REPORTS

Chinese Science Bulletin 2003 Vol. 48 No. 18 1982—1987

Involvement of Jasmonate-signaling pathway in the herbivore-induced rice plant defense

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Abstract **The expression patterns of eight defense-related genes in the herbivore-infested and jasmonate-treated (jasmonic acid, JA and its derivative MeJA) rice leaves were analyzed using RT-PCR. The results showed that *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) herbivory induced the expression of lipoxygenase (LOX) and allene oxide synthase (AOS) genes that are involved in the jasmonate-signaling pathway. Moreover, *S. litura* damage resulted in the expression of farnesyl pyrophosphate synthase (FPS), Bowman-birk proteinase inhibitor (BBPI), phenylalanine ammonia-lyase (PAL) and other rice defense-related genes that were also induced by aqueous JA treatment or gaseous MeJA treatment. These indicated that in rice leaves, the JA-related signaling pathway was involved in the *S. litura*-induced chemical defense. Mechanical damage and brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae) damage induced the expression of *LOX* gene, but both treatments did not induce the expression of *AOS* gene. However, BPH damage induced the expression of acidic pathogen-related protein 1 (*PR-1a*), Chitinase (*PR-3*), and *PAL* genes, which is involved in the salicylate-signaling pathway. It was suggested that salicylate-related signaling pathway or other pathways, rather than jasmonate-signaling pathway was involved in the BPH-induced rice plant defense.**

Keywords: rice, brown planthopper, *Spodoptera litura*, jasmonate signaling pathway, gene expression.

DOI: 10.1360/03wc0072

Jasmonic acid (JA) and its methyl ester (MeJA), collectively termed jasmonates, are fatty acid derived, naturally-occurring octadecanoid-based compounds which are synthesized from linolenic acid through the sequential action of a lipoxygenase (LOX), and an allene oxide synthase (AOS) and other enzymes[1—3]. Jasmonates are involved not only in plant growth and development, but also in defense responses against herbivore and pathogen attack[1—3]. Recent studies have indicated that JA functions as a key signal molecule in herbivore-induced plant chemical defense and wound signal transduction pathways[4—7]. Several lines of evidence support an essential role for the jasmonate-signaling pathway in plant chemical defense. First, endogenous levels of JA and its derivative MeJA increase rapidly in response to wounding and herbivore damage, and these signal molecules activate the plant defense-related genes to produce various chemical defenses[4—7]. Second, treatment of plants with exogenous JA (or MeJA) can induce the increase of the amounts of plant direct chemical defenses (such as nicotine, proteinase inhibitors), and result in a significant improvement of the plant resistance to herbivore damage[4,5,8,9]. Moreover, exogenous applications of JA and its methyl ester elicit the same volatiles release that follows herbivore attack in several plants, such as lima bean and cotton[10—12]. The jasmonate cascade and pathways that interact with it to activate direct defenses also appear to be involved in indirect defenses. All the evidence indirectly supports that jasmonate-signaling pathway is involved in the induction of plant chemical defense in response to herbivore attack. However, most of these researches are focused on the study of dicot plants. The roles of jasmonate-signaling pathway in the herbivore-induced defense of monocot plants are largely unknown.

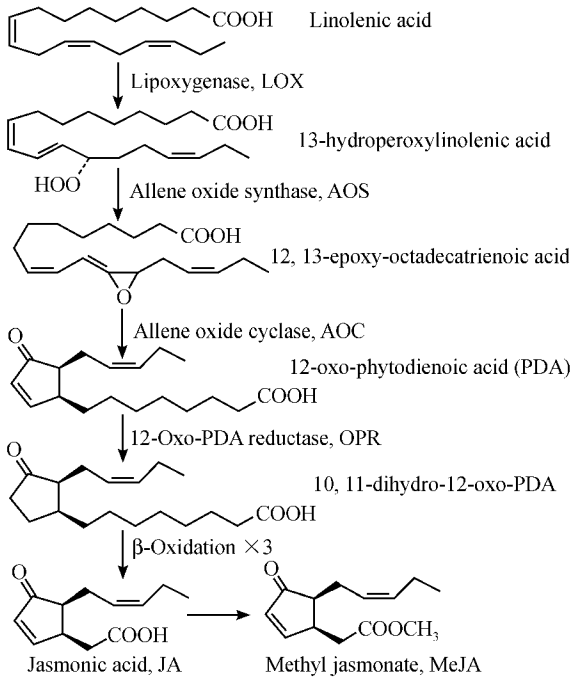

Fig. 1. Biosynthetic pathway of jasmonate[1—3].

Rice (*Oryza sativa* L.), a model monocot cereal crop species, and one of the most important food crops, is the major focus of our study towards understanding herbivore-induced plant chemical defense and its signal transduction pathways. Our previous studies showed that rice plants attacked by *S. litura* and BPH, released blends of volatiles that had repellent effects on BPH[13]. Chemical

analysis showed that these volatiles at least synthesized through fatty acid/lipoxygenase and three other biosynthetic pathways[13]. Lou et al.[14] studied herbivore-induced indirect defense of rice plants in detail. Their studies showed that rice volatiles can attract the natural enemies (*Anagrus nilaparvatae* Pang et Wang) of the BPH, and this attraction effect of rice volatiles was significantly increased when rice stems were infested by BPH[14]. This demonstrated that the damage by BPH induced rice synthesizing and releasing some volatile compounds[14]. Moreover, the emission of rice volatiles was dramatically induced by application of JA on mechanically damaged rice plants, including the increase in amount and number of volatiles, and changes in composition proportion[1)]. The volatiles profile from JA-induced rice plants was similar to that from *S. litura* damaged rice plants, although the rice varieties were not the same in these two studies[1,13]. This demonstrated that jasmonate-signaling pathway may be involved in herbivore-induced rice plant chemical defense.

In this study we explored whether the jasmonate-signaling pathway was involved in the herbivore-induced rice plant defense from two points. First, can herbivore-damage induce the expression of *LOX* and *AOS* genes which are involved in the synthesis of jasmonates? Second, were the expression patterns of rice defense-related genes the same or similar between jasmonate-induced rice plants and herbivore-damaged rice plants?

1 Materials and methods

(ⅰ) Rice variety. A standard susceptible rice variety TN1 was used in this study. Jasmonic acid can induce this rice variety to release parasitoid-attractive volatiles[1)]. Rice seeds were germinated in laboratory and planted in plastic pots (ϕ10 cm × 15 cm) with 50 seedlings per pot after germination. Rice seedlings were cultivated in the growth chamber at (27 ± 1)℃, 80% R.H., and 14 ∶ 10 (L ∶ D) and 20-d-old seedlings were used for experiments.

(ⅱ) Insects. BPH adults collected from rice fields in Dasha Town of Sihui City in Guangdong Province, China were reared on susceptible rice variety Qidaizhan more than 10 generations in screen cages (35 cm × 35 cm × 50 cm) separately in laboratory at (27 ± 1)℃, 80% R.H., 14 ∶ 10 (L ∶ D). 1-d-old adult BHP females were used in experiments. *S. litura* was reared with artificial diets in laboratory and the third instar caterpillars were used in experiments.

(iii) Plant treatment. Plastic pots with 50 seedlings were individually transferred into screen cases (35 cm × 35 cm × 35 cm) for the following treatments: ⅰ) BPH infested plants: 200 adult BPH females were introduced into the case and allowed to feed on plants for 24 h, and the females were removed immediately before extracting total RNA; ⅱ) *S. litura* infested plants. Two third-instar caterpillars were placed on each seedling and the larvae were allowed to feed on the plants for 24 h, and removed with their by-products immediately before extracting total RNA; iii) mechanically damaged plants (stem). Uninfested plants were mechanically damaged using a pin to pierce the stems (1—2 mm), 300 pin pricks were administered to the plant 24 h before extracting total RNA, and another 200 pin pricks were done immediately before the experiment; and ⅳ) mechanically damaged plants (leaf). About 1 cm^2 area of each leaf of undamaged healthy rice seedlings was cut with clean scissors before extracting total RNA; ⅴ) jasmonic acid treated rice plants. Jasmonic acid (Sigma-aldrich Company, St. Louis, MO) was dissolved in ethanol as a stock solution of 100 mmol/L, and diluted to 500 μmol/L with ddH_2O, and 2 mL of this solution were sprayed to the rice leaves 24 h before extracting total RNA; and ⅵ) methyl jasmonate treated rice plants. 7 μL methyl jasmonate (Sigma-aldrich Company, St. Louis, MO) were applied to the cotton ball, and transferred into a desiccator together with 50 potted undamaged rice seedlings and cultivated in a growth chamber for 24 h before extracting total RNA. Healthy rice seedlings with the same age and size were used as control plants.

(ⅳ) Analysis of gene expression. Expression patterns of defense-related genes and jasmonate-biosynthesis control genes in differently treated rice leaves were analyzed using reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from rice leaves using the Trizol Kit according to the protocol provided by manufacturer (GibcoBRL). The first-strand cDNA was synthesized from 1 μg of total RNA using the SuperScript Ⅱ Kit according to the protocol provided by manufacturer (Invitrogen). The first-strand cDNA was amplified by adding Taq DNA polymerase. Standard PCR-conditions were a hot start of 5 min at 94℃ followed by 30 cycles of 45 s at 94℃, 45 s at 55℃ and 45 s at 72℃, and further extended at 72℃ for 8 min. The gene-specific primers used in RT and PCR are listed in Table 1. An equal amount of PCR products (5 μL) were separated by electrophoresis in 1% agarose gels and detected by staining with ethidium bromide. All RNA analysis was performed in triplicate, using a new set of plants for each iteration.

2 Results

(ⅰ) Herbivore-induced expression of rice genes involved in jasmonate biosynthesis. Lipoxygenase (LOX) and Allene oxide synthase (AOS) are two important en-

1) Lou, Y. G., Role of infochemicals in the host selection behavior of parasitoid, Anagrus nilaparvatae Pang et Wang, Ph.D dissertation in Zhejiang University, 1999, 1—117.

Table 1 The specific primers in RT-PCR

Genes	Accession number in GenBank	Functions	Primers
LOX	AF095896	First enzyme in the biosynthesis of jasmonates and green leaf volatiles[1−3]	F: 5′-TGTACGTGCCGAGGGACGAG-3′ R: 5′-GCGAGCGTCTCCCTCGCGAACTC-3′
AOS	AY062258	A major control enzyme in the biosynthesis of jasmonates[1−3]	F: 5′-TGCCACAACCTGCTGTTCGC-3′ R: 5′-TGGTAGCCGAACAGCATCTC-3′
FPS	D85317	Key enzyme in the biosynthesis of sesquiterpene in the plants[15]	F: 5′-GGTTGGTGCATTGAATGGCT-3′ R: 5′-ATGTCCGTTCCAATCTTGCC-3′
PAL	X16099	First enzyme in the biosynthesis of salicylate and phenols[16]	F: 5′-CACAAGCTGAAGCACCACCC-3′ R: 5′-GAGTTCACGTCCTGGTTGTG-3′
BBPI	U76004	Insect resistant protein[17]	F: 5′-GCTCATCTGCGAGGACATCT-3′ R: 5′-TTCCTCATGGTCCACACAAG-3′
PR-1a	AJ278436	Disease resistance[18]	F: 5′-GTGGACCCGCACAACGCG-3′ R: 5′-GCCGATCGCCGTCGAGTC-3′
PR-2	AF443600	Disease resistance[19]	F: 5′-ACATCGCCGTCGGCAACGAG-3′ R: 5′-GGTTCTCGTTGAACATGGCG - 3′
Chitinase	AB006188	Resistance to herbivore and pathogens[19]	F: 5′-GGCGTCGACTTCGACATCGA-3′ R: 5′-CATGATGCCGCCGTACTTGG-3′
Actin	X15865	Internal standard of RT-PCR[17,18]	F: 5′-ACTGTCCCCATCTATGAAGGA-3′ R: 5′-CTGCTGGAATGTGCTGAGAGA-3′

zymes in the biosynthesis of jasmonates[1−3]. The expression patterns of these two genes were analyzed in herbivore-infested rice leaves, mechanically damaged rice leaves and other treatments of rice leaves using RT-PCR (Fig. 2). The expression of *LOX* gene was induced in rice leaves as consequence of mechanical wounding (at stem or leaf), BPH-infestation or *S. litura* feeding. In comparison to *LOX* expression induced by BPH or mechanical damage, *S. litura* feeding induced more strongly (Fig. 2). There was no difference found in the transcript levels between BPH infested rice leaves and mechanically damaged leaves. *S. litura* damage induced the expression of *AOS* after 24 h, but no *AOS* transcripts were detected in either mechanically damaged rice leaves or BPH infested rice leaves (Fig. 2). The effects of the exogenous JA or MeJA on transcript accumulation of *LOX* and *AOS* in rice leaves were also investigated. The results showed that aqueous JA treatment and gaseous MeJA treatment induced strong expression of *LOX* and *AOS* exactly as *S. litura* feeding did (Fig. 2).

(ii) Herbivore- and jasmonate-induced expression of rice defense genes. Terpenoids, green leaf volatiles and methyl salicylate are commonly found in the volatiles induced by herbivory[13−15]. These compounds are produced through different biosynthetic pathways. Terpenoids (monoterpene, sesquiterpene and their derivatives) are the main components in herbivore-induced rice and other plants volatiles and produced via isoprene biosynthetic pathway[13,14]. Farnesyl pyrophosphate synthase (FPS) is a key enzyme in the biosynthesis of sesquiterpenes[15]. Caterpillar *S. litura* feeding or application with exogenous JA induced the expression of FPS in rice leaves. MeJA treatment and BPH infestation induced a weak expression of *FPS*, but no *FPS* transcripts were detected in mechanically damaged rice leaves (Fig. 3).

Methyl salicylate is a volatile derivative of the plant hormone salicylic acid, which is produced via the phenyl propanoid pathway[16]. A key enzyme in the phenyl propanoid pathway is phenylalanine ammonia-lyase (PAL). Besides salicylate, many other plant defense chemicals are produced from this pathway, such as phenols and flavonoids[16]. The expression of *PAL* in rice leaves was induced by mechanical damage, herbivory, and jasmonate treatment separately (Fig. 3). *S. litura* feeding and exoge-

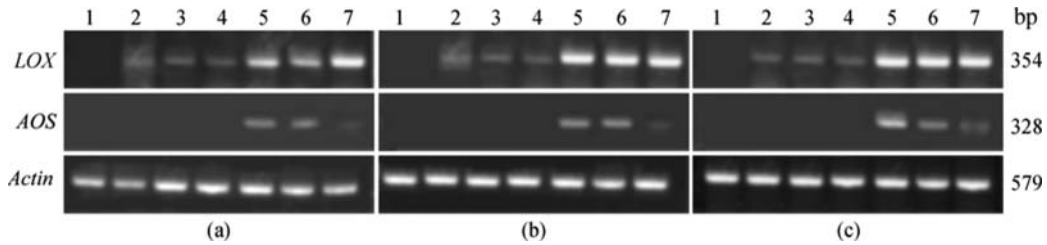

Fig. 2. Expression patterns of jasmonate-biosynthesis control genes in differently treated rice leaves. *LOX*, Lipoxygenase; *AOS*, Allene oxide synthase; *Actin*, internal standard of RT-PCR. 1, Healthy rice seedlings; 2, mechanically damaged rice seedlings (stem); 3, mechanically damaged rice seedlings (leaf); 4, adult *N. lugens* infested rice seedlings; 5, *S. litura* infested rice seedlings; 6, jasmonic acid treated rice seedlings; 7, methyl jasmonate treated rice seedlings. bp, base pairs. Three replicates were set up with each experiment. (a) Experiment 1; (b) experiment 2; (c) experiment 3.

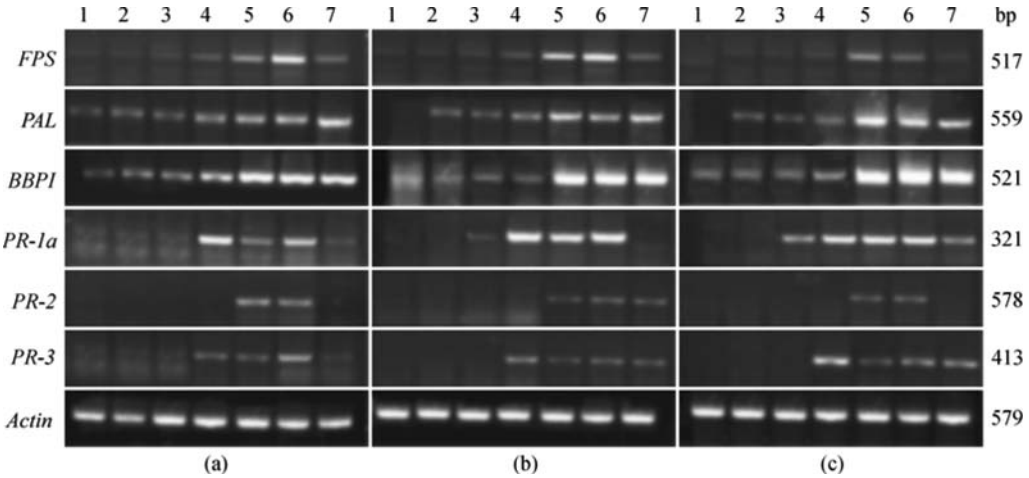

Fig. 3. Expression patterns of defense-related genes in differently treated rice leaves. *FPS*, Farnesyl pyrophosphate synthase; *PAL*, phenylalanine ammonia-lyase; *BBPI*, Bowman-proteinase inhibitor; *PR-1a*, acidic pathogenesis-related protein 1; *PR-2*, β-1,3-Glucanase; *PR-3*, Chitinase; *Actin*, internal standard of RT-PCR. 1, Healthy rice seedlings; 2, mechanically damaged rice seedlings (stem); 3, mechanically damaged rice seedlings (leaf); 4, adult *N. lugens* infested rice seedlings; 5, *S. litura* infested rice seedlings; 6, Jasmonic acid treated rice seedlings; 7, methyl jasmonate treated rice seedlings. bp, base pairs. Three replicates were set up with each experiment. (a) Experiment 1; (b) experiment 2; (c) experiment 3.

nous jasmonate treatment had a stronger inducing effects on the expression of *PAL* than BPH infestation and mechanical damage (Fig. 3).

There is strong evidence that Bowman-birk proteinase inhibitors (BBPI) play a role in the plant defense response against herbivorous insects via inhibition of insect proteolytic enzymes[8,17]. The transcript levels of *BBPI* were higher in rice leaves damaged by *S. litura* or treated with exogenous jasmonates (JA and MeJA) than in leaves that had been damaged mechanically or infested by BPH. There was no difference in the transcript levels of *BBPI* among BPH infested rice leaves, mechanically damaged leaves and undamaged healthy leaves, but *S. litura* feeding had a strong inducing effect on the expression of *BBPI* (Fig. 3).

Acidic pathogenesis-related protein 1 (PR-1a), β-1, 3-Glucanase (PR-2), Chitinase (PR-3) and many other pathogen defense proteins collectively referred to as the "pathogenesis-related proteins (PRs)"[18,19]. It has often been suggested that the collective set of PRs may be effective in the protection of the plants from pathogen infection and be responsible for the state of the systemic acquired resistance (SAR)[18,19]. The expressions of the genes for the PR-1a and PR-3 proteins were induced 24 h after herbivory by BPH or *S. litura*. These gene expressions were also induced within 24 h of JA treatment. However, they were not induced (or only weakly induced) within 24 h of MeJA treatment. The expression of the *PR-2* gene was induced by *S. litura* herbivory or JA treatment. However, we did not detect its expression in the rice leaves with BPH infestation and MeJA treatment.

3 Discussion

The importance of the signaling function of jasmonates in the herbivore-induced plant defense has been demonstrated by the study of several model dicot plants, such as *Arabidopsis thaliana*, tomato and tobacco. The role of the jasmonate signal pathway in regulating herbivore-induced defense has been confirmed in several ways. First, endogenous levels of JA increase rapidly in response to herbivory[4—7]. Second, exogenous JA and MeJA treatment induce the production of the plant defenses and the expression of genes for the enzymes involved in the biosynthesis of these defenses, and inhibitors of the jasmonate signal pathway, such as salicylic acid, block JA and herbivory activation of defensive genes[5—8]. Third, mutants defective in either the biosynthesis or perception of JA are dramatically compromised in resistance to numerous plant invaders[20—22]. In tomato, the *def1* mutant which does not up-regulate levels of JA after wounding also produces lower levels of PIs, and is more susceptible to be attacked by Lepidopteran insects[22]. In *Arapidopsis*, mutants existing either do not produce, or are insensitive to JA (*fad3-2 fad7-2 fad8*, *opr3/dde*), in both cases their re- sponses to herbivorous insects are impaired[20,21]. More- over, antisense-mediated depletion of the key enzymes involved in jasmonate biosynthesis, such as LOX and AOS, reduced herbivory induction of plant defenses and increases weight gain of insect pests[5,23]. A recent report by Howe's team, using mutants of tomato deficient in jasmonate synthesis or in jasmonate perception, has pro- vided a convincing case for jasmonate acting as mobile signal transmissible through graft junctions[24]. However, most of the evidence come from the study of model dicot plants. The roles of jasmonate-signaling pathway in the herbivore-induced defense of monocot plants are largely unknown.

Herbivory by *S. litura* clearly induced the expression of two key genes, *LOX* and *AOS*, which are involved in the biosynthesis of jasmonates (Fig. 2). Moreover, *S. litura* damage resulted in the expression of *FPS*, *BBPI*, *PAL* and other rice defense-related genes that were induced by aqueous JA treatment and gaseous MeJA treatment, respectively (Fig. 3). The expression profiles of these defense genes in *S. litura* damaged rice leaves were similar to the treatment with jasmonates. These indicated that the JA-related signaling pathway in rice leaves was involved in the *S. litura*-induced chemical defense. Mechanical damage and BPH damage induced the expression of *LOX* gene, but both treatments did not induce the expression of *AOS* gene (Fig. 2). However, BPH damage induced the expression of *PAL*, which is involved in the salicylate signaling pathway, as well as acidic pathogenesis-related protein 1 (*PR-1a*) and Chitinase (*PR-3*) genes (Fig. 3). It was suggested that salicylate-related signaling pathway or other pathways, rather than jasmonate-signaling pathway was involved in the BPH-induced rice plant defense.

Our previous study showed that herbivores with different feeding strategies and feeding habits can trigger distinctly different quantities of volatiles in rice plants[13]. The induction effects of the *S. litura* herbivory on the green leaf volatiles, terpenoids and methyl salicylate were stronger than that of BPH infestation[13]. The present study further confirmed that herbivores with different feeding strategies and feeding habits differently induced the expression of genes that was involved in the biosynthesis of these volatiles. Herbivory by *S. litura*, a folivorous chewing caterpillar, had a stronger induction effect on the expression of the *LOX*, *FPS* and *PAL* genes, which was involved in synthesis of green leaf volatiles, sesquiterpenes and methyl salicylate separately, than the infestation of BPH with a sucking habit (Figs. 2 and 3). Difference in gene expression profiles and herbivore-induced volatiles appeared to be due to different signaling pathways which were activated by herbivores with different feeding strategies and feeding habits.

In their natural habitat, plants are attacked by a number of organisms simultaneously during their life span, including insect herbivores and pathogens. They response to these attacks by inducing resistance that can protect themselves against future damage. Therefore, induced-resistance of plant to pathogens and to herbivores may interact with each other[25]. At present, the relationship between herbivore- and pathogen-induced resistance appears to be idiosyncratic and to depend upon the particular species of plant, insect herbivore and pathogen involved[25,26]. It is generally assumed that there are trade-offs (or antagonism) between herbivore- and pathogen-induced resistance. That is, herbivore damaged plant is more susceptible to pathogen and pathogen infested plant is more susceptible to herbivore[25,26]. Conversely, cross-resistance between herbivores and pathogens was reported in other studies[25]. Two suits of responses that are important in plant resistance have JA and salicylic acid (SA) as the key signal components according to the study of the model dicot plant[5—8,19]. Jasmonate-mediated responses and salicylate-mediated responses (also known as SAR) are necessary for plant resistance against herbivores and pathogens, respectively. Molecular interactions in the expression of the JA and SA pathways can prevent the simultaneous expression of each pathway[5—8,19]. However, the present study showed that *S. litura* and BPH herbivory and JA treatment induced the expression of the PRs genes and *PAL* gene, which is involved in the synthesis of the salicylates (Fig. 3). It was indicated that there were some pathogens in the herbivore oral secretions, which elicited the expression of these pathogen defense genes. On the other hand, it suggested a cross-talk between the JA and SA pathways in the herbivore- and pathogen-induced rice plant defense. Our next work will focus on the study of the interactions between herbivore- and pathogen-induced rice plant defense and the signaling pathways involved in these defenses.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Grant Nos. 30000114 and 30270137).

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(Received March 3, 2003; accepted May 21, 2003)