by mechanical damage and aphid infestation are involved in cotton plants communication, but the component, concentration of the volatiles and the potent signal responsible for plant communication need to be further analyzed and identified.

References

1 Karban R. and Baldwin I. T. Induced Responses to Herbivory. Chicago: The University of Chicago Press, 1997.

2 Koiwa H. and Bressan R. A. Regulation of protease inhibitors and defense. Trends Plant Sci., 1997, 2: 379—384.

3 Baldwin I. T. Short-term damage-induced increases in tobacco alkaloids protect plants. Oecologia, 1988, 75: 367—370.

4 Kahl J., Siemens D. H., Aerts R. J. et al. Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adopted herbivore. Planta, 2000, 210: 336—342.

5 Dick M., van Beek T. A., Posthumus M. A. et al. Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. J. Chem. Ecol., 1990, 16: 381—396.
6 Dick M., Gols R., Ludeking D. et al. Jasmonic acid and herbivory differentially induce carnivore attracting plant volatiles in lima bean plant. J. Chem. Ecol., 1999, 5: 1907—1922.
7 Turlings T. C. J., Tumlinson J. H. and Lewis W. J. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. Science, 1990, 250: 1251—1253.
8 Takabayashi J. and Dicke M. Plant-carnivore mutualism through herbivore-induced carnivore attractants. Trends Plant Sci., 1996, 1: 109—113.
9 De Moraes C. M., Lewis W. J., Pare P. W. et al. Herbivore-infested plants selectively attract parasitoids. Nature, 1998, 393: 570—573.
10 Dick M., Sabelis M. W., Takabayashi J. et al. Plant strategies of manipulating predator-prey interaction through allelochemicals: Prospects for application in pest control. J. Chem. Ecol., 1990, 16: 3091—3118.
11 Bruin J., Dicke M. and Sabelis M. W. Plant are better protected against spider-mites after exposure to volatiles from infested conspecifics. Experientia, 1992, 48: 525—529.
12 Arimura G., Ozawa R., Shimoda T. et al. Herbivore-induced volatiles elicit defense genes in lima bean leaves. Nature, 2000, 406: 512—515.
13 Arimura G., Ozawa R., Shimoda T. et al. Herbivore-induced volatiles induce the emission of ethylene in neighboring lima bean plants. Plant J., 2002, 29: 87—98.
14 Agrawa A. A. Communication between plants: this time it's real. Trends Ecol. Evol., 2000, 15: 446.
15 Farmer E. E. Surface-to-air signals. Nature, 2001, 411: 854—856.
16 Dick M. and Bruin J. Chemical information transfer between plants: back to the future. Biochem. Syst. Ecol., 2001, 29: 981—994.
17 Preston C. A., Laue G. and Baldwin I. T. Methyl jasmonate blowing in the wind, but can it act as a plant-plant airborn signal? Biochem. Syst. Ecol., 2001, 29: 1007—1023.
18 Yang D., Li Q., Hu Z. H. et al. Content changes of abscisic acid and jasmonic acid in damaged and neighboring healthy ashleaf maple seedlings. Journal of Beijing Forestry University (in Chinese), 2003, 25: 35—38.
19 Karban R., Baldwin I. T., Baxter K. J. et al. Communication between plants: induced resistance in wild tobacco plants following clipping of neighboring sagebrush. Oecologia, 2000, 125: 66—71.
20 Karban R. and Baxter K. J. Induced resistance in wild tobacco with clipped sagebrush neighbor: the role of herbivore behavior. J. Insect. Behav., 2001, 14: 147—156.
21 Karban R. Communication between sagebrush and wild tobacco in the field. Biochem. Syst. Ecol., 2001, 29: 995—1005.
22 Dubery I. A. and Smit F. Phenylalanine ammonia-lyase from cotton (*Gossypiunm hirsutum*) hypocotyls, properties of enzyme indued by a *Verticillium dahliae* phytotoxin. Biochim. Biophysi. Acta, 1994, 1207: 24—30.
23 Mercedes E. C., Cylvia V. C. and Victor H. A. Relationship between salicylic acid content, phenylalanine ammonia-lyase (PAL) activity, and resistance of barley to aphid infestation. J. Agric. Food Chem., 2003, 51: 2227—2231.
24 Arimura G., Ozawa R., Horiuchi J. et al. Plant-plant interactions mediated by volatiles emitted from plant infested by spider mites. Biochem. Syst. Ecol., 2001, 29: 1049—1061.
25 Baldwin I. T., Kessler A. and Halitschke R. Volatile signaling in plant-plant-herbivore interactions: what is real? Current Opinion in Plant Biology, 2002, 5: 1—4.
26 Koukol J. and Conn E. E. The metabolism of aromatic compounds in higher plant. J. Biol. Chem., 1961, 236: 2692.
27 Axelrod B., Cheesbrough T. M. and Leakso S. Lipoxygenase from soybeans. Methods in Enzymology, 1981, 7: 443—451.
28 Bradford M. M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 1976, 72: 248—254.
29 Mussell H. M. Endopolygalacturonase: evidence for involvement in *Verticillium* wilt of cotton. Phytopathology, 1973, 63: 62—70.
30 Venere R. J. Role of peroxidase in cotton resistant to blight. Plant Sci. Letts., 1980, 20: 47—56.
31 Leyva A., Jarillo J. A., Salinas J. et al. Low-temperature induces the accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs of *Arabidopsis* in a light-dependent manner. Plant Physiol., 1995, 108: 39—46.
32 Baldwin I. T. and Schultz J. C. Rapid changes in tree leaf chemistry induced by damage: evidence for communication between plants. Science, 1983, 221: 277—279.
33 Turlings T. C. J., Alborn H. T., Loughrin G. H. et al. Volicitin, an elicitor of maize volatiles in oral secretion of *Spodoptera exigua*: its isolation and bioactivity. J. Chem. Ecol., 2000, 26: 189—202.