Role of Salicylic Acid in Tomato Defense against Cotton Bollworm, *Helicoverpa armigera* Hubner

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We investigated the role of the salicylic acid (SA) signaling pathway in defense responses of tomato plants to the herbivore, cotton bollworm. After exposure to the cotton bollworm, tomato leaves rapidly accumulated a high level of SA. The transcription of *PR*1 and *BGL*2 genes, the marker genes of SA pathway, was up-regulated. An enhanced endogenous SA level was accompanied by an increase in the endogenous H_2O_2 level as compared with controls. Spraying tomato plants with a solution containing either SA or methyl salicylic acid (Me-SA), the H_2O_2 level dramatically increased. These data proved that the SA pathway was involved in the tomato plant defense responses to the herbivore.

Key words: Lycopersicon esculentum, Salicylic Acid Pathway, Plant Defense Response

Introduction

Plants are challenged by a variety of abiotic and biotic stresses. The differential activation of distinct sets of genes or gene products in response to these challenges is referred to as specificity. Several signaling pathways, including jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and probably hydrogen peroxide (H_2O_2) orchestrate the induction of defenses (Reymond and Farmer, 1998).

A large proportion of multicellular eukaryotes feed on plants, and a particularly common source of damage to plants are insect herbivores. Plants clearly respond differently to tissue damage caused by insects and pathogens, and the mechanisms responsible for this differential recognition and response may involve crosstalk among at least three different signal transduction pathways: JA, ethylene, and SA (Dong, 1998).

In plants, the JA pathway is generally considered to be required for defenses against necrotrophic pathogens and chewing insects, while the SA-related pathway is required for specific, resistance (R) gene-mediated defenses against both biotrophic and necrotrophic pathogens (Felton *et al.*, 1999). Numerous studies argue that SA is a vital component of the plant signal transduction pathways causing disease and pathogen resistance (Maleck and Dietrich, 1999).

Recently, accumulating reports indicate that the SA pathway is involved in a wide range of plant defense responses. For example, plant defense in response to microbial attack is regulated through a complex network of signaling pathways that involve three signaling molecules: salicylic acid, jasmonic acid and ethylene (Kunkel and Brooks, 2002). SA is a key regulator of pathogen-induced systemic acquired resistance (SAR), whereas JA and ET are required for rhizobacteria-mediated induced systemic resistance (ISR) (Ton *et al.*, 2002). Branch *et al.* (2004) found that SA is an important component of the signaling that leads to root-knot nematode resistance and the associated hypersensitive response.

The SA involved plant defense responses are characterized as species specific. Even in two phylogenetic closely related plant species such as tomato and tobacco, the SA-dependent defense pathway does not trigger the same defense responses. It also means that the outcome of a BTH (benzothiodiazole) treatment cannot be predicted and has to be tested for each plant-pathogen combination.

Although both SA and JA are involved in plant defenses, the antagonistic interactions between the SA and JA signaling mechanisms modulate defense gene expression and the activation of RCY1-

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conferred gene-for-gene resistance to CMV(Y) (Takahashi *et al.*, 2004). Plant defenses against pathogens and insects are regulated differentially by cross-communicating signal transduction pathways in which salicylic acid and jasmonic acid play key roles (Spoel *et al.*, 2003). The SA and JA signaling pathways are mutually antagonistic (Kunkel and Brooks, 2002).

A few available reports mentioned the function of SA in defense responses to insects (Stotz *et al.*, 2002; Martinez *et al.*, 2003). The aphid feeding on potato involves both SA and JA/ethylene plant defense signaling pathways (Martinez *et al.*, 2003). However, the aphid is a kind of sucking insects, its feeding behavior is different from chewing insects. In this paper, our objective was to confirm whether the SA pathway is involved in tomato plant defense against feeding by cotton bollworm larvae. We first measured the endogenous SA and H_2O_2 levels of tomato seedlings injured by cotton bollworm larvae, and then documented gene expression patterns in the downstream and upstream SA pathway.

Materials and Methods

Plant growth

Tomato plant (*Lycopersicon esculentum* cv. *Castlemart*) seeds were germinated in soil and seedlings grew for 2 weeks in a growth chamber in a greenhouse under a 16-h photoperiod on a 28/23 °C day/night cycle. Experiments were initiated when plants were in the late rosette stage of growth.

Insect rearing

Helicoverpa armigera (Lepidoptera: Noctuidae) larvae (cotton bollworm, CBW) were collected from a cotton field in Anyang, Henan Province in July 2002 and reared on artificial diets at 24 ± 1 °C and 80 ± 5% relative humidity with a 14 h/10 h light/dark regime. Fourth instar cotton bollworms were placed on the leaves of tomato seedlings until the experiment ended regardless of the amounts of feeding. Damaged tomato plants were harvested and analyzed at different times (t = 0, 3, 6, 12, 24 h). Three replicates for each sample were measured and three tomato seedlings were used for each replicate.

Exogenous application of SA and Me-SA to tomato plants

Plants were directly sprayed with 0.5 mM SA (Sigma) solution, and control plants were sprayed with water using the same method. Methyl salicylic acid (Me-SA; Sigma) was dissolved in pure dichloromethane (1 μg per 1 μl solution), and 10 μg of Me-SA were then impregnated into a piece of cotton wool. After evaporation of the solvent, each piece of cotton wool was enclosed in a glass container together with two tomato seedlings. A piece of cotton wool containing only dichloromethane was used as the control. A clean container was used for each experiment to exclude possible contamination. The samples were harvested at different time points (t = 0, 3, 6, 12, 24 h).

Extraction and GC analysis of SA

The aboveground tissues of seedlings were harvested at various times, immediately frozen in liquid nitrogen and stored at −70 °C and subsequently extracted according to the method reported by Nordin *et al.* (1991). Frozen seedlings (0.5 g) were ground to fine powder in liquid nitrogen and homogenized with 1 ml 80% cooled ethanol. The extract was centrifuged at 10,000 × *g* for 10 min. The supernatant was collected and processed immediately or quick-frozen at −70 °C until further analysis. Total SA was purified using the method described by Rasmussen *et al.* (1991). The purified SA was esterized to Me-SA, subjected to GC analysis, and quantified using pure Me-SA (Sigma) as an external standard. Esterification of SA was performed at 85 °C for 2 h with methanol, benzene and the oil of vitriol in a ratio of 100:50:3.

A gas chromatograph (Shimadzu GC-17A, Kyoto, Japan) fitted with a flame ionization detector (FID) and a split/splitless injector was employed to analyze the Me-SA on capillary columns (DB-Wax, 30 m × 0.25 mm). The oven temperature was controlled as follows: 100 °C for 2 min, programmed to 230 °C at 10 °C/min. The injector and detector temperatures were 250 °C. The carrier gas was nitrogen. The concentration was determined using a linear range of calibration standards consisting of 0 to 1.3 μg/50 μl of Me-SA (Sigma-Aldrich, St. Louis). In turn, the SA concentration was expressed in micromole per gram fresh weight.

Table I. Primers used in the RT-PCR.

Gene	Genbank Access No.	Primers
*PR*1	AJ011520	F:5′-ATC TCA TTG TTA CTC ACT TGT C 3′ R:5′-AAC GAG CCC GAC CA 3′
*BGL*2	M80680	F:5′-CAC CAA CAT TCA CAT AAC AGA GGC 3′ R:5′-CAG GGC TGA TTT CAT TAC CAA C 3′
PAL	M833314	F:5′-TTC AAG GCT ACT CTG GC 3′ R:5′-CAA GCC ATT GTG GAG AT 3′

Measurement of H_2O_2

H_2O_2 was determined by measuring the absorbance of the Fe^{3+}-xylenol orange complex at 560 nm following a modification of the method described by Jiang (1990). Absorbance values were calibrated to a standard curve using known concentrations of H_2O_2 (Sigma).

RNA extraction and RT-PCR analysis

At 0.5–24 h after injured by cotton bollworms, tomato seedlings, leaves and stems were excised, immersed in liquid nitrogen, and stored at −70 °C until use. Total RNA was extracted using the cooled phenol method (spring laboratory manufactures). 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 analyzing for specific mRNAs.

The reverse transcription reaction was carried out as recommended by the manufacturer of the kit (Takara R019A, Osaka, Japan). The products of reverse transcription were used as templates for PCR analysis. After the initial denaturing step at 94 °C for 5 min, different cycles were performed. Each cycle consisted of a denaturing step at 94 °C for 1 min, annealing at corresponding temperature for 45 s, and elongation at 72 °C for 1 min. A final extension step was performed at 72 °C for 5 min. Cycle number varied depending on the primers used and was within the linear range. The primers used in the PCR were listed in Table I.

Statistical analysis

Statistical analysis was performed by the treatment of one way ANOVA with Posthoc Tukey HSD tests ($p < 0.05$). The data shown were mean ± SE. Levels of significance were represented by $p < 0.05$.

Results

Accumulation of SA in tomato seedlings after injured by cotton bollworm

Cotton bollworms were allowed to feed on tomato seedlings at the four-leaf stage and samples were collected at different time points as described in Materials and Methods. The production of SA was measured. As shown in Fig. 1, the kinetic was biphasic: phase I peaked at 0.5 h and phase II at 3 and 6 h. The concentration of SA in control (undamaged) plants was relatively lower with a significant difference ($p < 0.05$), whereas a peak of 0.68 ± 0.02 μM/g FW at 0.5 h was observed in caterpillar-damaged plants. The variation of the SA amount within 24 h indicated that the SA pathway responded strongly to cotton bollworm feeding.

Expression of defense related genes induced by cotton bollworm feeding in tomato seedlings

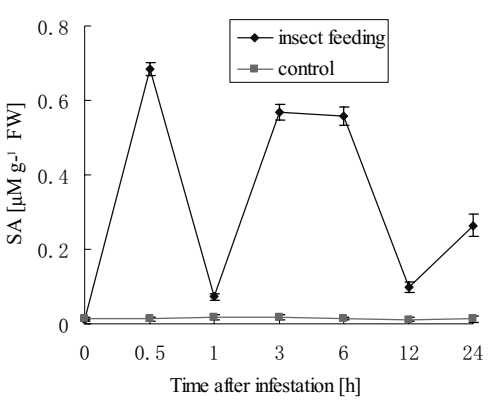

Fig. 1. Kinetics of salicylic acid (SA) accumulation in wounded leaf tissue of tomato seedlings. SA was extracted from the tomato seedling damaged by cotton bollworms according to "Materials and Methods". Bars represent the means ± SE of three independent experiments. FW, fresh weight.

Undamaged tomato seedlings showed very low levels of *PR*1, *BGL*2 and *PAL* mRNA. However, in seedlings injured by cotton bollworms, the transcription of *PR*1, *BGL*2 and *PAL* was strongly induced 1 h after feeding and kept increasing for 3–6 h. After 6 h, transcription levels began to decrease. *PR*1 and *BGL*2 were the common marker genes for the SA pathway, which function downstream in the SA pathway (Yalpani *et al.*, 1994). PAL is an upstream component in the SA biosynthetic pathway, which has been shown to play an important role in plant resistance (Mauch-Mani, 1996). *PAL* is also a key enzyme involved in the biosynthesis of the signal molecule SA (Mauch-Mani and Slusarenko, 1996). The expression of these components of SA pathway suggested that the SA pathway involved in defense responses of tomato plants functions in response to the cotton bollworm (Fig. 2A, B, and C).

H_2O_2 production after cotton bollworm feeding in tomato seedlings

The sample preparation was the same as SA treatment. The production of H_2O_2 was measured. When tomato plants were exposed to cotton bollworm for 0.5 h, a rapid increase in the amount of H_2O_2 in leaves was observed (Fig. 3A), with another peak appearing after 3 h, and this pattern was similar to the SA kinetic pattern. Thus, H_2O_2 and SA levels in plant tissues might have a close positional relationship in the SA pathway.

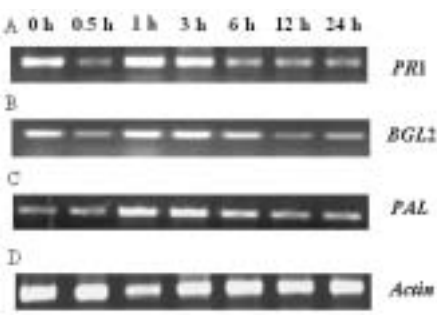

Fig. 2. Time course of expression of *PR*1, *BGL*2, and *PAL* genes in response to cotton bollworm feeding. The samples were harvested 0, 0.5, 1, 3, 6, 12 and 24 h after feeding. The expression was analyzed by RT-PCR using total RNA isolated from leaves and specific primers for *PR*1, *BGL*2, and *PAL* gene amplifications. As a control, actin mRNA was also amplified.

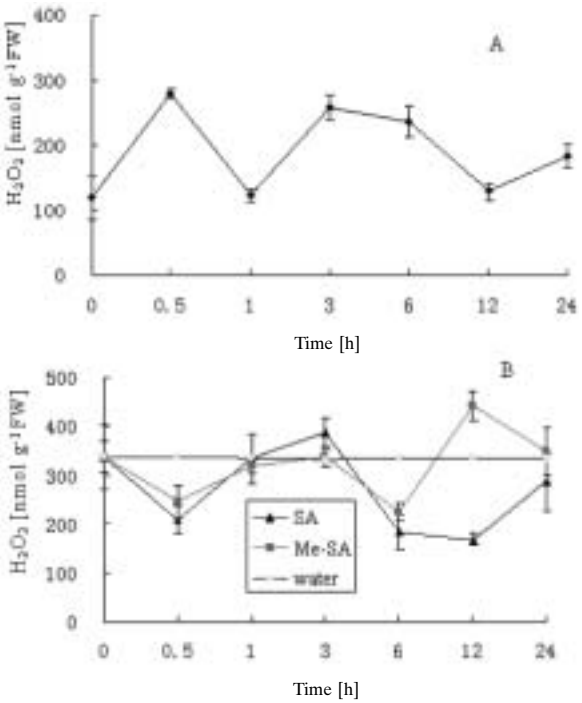

Fig. 3. (A) Cotton bollworm-induced changes of H_2O_2 concentration in tomato seedlings. Cotton bollworms were inoculated to tomato seedlings. When cotton bollworms began to feed the tomato leaves, samples were collected at indicated times. Data are means ± SE, n = 3. (B) Comparison of the SA and Me-SA induced H_2O_2 dynamic pattern in tomato seedlings. The triangular symbol represents the SA-treated sample, the square symbol denotes Me-SA-treated samples, and the circle symbol denotes the water-treated samples. Data are represented by means ± SE, n = 3.

Accumulation of H_2O_2 induced by exogenous SA and Me-SA

Because SA and Me-SA are considered to be signal molecules that induce a variety of plant genes involved in wound or defense responses (Senaratna *et al.*, 2000), we examined whether these chemicals also affected the H_2O_2 production in intact tomato plants. As shown in Fig. 3B, the concentration of H_2O_2 in control plants remained stable over a 24 h period, whereas that of treated with SA and Me-SA showed a fluctuated trend, with the highest value at 3 and 12 h post-treatment. At 24 h after treatment, the H_2O_2 content in tomato leaves returned to the control levels.

As compared to SA, Me-SA had a similar effect on H_2O_2 production for the first 12 h. However, it differed at 12 h, and returned to a similar level at 24 h post-treatment. The reason for this change is

unknown, but it is possibly due to the different chemical structures.

Dose-dependent and time-dependent effect of SA on the SA-dependent genes expression

Spraying tomato seedlings with SA solutions between 0.5 and 10 mM the transcription level of several downstream components increased dramatically in the SA pathway (Fig. 4). The effects of SA were concentration-dependent, and there was a positive correlation between strong gene expression and high concentration of SA up to a concentration of 9 mM. However, a SA concentration > 9 mM inhibited the genes expression.

Fig. 4 shows that *PR*1 and *BGL*2 genes expression in tomato leaves was positively correlated with the concentration of exogenous SA. With respect to the increasing expression of *BGL*2, a significant difference was found for the treatment with 0.5 mM, 1.0 mM, 5.0 mM of exogenous SA at different time points whereas the expression of *PR*1 gene did not seem to correlate with the SA level. We also measured the H_2O_2 levels, and the amplitude of the H_2O_2 increase was also similar to that reported by Chen and Silva (1993). The results shown in Fig. 4 indicated that 0.5 ~ 9 mM SA significantly induced the SA-dependent genes expression, suggesting that SA plays an active role in the defense response to the herbivore.

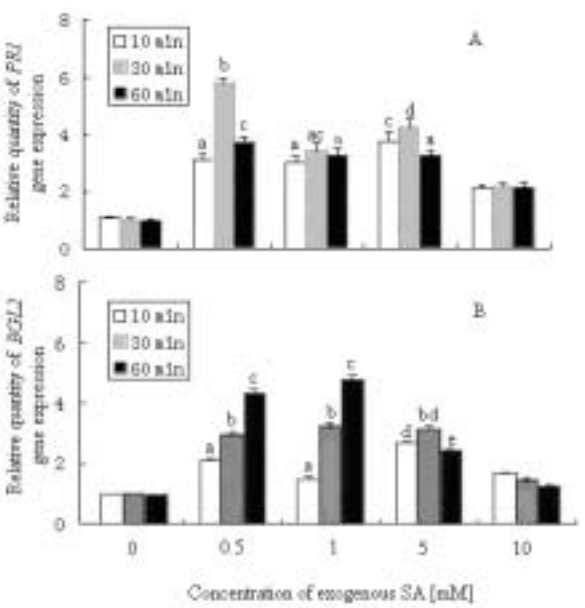

Fig. 4. Dose- and time-dependent effects of exogenous SA on the expression of salicylic acid pathway related genes. The data shown are mean values ± SE. Different letters represent significantly difference at the level of $p < 0.05$.
(A) Effects of exogenous SA on the *PR*1 gene expression. Samples were harvested treated with the indicated concentration at the indicated time, respectively. Each group represents a certain concentration and three different time points (10 min, 30 min, 60 min). The relative quantity was accounted as the relative intensity of gene expression compared with that of control. (B) Effects of exogenous SA on the *BGL*2 gene expression. All the treatments were the same as that of *PR*1 genes.

Discussion

Previous evidence has confirmed that SA plays a key role in the signal transduction pathway leading to systemic acquired resistance (SAR) (Enyedi *et al.*, 1992). Nonetheless, these experiments did not address clearly whether SA is involved in defense response to herbivore attacks. Our results demonstrate that the attack by cotton bollworm would induce the defense responses of tomato plants, including the accumulation of H_2O_2, the production of SA and expression of genes active in several steps of the SA signal pathway.

An oxidative burst is the first evidence of the SA pathway involving the tomato response to cotton bollworm feeding (Levine *et al.*, 1994; Lamb and Dixon, 1997). Previous studies have demonstrated that plants infected by pathogens produce an oxidative burst and induce the SA pathway related response thereafter. It is reasonable to hypothesize that the SA pathway is also involved in defense against insect feeding, because H_2O_2 can damage the digestive system of insects and suppress their development. Feeding on previously wounded foliage causes oxidative damage in the insect mid-gut and disrupts the absorption and transport systems of herbivore insects (Felton and Summers, 1993; Felton *et al.*, 1994). Our results demonstrate that H_2O_2 levels rise sharply in tomato plants in response to a cotton bollworm attack (Fig. 3A), suggesting that the SA pathway is involved in defense against insect injury.

Benzoic acid, the direct substrate for SA biosynthesis, is derived from phenylpropanoid pathways in plants with *PAL* as key regulatory enzyme (Dixon, 2001). Fluctuation in the quantity of specified metabolite may be involved in the pathway flux controlled by the activity of its rate-control enzyme and substrate available (Kolosova *et al,*, 2001). The sudden drop in the SA level at the time

of 1 h may be caused by rapid depletion of its substrate in pool. Corresponding to the increasing expression of *PAL* as shown in Fig. 2C, it is suggested that with a more supply of substrate, benzoic acid will compensate for a later increase of SA level.

We showed here that *PR*1 and *BGL2* genes, active in the SA pathway, were up-regulated after damaged by the cotton bollworm. Aphid feeding was also reported to induce the activity in the *PR*1 and *BGL2* genes in *Arabidopsis thaliana* seedlings (Moran and Thompson, 2001). In the tomato, the expression of *PR*1 gene is induced by pathogen infection. In our study, cotton bollworm feeding up-regulated the expression of *PR*1 and *BGL2* genes in tomato. As showed in Fig. 2, *PR*1 genes associated with the SA-dependent response pathway were induced after feeding by cotton bollworm larvae. In contrast with pathogen or elicitor treatment, the induction was not apparent for either gene when leaves were harvested 30 min after cotton bollworm feeding.

The direct evidence comes from the accumulation of SA after the damage by the cotton bollworm. At 0.5 h after injuring, the first peak of SA appeared in the tested plants. However, the SA amount in the uninjured plants was kept at a low level. It indicates that injuring was closely correlated to the insect feeding.

A volatile analogue of SA, Me-SA, is a common component of the blend emitted by herbivore-infested plants (Van Poecke *et al.*, 2001). As a single compound it can attract predatory mites (Dicke *et al.*, 1993). In this study we have attempted to assess the role of Me-SA in herbivore attack by application of chemically synthesized SA and Me-SA to tomato seedlings. Exogenous applications of SA, either by direct injection or by spraying, have been reported to cause a multitude of effects on the morphology and physiology of plants (Pancheva *et al.*, 1996). In our experiments, SA and Me-SA exogenously applied to tomato seedlings increased the H_2O_2 level, suggesting that SA and Me-SA activated the production of H_2O_2.

In this study, we assessed whether the SA pathway was involved in defense responses of tomato seedlings grazed by cotton bollworm larvae. Although we did not measure the activity in the JA pathway; the role of the JA pathway in direct plant defenses against herbivores has been well established (Li *et al.*, 2002). The signaling pathways that allow plants to mount defenses against insect herbivores are known to be complex. Cooperative interactions between signaling response pathways may be regarded as means developed by plant species to increase the number of distinct gene repertoires that can be controlled by a limited set of signaling molecules and hence to increase behavioral plasticity.

In conclusion, the major evidence for a role of the SA pathway in herbivore resistance in tomato is summarized as follows: (i) SA-dependent genes such as *PR*1 and *BGL2* are up-regulated after herbivore attack; (ii) an increase in endogenous SA is correlated with the expression of defense-related genes and H_2O_2 accumulation; (iii) application of exogenous SA and Me-SA induce the same set of genes that are activated systemically after herbivore attack.

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