




Hydrogen peroxide regulated salicylic acid- and jasmonic acid-dependent systemic defenses in tomato seedlings

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Abstract

To investigate the regulation of hydrogen peroxide (H_2O_2) in SA- and JA-dependent systemic defense pathways in tomato seedlings damaged by cotton bollworm. The lower two leaves of four-leaf tomato plant seedlings were treated with cotton bollworms. The upper leaves were then removed at various time points and assessed for systemic changes in H_2O_2 levels, enzyme activity and defense-related genes expression. Wild-type seedlings, *def-5* mutant seedlings (deficient in JA accumulation) and transgenic *nahG* seedlings (deficient in SA content) were constructed at four-leaf stage. Wild-type plants were further treated with dimethylthiourea (DMTU) and 2,5-dihydroxycinnamic acid methyl ester (DHC). Bollworm feeding in the lower leaves induced rapid systemic accumulation of H_2O_2 in the upper leaves. H_2O_2 accumulation further increased activities for SA- and JA-dependent enzymes including proteinase inhibitors (PIs), pathogenesis-related gene 1 (PR-1), cathepsin D inhibitor (CDI), β -1,3-glucanase (BGL) and polyphenol oxidase (PPO). Furthermore, H_2O_2 accumulation enhanced mRNA expression of BGL-2, PR-1, PI, CDI and PPO genes. Suppression of H_2O_2 accumulation using H_2O_2 scavengers substantially diminished these effects. mRNA expression of PR-1 was not induced in transgenic *nahG* plants. SA- and JA-dependent signaling pathways are involved in the tomato systemic defense responses to herbivores, and that H_2O_2 generation is required for both systemic pathways.

Keywords: *Heliothis armigera*; hydrogen peroxide; jasmonic acid; salicylic acid; tomato seedling; defense response.

Practical Application: mRNA expression of PR-1 was not induced in transgenic *nahG* plants. SA- and JA-dependent signaling pathways are involved in the tomato systemic defense responses to herbivores, and that H_2O_2 generation is required for both systemic pathways.

1 Introduction

Plants can utilize both constitutive and inducible defense mechanisms to protect themselves against attack from pathogens and herbivores (Schulze et al., 2019; Mason et al., 2017; Mayrose et al., 2004; Chen, 2008; Rasmann & Agrawal, 2009; Agrawal et al., 2015). Defense responses induced by insects include a local hypersensitivity response and the production of signaling molecules, which then systemically activate defense-related genes and enzyme pathways generating biomolecules such as toxins, repellents and natural enemy attractants (Smith et al., 2009; Fan et al., 2012). These molecules can harm insects via physiological disruption, inhibition of digestion and assimilation, repel insects or attract their natural enemies (Erb & Reymond, 2019; War et al., 2012; Fürstenberg-Hägg et al., 2013).

Several signaling molecules, including reactive oxygen species (ROS), salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), have been implicated in these defense responses and work together as a network (Qi et al., 2018; Waterman et al., 2019; Grant & Lamb, 2006). JA is synthesized from linolenic acid via the octadecanoid pathway, and is considered to be a key regulator for stress-induced gene expression in all plants (Browse, 2009; Koo & Howe, 2009). Both mechanical wounding and insect feeding can induce accumulation of JA and increase the activities of proteinase inhibitors (PIs), cathepsin D inhibitor (CDI) and polyphenol oxidase (PPO) in tomato seedlings (Orozco-Cárdenas et al., 2001; Tholl et al., 2011). Plants defective in JA synthesis are

highly susceptible to herbivores (Stotz et al., 2002; Ellis et al., 2002). Similarly, SA-dependent signaling is essential for local hypersensitivity responses and resistance against many plant pathogens (Shine et al., 2019). SA mediates the transcription of β -1,3-glucanase gene 2 (BGL-2) and pathogenesis-related gene 1 (PR-1) in tobacco seedlings following pathogenic infection. SA treatment also enhances herbivore defense in wheat after aphid feeding (Mohase & Van der Westhuizen, 2002).

Among ROS produced in plants, hydrogen peroxide (H_2O_2) is particularly toxic to pathogens, including insects (Zhu-Salzman et al., 2005). H_2O_2 is involved in various signal transduction pathways and serves as a signaling molecule, activating defense responses in plants (Sharma et al., 2019; Zurbruggen et al., 2010). JA can induce accumulation of H_2O_2 and activation of the octadecanoid pathway in wounded tomato seedlings (Orozco-Cárdenas et al., 2001). In aphid-infested barley, H_2O_2 accumulates rapidly and is involved in ET production, suggesting an important role of H_2O_2 in defense responses (Argandoña et al., 2001). SA can specifically inhibit the activity of catalase *in vitro* and cause an increase in H_2O_2 concentrations *in vivo* (Chen et al., 1993). H_2O_2 itself induces accumulation of free benzoic acid and SA in tobacco leaves (Qi et al., 2018). SA can suppress the octadecanoid pathway and accumulation of PI-I in JA-treated tomato seedlings (Spoel et al., 2003).

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To date, most of the research in this area has focused on the roles of H₂O₂, SA and JA in plant defense responses to local pathogen infection or mechanical wounding; however, the molecular mechanism involved in H₂O₂ regulation of plant systemic defense responses against infection is not clear. In the present study, we used the cotton bollworm-treated tomato seedlings as a model to determine the contribution of H₂O₂, JA and SA signaling to systemic defense responses.

2 Materials and methods

2.1 Tomato seedlings

Tomato plants (*Lycopersicon esculentum* cv. Castlemart) were grown in a growth chamber with a photoperiod of 18 h light [300 µE/m²/sec] at 28 °C, and 6 h dark at 23 °C. Mutant *def-5* plants and transgenic plants expressing *nahG* were grown under the same conditions. Seedlings at the four-leaf stage (containing two upper leaves and two lower leaves) were used in all experiments.

2.2 Cotton bollworm feeding

Cotton bollworms (*Heliothis armigera*; Lepidoptera: Noctuidae) were collected in Jingzhou, Hubei Province, China in 2009 and thereafter mass-reared on artificial diets at 26 ± 1 °C and 80 ± 5% relative humidity with a 14-h light and 10-hr dark regime. During experiments, fourth-instar bollworms were placed on the lower (local) leaves of wild-type, mutant and transgenic seedlings. When 80% of the two lower leaves were consumed (-6 h), the cotton bollworms were removed. Upper (systemic) leaves were harvested at various times ranging from 3 to 37 h after initiation of feeding, flash-frozen in liquid nitrogen and stored at -70 °C until analysis.

2.3 H₂O₂ scavenging

To remove H₂O₂, seedlings were watered with the H₂O₂ scavengers DMTU (0.1 mM) or DHC (10 µM) daily for 3 days prior to treatment with caterpillars (Levine et al., 1994). Tomato leaves were also sprayed with the same solution throughout the experimental period.

2.4 Measurement of H₂O₂ concentration

Frozen leaves (0.2 g) were ground to a fine powder in liquid nitrogen and homogenized with 1 mL of 0.2 M perchloric acid at 4 °C. The extract was kept on ice for 5 min and then centrifuged at 10,000 × g for 10 min at 4 °C. Then the supernatant was collected and the concentration of H₂O₂ was measured as described by Chen et al. (1993).

2.5 Enzyme activity assays

Frozen upper leaves were homogenized in 10 mM Tris-HCl, pH 8.0 to obtain 1 g leaves (fresh weight, FW) per 5 mL buffer at 4 °C, and then centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant was collected for enzyme activity assays at room temperature on a DU800 spectrophotometer (Beckman

Coulter, Inc., CA, USA). All experiments were performed using 3 replicates.

BGL activity was assayed using laminarin (Sigma, St Louis, USA) as the substrate (Fink et al., 1988). Enzyme extract (100 µL) was incubated with 0.25 mL 10 mM Tris-HCl, pH 8.0, containing 2 mg/mL laminarin for 30 min at 37 °C. The absorbance at 550 nm was monitored over time with a DU800 spectrophotometer (Beckman Coulter, Inc., CA, USA). BGL activity was expressed as the change in absorbance per minute per mg FW (ΔOD/min/mg FW).

PPO activity was measured as the rate of formation of melanin-like material from phenolic substrates (Stout et al., 1998). Enzyme extract (10-30 µL) was added to 1 mL of 2.92 mM caffeic acid in 10 mM Tris-HCl, pH 8.0, and ΔOD was measured at 470 nm for 1 min. PPO activity was expressed as ΔOD/min/g FW.

For analysis of CDI activity, aspartic proteinase (pepsin) and its substrate biopodyl-labeled casein (BP-casein) were used as reported in a previous study (Christeller et al., 1998). Briefly, 100 µL assay buffer (30 mM Tris-acetic acid, pH 2.0, containing 10 ng pepsin) and 25 µL enzyme extract or 25 µL distilled water (as the control) were incubated in fluorometric tubes. The assay was initiated by addition of substrate (0.5 µg BP-casein), and the release of fluorescent peptide was detected by emission at 538 nm. CDI activity was defined as the percent inhibition of pepsin activity (percent reduction in fluorescence emission) relative to the control.

PIs of plant extracts were assayed using the fluorometric method through detecting the degradation of the peptide substrate benzoyl tyrosine ethyl ester (BTEE) by the proteinase chymotrypsin (Stout et al., 1998). Briefly, enzyme extract (25 µL) was added to 25 µL of 1 mM HCl containing 1.5 µg chymotrypsin. Following mixture, 2.9 mL of 0.5 mM BTEE in 12:13 (v/v) methanol/Tris-HCl buffer, pH 8.0, was added, and the absorbance was monitored at 256 nm for 10 min. For every sample run, a control was run without enzyme extract. PI activity was expressed as the percent inhibition of chymotrypsin relative to the control.

2.6 Measurement of gene expression

Total RNA was extracted from upper leaves and subjected to Northern blot analysis as described by Orozco-Cárdenas et al. (2001). Briefly, RNA (30 µg) was electrophoresed on a 1.2% [w/v] agarose gel with 0.67 M formaldehyde. After separation, the RNA was transferred using 10×saline sodium citrate (SSC; 1.5 mM NaCl, 150 mM trisodium citrate) onto Hybond-N membrane (Amersham Pharmacia Biotech, Amersham, UK). The membrane was then incubated at 62 °C for 2 h in prehybridization solution consisted of 10× Denhardt's solution (1% [w/v] polyvinylpyrrolidone, 1% [w/v] bovine serum albumin (BSA) fraction V, 1% [w/v] Ficoll 400), 3× SSC, 10% dextran sulfate, 7% sodium dodecyl sulfate and 167 µg/mL single-stranded salmon sperm DNA. Labeled gene probes were added to the prehybridization solution and blots were incubated for 18 to 36 h at 60 °C. Hybridized membranes were treated with the Dig DNA Labeling and Detection Kit (cat. no. 1 093 657, Roche Diagnostics GmbH, Mannheim, Germany). Equal mRNA loading was verified by

hybridization with a labeled Actin cDNA fragment. Digoxigenin-labeled probes were generated from tomato cDNAs encoding PR-1, BGL-2, PIs, CDI and PPO (Orozco-Cárdenas et al., 2001) by random priming using the Dig DNA Labeling and Detection Kit according to manufacturer's recommendations.

2.7 Statistical analysis

The statistical analysis was performed using commercially available Statistical Product and Service Solutions (SPSS) software version 17.0 (SPSS; SPSS Inc., Chicago, IL, USA). Data gained from this study was expressed as mean \pm standard deviation (SD). Differences between different groups were compared using *t* test. $P < 0.05$ considered indicative of a significant difference.

3 Results

3.1 Induction of systemic H_2O_2 accumulation in tomato seedling leaves following cotton bollworm feeding

Systemic levels of H_2O_2 were determined in the upper leaves of tomato seedlings at various time points (0 to 37 h) at the start of bollworm feeding (Figure 1). In control plants (undamaged), the concentration of H_2O_2 remained at low levels over the entire period. In contrast, compared to the control, the H_2O_2 concentration in bollworm-damaged plants exhibited a significant elevation, and reached a peak of $0.84 \mu\text{M/g FW}$ at 12 h ($P < 0.05$). The concentrations of H_2O_2 in the upper leaves of DMTU- and DHC-treated seedlings that were challenged with cotton bollworms were slightly higher than those in the control, but they were much lower than those in non-cotton bollworm-exposed seedlings ($P < 0.05$). Preliminary experiments showed that the scavenger DMTU or DHC alone did not affect insect feeding or normal seedling growth (data not shown).

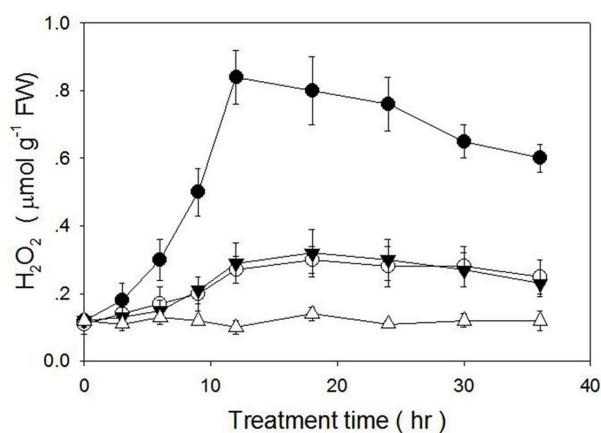


Figure 1. Induction of systemic H_2O_2 production in cotton bollworm feeding tomato seedlings. Samples were collected at the indicated time points, where time = 0 h corresponds to the onset of cotton bollworm feeding tomato leaves. Bollworms were removed when 80% of lower leaves were eaten (-6 h). For H_2O_2 scavenging, tomato seedlings were watered with DMTU (0.1 mM) or DHC (10 μM) daily for 3 days prior to cotton bollworm feeding and applied by spraying daily throughout the experimental period. ● = cotton bollworm feeding; ○ = cotton bollworm feeding plus DMTU (0.1 mM); ▼ = cotton bollworm feeding plus DHC (10 μM); Δ = control (no bollworm or scavenger treatment). Data presented as mean \pm SD, $n = 10$.

These findings suggest that insect feeding induced systemic accumulation of H_2O_2 , and that this accumulation can be effectively reversed by H_2O_2 scavengers.

3.2 Induction of defense-related enzyme activity following cotton bollworm feeding

In upper leaves of undamaged tomato seedlings, the activities of PI, CDI, BGL and PPO all remained low over a 120- to 150-h period (Figures 2 and 3). Moreover, the upper leaves of bollworm-damaged seedlings exhibited the elevated activities of all four enzymes within 50 h from the start of bollworm feeding, and these levels remained high for the duration of the experiment in comparison with the control (Figures 2 and 3; $P < 0.05$). All increases in enzyme activities were substantially inhibited in bollworm-damaged seedlings treated with DMTU or DHC, indicating that H_2O_2 played an important role in the induction of defense-related enzyme activity. In the present study, DMTU and DHC alone did not influence the activities of these enzymes (data not shown).

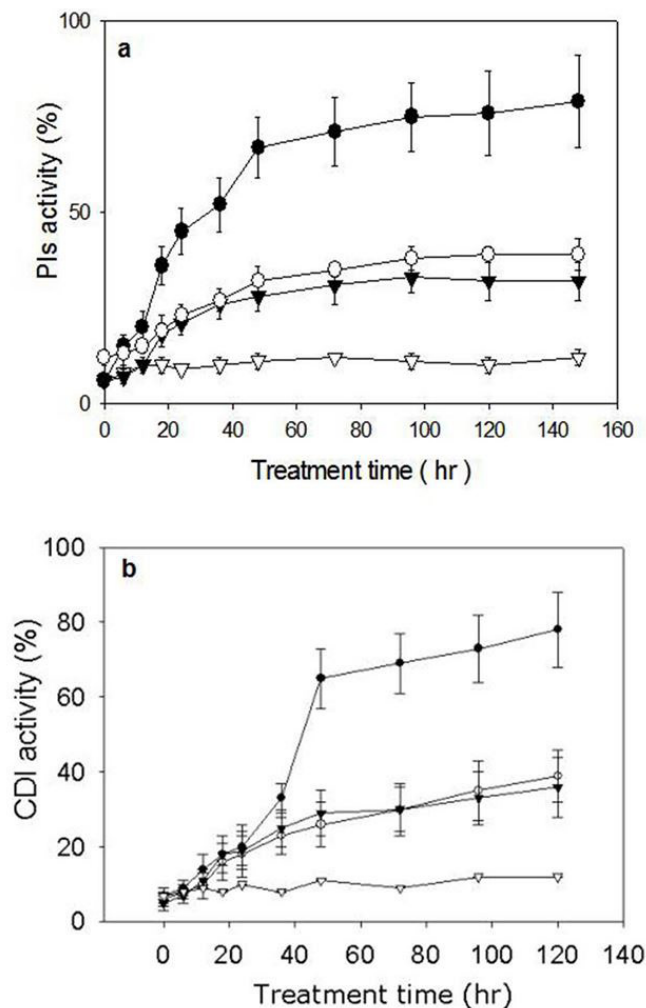


Figure 2. Induction of systemic increases in PI (a) and CDI (b) activity in cotton bollworm feeding tomato seedlings. ● = cotton bollworm feeding; ○ = cotton bollworm feeding plus DMTU (0.1 mM); ▼ = cotton bollworm feeding plus DHC (10 μM); Δ = control (no bollworm or scavenger treatment). Data presented as mean \pm SD, $n = 8$.

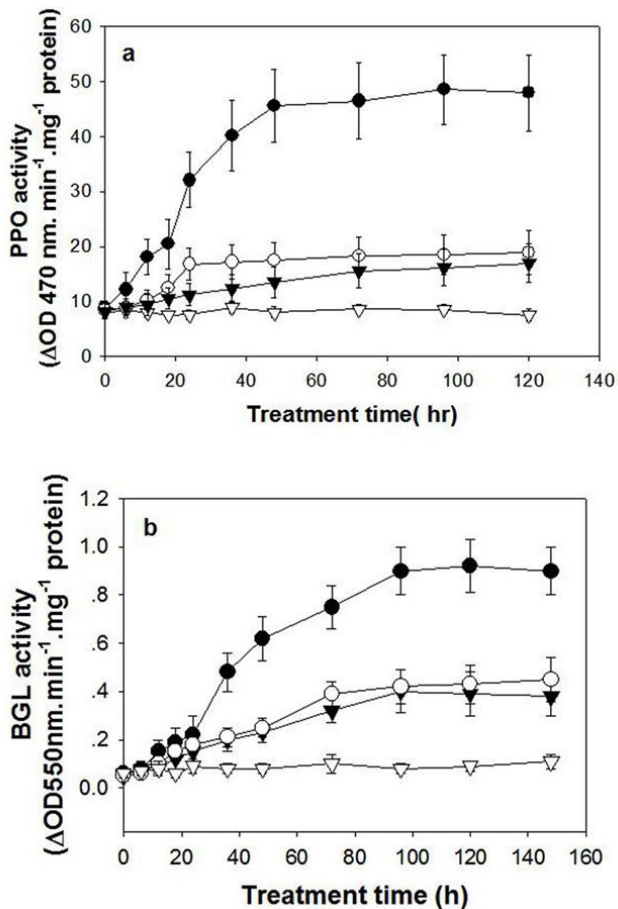


Figure 3. Induction of systemic increases in PPO and BGL activity in cotton bollworm feeding tomato seedlings. PPO (a) and BGL (b) activity were determined in cotton bollworm feeding, cotton bollworm feeding plus DMTU, cotton bollworm feeding plus DHC and the control groups. ● = cotton bollworm feeding; ○ = cotton bollworm feeding plus DMTU (0.1 mM); ▼ = cotton bollworm feeding plus DHC (10 μM); △ = control (no bollworm or scavenger treatment). Data presented as mean ± SD, n = 8.

3.3 Induction of defense-related gene expression following cotton bollworm feeding

The upper leaves of undamaged tomato seedlings contained only very low amounts of PR-1, BGL-2, PI, CDI and PPO mRNA (Figure 4a). Compared to the control without damage, mRNA expression of PR-1, BGL-2, PI, and CDI in the upper leaves of bollworm-damaged seedlings was significantly promoted within 6 h of feeding. Increased expression of PPO was detected after 12 h, and the highest expression was observed at 24 h (Figure 4a; $P < 0.05$). In the *def-5* seedlings (which lack JA), mRNA expression of PR-1 (a product of the SA-dependent pathway) was found, but mRNA expression of PIs (products of the JA-dependent pathway) were not observed (Figure 4b). However, in transgenic *nahG* seedlings (which lack SA), PI expression was normal, and PR-1 expression was elevated (Figure 4b). Expression of these genes was suppressed to varying degrees by DMTU and DHC treatment (Figure 5), indicating a requirement for H₂O₂ in their activation.

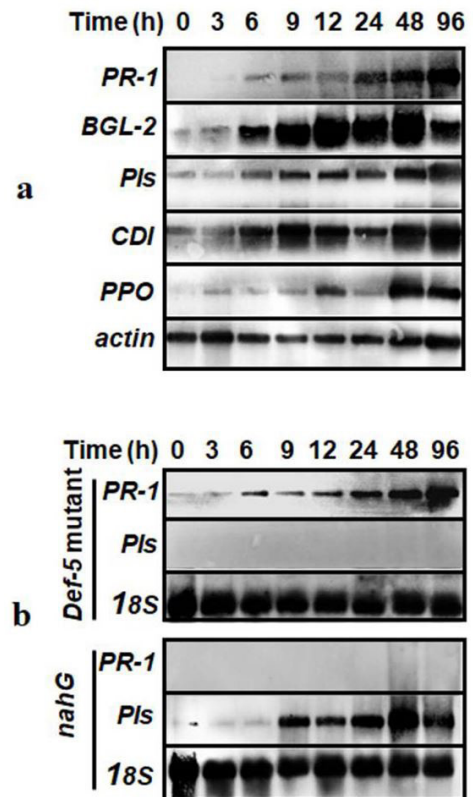


Figure 4. Expression of defense-related genes in tomato seedlings following cotton bollworm feeding. (a) mRNA expression of PR-1, BGL-2, CDI, PI and PPO genes in wild-type seedlings, and (b) PR-1 and PI genes in *def-5* mutant and transgenic *nahG* seedlings were detected by Northern blot. β -actin and 18S were used as the loading controls.

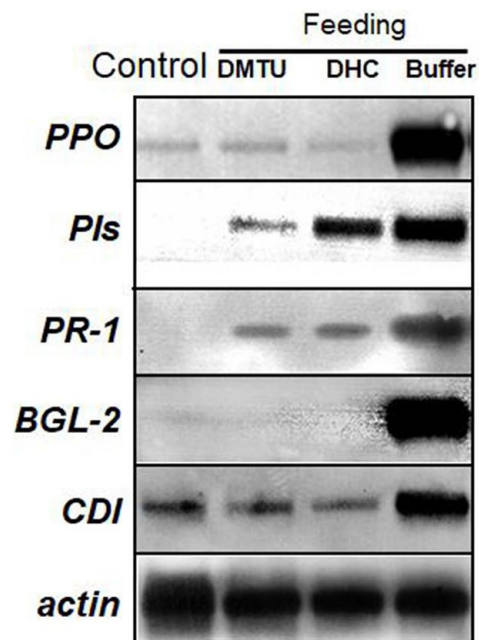


Figure 5. Effects of DMTU and DHC on expression of defense-related genes induced by cotton bollworm feeding. Tomato seedlings were treated with DMTU (10 mM) or DHC (10 μM) by daily watering for 3 days prior to cotton bollworm feeding. For the control, tomato seedlings were harvested without bollworm treatment.

4 Discussion

Higher plants respond to insect attack and wounding by activating the expression of genes involved in herbivore deterrence, wound healing, and other defense-related processes. Defense responses occur at the site of wounding (local response) and in undamaged leaves located distal to the site of attack (systemic response) (Li et al., 2002). The wound response of tomato plants has been extensively studied and provides a useful model to understand the signal transduction events leading from injury to marker gene expression (Sun et al., 2011). Our results suggest that cotton bollworm feeding elicits a strong systemic defense response in tomato seedlings. Damage to the lower leaves by cotton bollworms causes an increase in the activities and mRNA expression of defense-related genes in the undamaged upper leaves, indicating that the effect was systemic.

PR-1 and BGL-2 are reporters of the SA-dependent pathway, which are normally involved in defense against pathogens. Moran & Thompson (2001) reported that aphid feeding induces expression of PR-1 and BGL-2 (Moran & Thompson 2001). Our data indicate that cotton bollworm feeding triggers the SA-dependent signaling pathway, since expression of the PR-1 gene is not induced in transgenic *nahG* tomato seedlings. Our findings are consistent with previous findings that H₂O₂ does not function downstream of SA in the induction of PR protein expression (Bi et al., 1995). The role of JA and its methyl ester in plants damaged by wounding, pathogens or insects has been the focus of some studies (Stout et al., 1998; Ruuhola & Yang, 2006). Herbivore feeding is suppressed in pre-wounded tomato leaves via the accumulation of PIs induced by JA (Schulze et al., 2019). In *Arabidopsis*, aphid feeding induces expression of an array of defense genes involved in the JA signaling pathway, although aphids do not activate the same intensity of JA signaling as wounding (Bi et al., 1995).

H₂O₂ acts as the second messenger for activation of PIs and the defense response in mesophyll cells of tomato plants (Orozco-Cárdenas et al., 2001). A previous study provided the evidence that damage to a single leaflet by mechanical wounding or insects results in local and systemic accumulation of defensive PIs within 2 h in tomato (Sun et al., 2011). Another study found that wound-inducible PIs in tomato plants provide a useful model system to elucidate the signal transduction pathways that regulate systemic defense response (Ryan, 2000). In our study, cotton bollworm feeding in upper leaves systemically induced higher expression of PI, CDI and PPO in lower leaves. In *def-5* seedlings deficient in JA synthesis, cotton bollworm feeding do not increase the level of PIs. Thus, it is likely that cotton bollworm feeding induces the JA-dependent signaling pathway in tomato seedlings. Most reports indicate a mutually antagonistic interaction between SA- and JA-dependent signaling, such as the suppression by SA of the JA-dependent resistance of *Arabidopsis* to Egyptian cotton worm (Stotz et al., 2002). Our study found that cotton bollworm feeding activates both SA- and JA-dependent signaling. This is consistent with the findings of Moran and Thompson (Stout et al., 1998), which reported that these two pathways are also activated by aphid challenge in *Arabidopsis*.

There is evidence that the oxidative burst is a mechanism of plant resistance to pathogen infection and herbivore feeding

(Orozco-Cárdenas et al., 2001). H₂O₂ can damage insect digestive systems and suppress insect development. Feeding on previously wounded foliage results in oxidative damage to the insect midgut, and disrupts absorption and transport systems (Bi et al., 1995). Our results demonstrate that H₂O₂ levels rise sharply in the upper leaves of tomato plants in response to bollworm feeding on lower leaves. Whether H₂O₂ is transported systemically or is generated in the upper leaves in response to another systemic signal remains to be determined (Orozco-Cárdenas et al., 2001). H₂O₂ can act as a secondary messenger to induce wound or defense responses, as well as to upregulate the expression of a large number of genes (Orozco-Cárdenas et al., 2001; Levine et al., 1994). In this study, suppression of H₂O₂ with DMTU or DHC reduces the activity of CDI, BGL and PPO, the accumulation of PIs, and the expression of SA- and JA-dependent genes, demonstrating that H₂O₂ can mediate a variety of defense responses in bollworm-wounded tomato seedlings.

5 Conclusion

Taken together, our data indicate that H₂O₂ is an essential component of both JA- and SA-dependent systemic pathways involved in defense against cotton bollworm feeding tomato seedlings. Further understanding of the JA and SA signaling pathways promises to provide new insights into the basic mechanisms of plant defenses to wounding and insect stress.

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