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Salicylic acid and ethylene signaling pathways are involved in production of rice trypsin proteinase inhibitors induced by the leaf folder *Cnaphalocrocis medinalis* (Guenée)

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The roles of signaling pathways in the production of trypsin proteinase inhibitors (TrypPIs) in rice infested by the leaf folder (LF) *Cnaphalocrocis medinalis* were studied. Infestation by LF increased TrypPI levels in the leaves of rice plants at the tillering, booting and flowering stages but decreased TrypPI levels at the ripening stage; TrypPI levels in rice stems did not increase at any developmental stage. Infestation by LF at the tillering stage systemically increased TrypPI levels in leaves but not in stems; it also enhanced salicylic acid (SA) levels in leaves and stems, and the ethylene level released from plants. However, LF infestation did not increase JA concentrations. Exogenous application of SA or ethylene enhanced TrypPI levels in the leaves and stems of plants at the tillering stage, whereas treatment with both SA and ethylene induced lower levels of TrypPIs than treatment with SA or ethylene alone, suggesting an antagonistic effect of SA and ethylene on TrypPIs induction. The results suggest that both SA and ethylene signaling pathways are involved in the production of TrypPIs in rice induced by LF; moreover, the antagonistic effect of SA and ethylene may explain the changes in TrypPI levels seen at different plant developmental stages and in different organs.

rice, *Cnaphalocrocis medinalis*, jasmonic acid, salicylic acid, ethylene, trypsin proteinase inhibitor, herbivore-induced defense response

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Plant proteinase inhibitors (PIs) are a group of small proteins, widespread in plants, with a molecular mass of between 8 and 20 kD. PIs are well known to reduce the growth of some lepidopteran and coleopteran larvae by inhibiting the activity of digestive enzymes in larval midguts [1–5]. Moreover, some plant PIs specifically inhibit the proteases of pathogens [6,7]. Therefore, PIs are regarded as an essential part of the plant's natural defense system against pests [8–10].

PIs can be induced by herbivore infestation, pathogen infection or mechanical wounding [11–16]. The induced PIs represent either local [7,17] or systemic [18–21] responses, and either age-dependent [22] or organ-specific [8,18] traits. Application of chemical signals, such as jasmonic acid (JA), abscisic acid (ABA) and ethylene or salicylic acid (SA) can manipulate the production of PIs in plants [13,14,23,24], but the effects of each compound differ for different PIs. For example, mRNA levels of *OsPIN* (one of the PI genes found in rice) in rice plants were significantly up-regulated by SA, JA and H_2O_2 but not by ABA and ethylene [25], whereas the expression levels of *OsBBPI* were increased by JA and ethylene but inhibited by SA and ABA [12]. Thus, the induced PI levels in plants depend on cross-talk among multiple signaling pathways that are elicited by the herbivore.

Leaf folder (LF) *Cnaphalocrocis medinalis* (Guenée) is one of the most important rice pests. Because its larvae, which feed on the mesophyll cells of the leaf, cause a decrease in leaf area, the yield loss that results from infestation is dramatic [26]. Previous studies have shown that LF caterpillar infestation increases the levels of SA, H_2O_2 and

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trypsin PIs (TrypPIs) but not JA in plants [27]. In contrast, LF caterpillar feeding induced lower TrypPI levels in rice as-*lox* mutants with low elicited JA levels than in wild-type plants, and the exogenous application of JA partially restored the induction of TrypPIs in as-*lox* mutants [28]. These results raise the question: which signaling pathways are responsible for the production of TrypPIs in rice induced by LF?

To answer this question, we first correlated the changes in LF-induced TrypPI levels with developmental stage in rice. Second, we measured local and systemic changes in levels of SA, JA and TrypPIs, and changes in the concentration of ethylene released from LF-infested plants. Finally, to determine if the exogenous application of chemical signals enhanced TrypPI levels in plants, we measured the TrypPI levels in plants treated with SA, ethylene, or both phytohormones.

1 Materials and methods

1.1 Plant materials

The rice cultivar Xiushui 11 was used in the experiments. Pregerminated seeds were sown in a greenhouse; after 20 d, the seedlings were transplanted into small (8 cm diameter × 10 cm height, each with one plant) or large (15 cm diameter × 10 cm height, each with 3 or 15 plants) clay pots. All plants were placed in a controlled climate room maintained at 28±2°C, 70%–80% RH (relative humidity), and 12 h photoperiod. Plants were watered daily and each pot was supplied with 10 mL nutrient solution (urea, 1 g L^{-1}) every week. Plants were used for experiments 25–30 d after transplanting.

1.2 Insects

Leaf folder larvae in their first or second instars were captured in the field in Hangzhou, and then reared on Xiushui 11 rice plants. Third-instar larvae were used in the experiments.

1.3 Plant treatments

(i) LF treatment. Plants (one per pot) were individually infested at noon (12∶00) with two third-instar larvae of LF that had been starved for 2 h and then placed on the leaves at nodes 3 and 4 (the youngest fully expanded leaf was defined as leaf node 1; thus, nodes 3 and 4 refer to the 3rd and 4th fully expanded leaves, which are older than leaf node 1) (LF). Control plants (C) were not manipulated.

(ii) Mechanical wounding. Two leaves (at nodes 3 and 4) per plant (one per pot) were damaged by rolling a fabric pattern wheel over the leaf surface to create two rows of standardized puncture wounds (each row 18 cm long) on each leaf (W). Control plants were not manipulated (C).

(iii) SA treatment. Pots with one plant were used for the experiments. Plants were individually sprayed with 2 mL SA (25, 50 or 100 μg mL^{-1}) in 50 mmol/L sodium phosphate buffer (titrated with 1 mol/L citric acid to pH 8, with 0.01% Tween) (SA). Controls were sprayed with 2 mL of the buffer (Buf).

(iv) Ethephon treatment. Plants (3 per pot) were enclosed in a sealed, transparent, plastic cage (14 cm diameter × 55 cm height), after which 20 mL ethephon (Sigma-Aldrich, St Louis, MO, USA; 3 concentrations in MilliQ water, pH 7.2) was placed in the cage for 12 h (Eth). After 12 h, the ethylene concentrations in the cages were determined using the method described below; concentrations were 0.034, 0.039 and 0.056 ppm (1 ppm=1 mg/L), respectively, corresponding to the three ethephon concentrations. Controls were similarly treated with 20 mL distilled water (Water).

(v) SA+Eth treatment. Pots with 3 plants were used for the experiment. Plants were treated with SA (50 μg mL^{-1}) and, after the SA solution had dried, with ethephon (0.056 ppm ethylene) for 12 h as described above (SA+Eth). Three groups of control plants were set up: (1) those treated with the buffer and, after the buffer dried, with ethephon (0.056 ppm ethylene) (Buf+Eth); (2) those treated with the buffer, and then with water (Buf+Water); and (3) control plants (C).

1.4 JA and SA analysis

Plants (1 per pot) were randomly assigned to 2 treatment groups, LF and C. The leaves at nodes 2, 3 and 5, and the leaf sheath corresponding to the leaf at node 3 (3 cm in length) from each plant were harvested 0, 0.5, 1, 2, 4, 12, 24, or 48 h after the start of treatment. Each treatment at each time interval was replicated 5 times. Samples were immediately immersed in liquid nitrogen and stored at –80°C. Jasmonic acid and SA were extracted for gas chromatography–mass spectrometry (GC-MS) analysis using labeled internal standards (328 ng D3-JA, kindly supplied by Ian T. Baldwin, Max Planck Institute of Chemical Ecology, Jena, Germany, and 345 ng D6-SA, by Cambridge Isotope Laboratory, Cambridge, MA, USA) as described by Lou and Baldwin [29].

1.5 Ethylene determination

Potted plants (15 per pot) were randomly assigned to 2 treatment groups, LF and C. Each pot was covered with a sealed, transparent, plastic cage (14 cm diameter × 55 cm height). Ethylene production was determined by sampling 5 mL of headspace from the cage using a syringe at 2, 4, 6, 8, 10, 12, 16, 20, 24, 28, and 32 h after the start of treatment. Each treatment was replicated 3 times. The ethylene sam-

ples were analyzed by gas chromatography as described by Wang et al. [27].

1.6 Effects of developmental stage of rice plants on induction of TrypPIs by LF

Plants (1 per pot) at different developmental stages (tillering, booting, flowering or ripening) were randomly assigned to 2 treatment groups, LF and C. The leaf at node 3 and the leaf sheath corresponding to the leaf at node 3 (3 cm in length) from each plant were harvested on days 1, 3, and 5 after the start of treatment. Each treatment at each time interval was replicated 6 times. Samples were immediately immersed in liquid nitrogen and stored at −80°C until TrypPI levels could be determined. TrypPI concentrations were measured by the radial diffusion assay as described by Lou and Baldwin [16] and expressed as nmol per mg of total protein.

1.7 Local and systemic changes in TrypPI levels in rice plants infested by LF

Plants (one per pot) were randomly assigned to 3 treatment groups: LF, W and C. The leaves at nodes 2, 3 and 5, and the leaf sheath (corresponding to the leaf at node 3) (3 cm in length) from each plant were harvested on days 1, 3, and 5 after the start of treatment. Each treatment at each time interval was replicated 6 times. Samples were immediately immersed in liquid nitrogen and stored at −80°C until TrypPI levels were determined.

1.8 TrypPI levels in rice plants treated by SA, ethephon, or both

Plants (3 per pot) were randomly assigned to 8 treatment groups: SA (25, 50 or 100 $\mu g\, mL^{-1}$), Buf, Eth (0.034, 0.039 and 0.056 ppm), water, SA+Eth, Buf+Eth, Buf+water and C. The leaves at nodes 3 and 4, and the corresponding leaf sheaths were harvested at days 1, 3, and 5 after the start of treatment (for SA+Eth, Buf+Eth, B+water and their corresponding controls, samples were harvested 3 d after treatment). Each treatment at each time interval was replicated six times. Samples were immediately immersed in liquid nitrogen and stored at −80°C until TrypPI levels were determined.

1.9 Data analysis

Differences in JA, SA and ethylene production, and TrypPI levels in plants at different developmental stages were determined using Student's *t*-test. Other data were analyzed by one-way ANOVA; if the ANOVA analysis was significant ($P<0.05$), Duncan's multiple range test was used to detect significant differences between groups. Data were analyzed using Statistica (Star Soft, Tulsa, OK, USA).

2 Results

2.1 Effects of developmental stage of rice plants on induction of TrypPIs by LF

Levels of TrypPIs in leaves and stems induced by LF depended on the plant's developmental stage (Figure 1). The level increased significantly in local leaves of plants at the tillering, booting or flowering stages when they were infested by LF, but decreased at the yellow ripening stage. In contrast, at all tested developmental stages, TrypPI levels in the stems of infested plants were lower than levels in the stems of non-infested plants, especially at the booting, flowering and yellow ripening stages.

2.2 Local and systemic changes in TrypPI levels in rice plants infested by LF

Levels of TrypPIs increased in infested leaves at node 3 on days 1, 3, and 5 after the start of treatment compared to those of control and mechanically wounded plants (Figure 2). TrypPI levels peaked on the third day following treatment (Figure 2(b)). Similar increases of TrypPI levels were found in the non-infested leaves at nodes 2 and 5 of infested plants. The levels of TrypPIs in the non-infested leaves at node 2 of infested plants significantly increased on days 3 and 5 after LF infestation compared to the levels in control and mechanically wounded plants (Figure 2(a)), and the levels of TrypPIs in leaves at node 5 only significantly

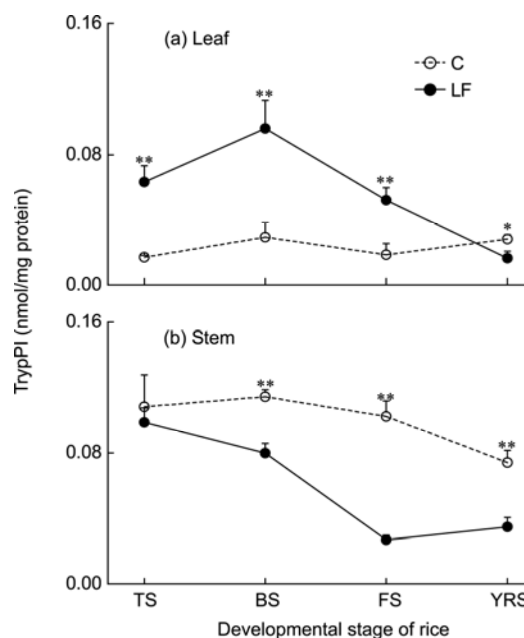

Figure 1 Mean (+SE) levels of TrypPIs at different developmental stages of rice plants infested by LF. (a) Leaf; (b) stem. C (control), non-manipulated plants; LF, plants infested by LF; TS, tillering stage; BS, booting stage; FS, flowering stage; YRS, yellow ripening stage. Asterisks indicate significant differences between C and LF at the same developmental stage (*, $P<0.05$; **, $P<0.01$; Student's *t*-test).

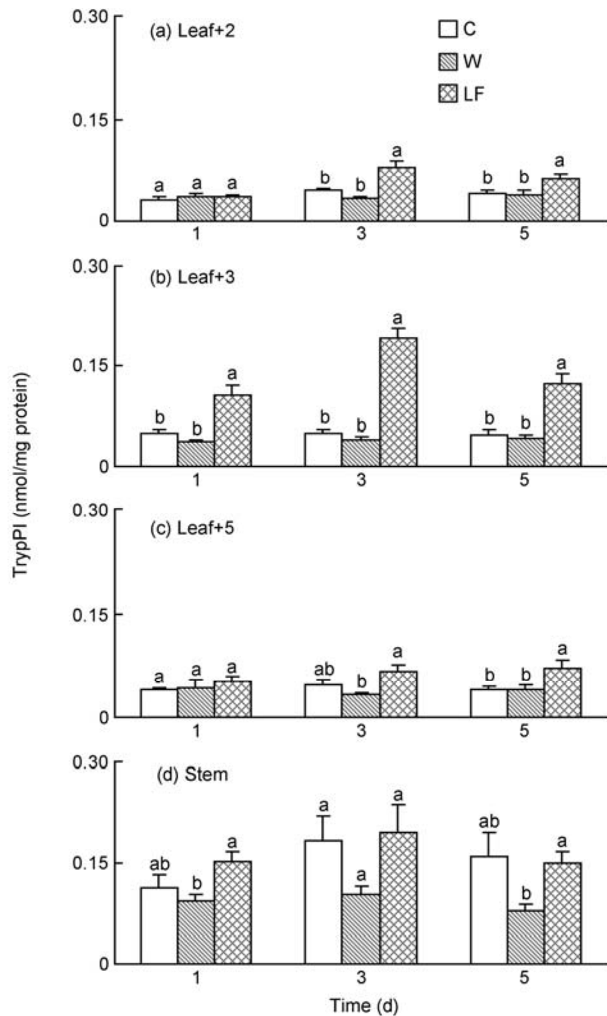

Figure 2 Mean (+SE) levels of TrypPIs in leaves and stems of rice plants infested by LF. (a) The leaf at node 2; (b) the leaf at node 3; (c) the leaf at node 5; (d) stem. C (control), non-manipulated plants; W, mechanically wounded plants; LF, plants infested by LF at nodes 3 and 4. Letters indicate significant differences among treatments at the same treatment time ($P < 0.05$, Duncan's multiple range test).

increased by day 5 of the treatment (Figure 2(c)). There was no significant difference between TrypPI levels in leaves from control and mechanically wounded plants (Figure 2(a)–(c)).

Levels of TrypPIs in LF-infested plant stems were significantly higher than those in mechanically wounded plants but not in control plants at days 1 and 5 after treatment (Figure 2(d)).

2.3 Changes in JA, SA and ethylene levels of rice plants infested by LF

Levels of JA in LF-infested plants were not higher than those in control plants (Figure 3). In contrast, JA levels were significantly decreased in some leaves of infested plants, namely the infested leaves (at node 3) at 2 h and the non-infested leaves at node 5 at 1 h after the start of treatment (Figure 3).

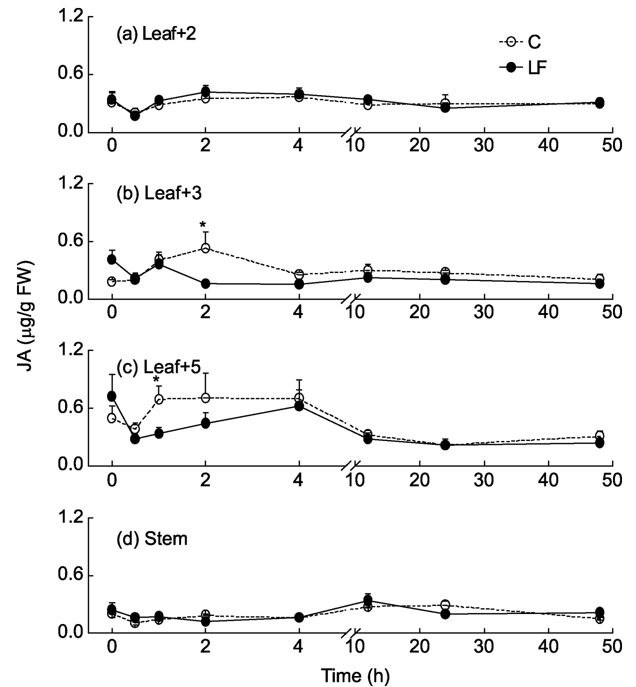

Figure 3 Mean (+SE) levels of JA in leaves and stems of rice plants infested by LF. (a) The leaf at node 2; (b) the leaf at node 3; (c) the leaf at node 5; (d) stem. C (control), non-manipulated plants; LF, plants infested by LF at nodes +3 and +4. Asterisks indicate significant differences between C and LF at the same treatment time (*, $P<0.05$; **, $P<0.01$; Student's *t*-test).

Infestation by LF increased the levels of SA in rice plants, depending on the timing of measurements during treatment (Figure 4). Levels of SA in infested leaves at node 3

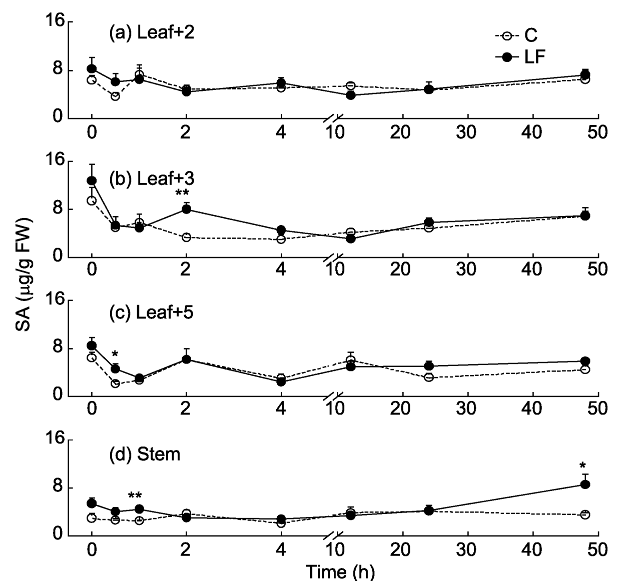

Figure 4 Mean (+SE) levels of SA in leaves and stems of rice plants infested by LF. (a) The leaf at node 2; (b) the leaf at node 3; (c) the leaf at node 5; (d) stem. C (control), non-manipulated plants; LF, plants infested by LF at nodes +3 and +4. Asterisks indicate significant differences between C and LF at the same treatment time (*, $P<0.05$; **, $P<0.01$; Student's *t*-test).

significantly increased at 2 h after treatment when compared to SA levels in control plants (Figure 4(b)). Similarly, in non-infested leaves at node 5 of the infested plants, SA levels were significantly increased at 0.5 h after treatment (Figure 4(c)). However, in leaves at node 2, the levels of SA were not as drastically altered as those in leaves of control plants (Figure 4(a)). In stems, LF infestation also significantly increased the levels of SA at 1 h after treatment (Figure 4(d)).

Significant increases in ethylene levels in LF-infested plants compared to non-infested plants were observed 2 to 32 h after treatment (Figure 5). Because the container was airtight, levels of ethylene in the container increased with treatment time.

2.4 TrypPI levels in rice plants treated with SA, ethylene or both

Levels of TrypPIs in SA-treated rice plants were enhanced compared to levels in plants in treatment groups Buf and C, depending on the SA concentration and plant tissue sampled (Figure 6). In leaves, 50 μg mL^{-1} SA solution significantly enhanced TrypPI levels on days 1 and 3 after treatment, yet 100 μg mL^{-1} SA significantly increased TrypPI levels only at day 3; in contrast, 25 μg mL^{-1} SA had no effect on TrypPI levels (Figure 6(a)). In stems, 25 μg mL^{-1} SA treatment increased TrypPI levels at day 1 after treatment and 50 μg mL^{-1} SA increased TrypPI levels at day 3, but 100 μg mL^{-1} SA had no effect (Figure 6(b)).

Ethephon treatment also increased the levels of TrypPIs in the leaves of rice plants, and the induced TrypPI levels were positively correlated with the concentration of applied ethylene (Figure 7(a)). For stems, only treatment with 0.056 ppm ethylene significantly enhanced TrypPI levels 5 d after treatment (Figure 7(b)). Treatment with 0.034 ppm ethylene decreased TrypPI levels in stems at day 1 after treatment.

Similarly, the levels of TrypPIs in leaves treated with Buf+0.056 ppm Eth increased compared to the levels of TrypPIs in control leaves or in leaves treated with Buf+Water (Figure 7(c)). However, there was no difference between TrypPI levels in the leaves of plants treated with SA (50 μg mL^{-1})+Eth (0.056 ppm) and of plants treated with Eth (Buf+Eth) alone. In stems, treatment with SA (50 μg mL^{-1}) +Eth (0.056 ppm) did not increase TrypPI levels more than in control plants or those treated with Buf+Water.

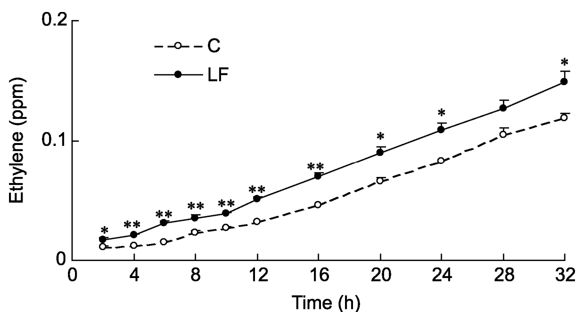

Figure 5 Mean (+SE) levels of ethylene released from rice plants infested by LF. C (control), non-manipulated plants; LF, plants infested by LF at nodes 3 and 4. Asterisks indicate significant differences between C and LF at the same treatment time (*, $P<0.05$; **, $P<0.01$; Student's t-test).

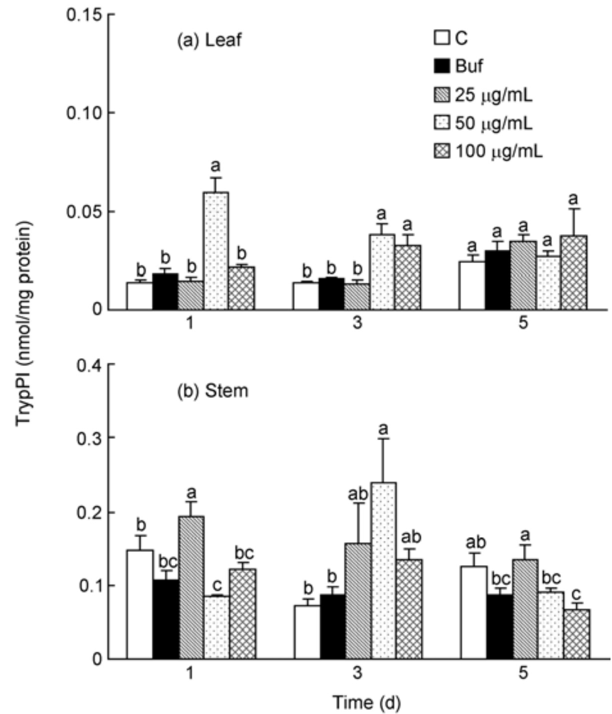

Figure 6 Mean (+SE) levels of TrypPIs in leaves and stems of rice plants treated by SA. (a) Leaf; (b) stem. C (control), non-manipulated plants; Buf, plants individually treated with 2 mL of 50 mmol/L Na_2HPO_4 buffer (pH 8.0); 25 μg mL^{-1}, plants individually treated with 2 mL of 25 μg mL^{-1} SA; 50 μg mL^{-1}, plants individually treated with 2 mL of 50 μg mL^{-1} SA; 100 μg mL^{-1}, plants individually treated with 2 mL of 100 μg mL^{-1} SA. Letters indicate significant differences among treatments at the same treatment time ($P<0.05$, Duncan's multiple range test).

3 Discussion

The systemic induction of herbivore-induced defense responses, including changes in levels of volatile and non-volatile chemicals, has been reported in many plant species, such as tomato and *Nicotiana attenuata* [30]. Similarly, we found that LF infestation resulted in a systemic increase of TrypPI levels in the leaves of rice plants at the tilling stage, and this response was more rapid and stronger in young leaves than in old leaves (Figure 2). However, LF infestation did not enhance the levels of TrypPIs in rice stems at the tillering stage, though it decreased the levels in stems at the booting, flowering and yellow ripening stages (Figures 1 and 2). This indicates that induction of TrypPIs in rice plants by LF infestation is in part systemic and transportation of the wound signals follows source–sink relationships, moving

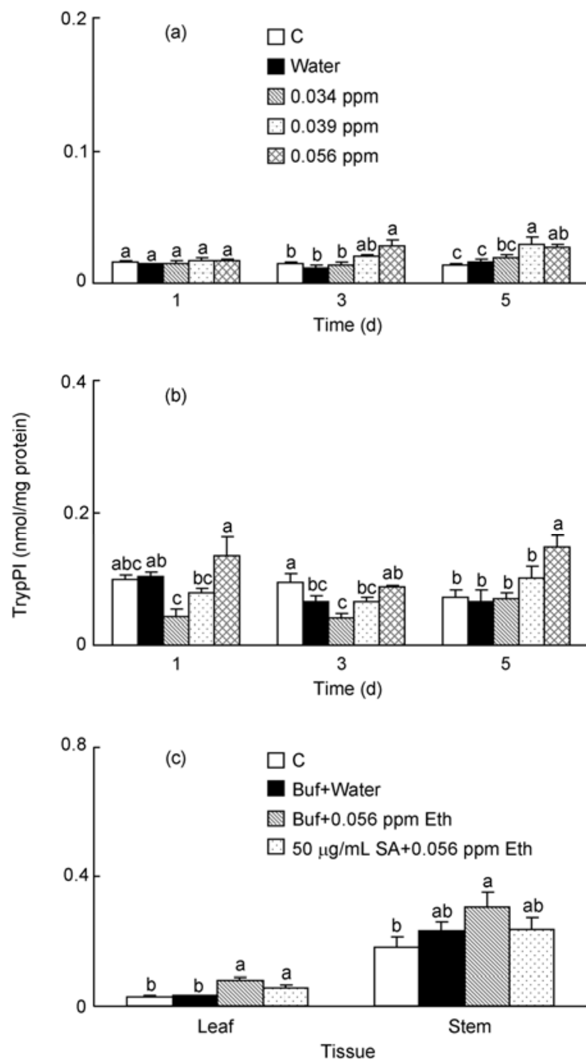

Figure 7 Mean (+SE) levels of TrypPIs in rice plants treated with ethephon, SA or both. (a, b) levels of TrypPIs in leaves (a) or stems (b) of plants treated with ethephon; (c) levels of TrypPIs in leaves and stems of plants treated with SA, ethephon or both. C (control), non-manipulated plants; Water, plants fumigated with 20 mL of ddH_2O; 0.034 ppm, plants fumigated with 20 mL ethephon solution in a plastic cage resulting in 0.034 ppm ethylene in the cage 12 h after treatment; 0.039 ppm, plants fumigated with 20 mL ethephon solution in a plastic cage resulting in 0.039 ppm ethylene in the cage 12 h after treatment; 0.056 ppm, plants fumigated with 20 mL ethephon solution in a plastic cage resulting in 0.056 ppm ethylene in the cage 12 h after treatment. Buf+Water, plants first individually treated with 2 mL of 50 mmol/L Na_2HPO_4 buffer (pH 8.0) and then fumigated with 20 mL of ddH_2O; Buf+0.056 ppm Eth, plants first individually treated with 2 mL of 50 mmol/L Na_2HPO_4 buffer (pH 8.0) and then fumigated with 20 mL ethephon solution resulting in 0.056 ppm ethylene in the cage 12 h after treatment; 50 μg mL^{-1} SA+0.056 ppm Eth: plants first individually treated with 2 mL of 50 μg mL^{-1} SA and then fumigated with 20 mL ethephon solution resulting in 0.056 ppm ethylene in the cage 12 h after treatment. Letters indicate significant differences among treatments at the same treatment time (P<0.05, Duncan's multiple range test).

in the phloem together with sucrose [13].

It is well documented that herbivore infestation may elicit JA, SA and ethylene signaling pathways in plants; moreover, these pathways are known to play an important role in shaping plant defense responses [16,31]. In this study, we found that LF infestation increased SA and ethylene levels in rice plants, but, surprisingly, decreased JA levels (Figures 3–5). This indicates that LF infestation activates the SA and ethylene signaling pathways but not the JA signaling pathway in rice plants, which is different from other chewing herbivores whose infestation generally activates the JA-mediated pathway in plants [30,32]. It is reported that SA suppresses JA accumulation and signaling in several plant species, such as *Arabidopsis*, *N. attenuata* and rice [33,34]. Thus, the decrease in JA levels in LF-infested rice plants might be because of an increase in SA levels. Unlike some chewing herbivores, LF larvae feed only on the epidermis of the leaves; this behavior damages plants less than if entire leaves are browsed. Whether non-activation of the JA signaling pathway in plants infested by LF is related to the feeding habits of LF remains to be elucidated.

Like TrypPI levels, SA levels in plants infested by LF were also systemically increased (Figure 4). In addition, much higher levels of ethylene were released in LF-infested plants than in control plants (Figure 5); the compound might be airborne or transported through the plant. The exogenous application of SA or ethylene at appropriate concentrations to rice plants enhanced the levels of TrypPIs in leaves and stems (Figures 6 and 7). Furthermore, treatment with both SA and ethylene resulted in relatively lower levels of TrypPIs in leaves and stems than treatment with SA or ethylene alone, and TrypPI levels in stems were similar to those of controls (C or Buf+Water) (Figure 7(c)). This indicates that both SA and ethylene signaling pathways are involved in the production of TrypPIs in rice induced by LF. The data also imply that SA and ethylene play antagonistic roles in the induction of TrypPIs in rice plants, particularly in rice stems. The antagonistic effect between SA and ethylene on the production of total TrypPIs in rice might result from the induction of the two opposing signal molecules on the same TrypPI; this is the case for *OsBBPI,* whose mRNA levels were increased by ethylene but inhibited by SA [12]. On the other hand, SA and ethylene may enhance the levels of the same TrypPI through two pathways that work in opposition to one another. Thus, plants treated with both SA and ethylene may have lower levels of TrypPIs than those treated with SA or ethylene alone. The antagonistic effect of SA and ethylene on the induction of TrypPIs may explain why LF infestation did not enhance TrypPI levels in the stems of plants (Figures 1 and 2).

As reported in other plants, for example, *N. attenuata* [13] and *N. plumbaginifolia* [35], the developmental stage of rice plants also influenced the degree to which TrypPIs were inducible by LF (Figure 1). Compared to those in non-infested plants, for example, TrypPI levels in infested leaves were significantly increased at the tillering, booting or flowering stages of plants, whereas levels were decreased at the ripening stage (Figure 1). Changes in the inducibility of TrypPIs in leaves or stems at different developmental stages

might be related to plant energy allocation [13]. For instance, at the ripening stage, most of a plant's energy has been allocated to production; therefore little energy remains for defense responses, which cause the levels of TrypPIs in leaves to increase or decrease. Plant energy allocation is a complex physiological process in which phytohormones play an important role [36]. On the other hand, constitutive and elicited levels of phytohormones, such as JA, SA and ET, change with plant developmental stage [37–39]. Therefore, changes in the inducibility of TrypPIs in rice leaves or stems at different developmental stages may be shaped by interactions among plant hormones, such as SA and ethylene. The relative amount of SA and ethylene in tissues or organs, and the sensitivity of the tissues or organs to SA and ethylene, might change with developmental stage, resulting in changes in the inducibility of TrypPIs.

In summary, LF infestation elicited SA and ethylene signaling pathways in rice, both of which, through crosstalk, manipulated the production of TrypPIs in plants. The levels of SA and ethylene in tissues or organs, the sensitivity of the tissues or organs to SA and ethylene, and the antagonistic effect of SA and ethylene on the induction of TrypPIs all influenced the production of TrypPIs in LF-induced rice plants. The interaction of these factors determined the levels of LF-induced TrypPIs in different tissues and organs and at different developmental stages.

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