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The Role of p38 MAPK, JNK, and ERK in Antibacterial Responses of *Chilo suppressalis* (Lepidoptera: Crambidae)

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Abstract

The mitogen-activated protein kinases (MAPKs) are conserved signal transduction pathways and broadly responsible for bacterial infection from yeast to mammals, and virus, fungi, and bacteria, specifically *Bacillus thuringiensis*, to insects. But little is known about the MAPK pathways in antibacterial responses in *Chilo suppressalis* (Walker), an important lepidopteran pest of rice. In this study, we used the bacteria of *Bacillus thuringiensis*, *Escherichia coli*, and *Staphyloccocus aureus* to infect *C. suppressalis* larvae, and the responses of MAPK pathways were analyzed. The results showed that *E. coli* infection induced the up-regulated expression of *Csp38* and *CsERK1* at 24 h postinfection (pi). Meanwhile, injection of *B. thuringiensis* and *S. aureus* resulted in strong activation of *Cs*JNK phosphorylation at 3 h pi. These results suggest that MAPK signaling pathways play important functional roles in antibacterial responses in *C. suppressalis* larvae.

Key words: p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, extracellular signal-regulated kinase, *Chilo suppressalis*, antibacterial responses

Mitogen-activated protein kinase (MAPK) cascades are serine–threonine protein kinases that regulate a variety of cellular processes (Zhang and Liu 2002, Roux and Blenis 2004, Huang et al. 2009). It had been well characterized that multicellular organisms have three subfamilies of MAPKs containing extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (Johnson and Lapadat 2002). The p38 MAPK is generally thought to be responsible for transmitting the signal induced by inflammatory cytokines and environmental stresses (Ono and Han 2000, Ashwell 2006). c-Jun N-terminal kinase is involved in immune defense to virus or bacteria, and ERKs are resistant to abiotic stresses in multicellular organisms (Katsuma et al. 2007, Li et al. 2012).

Mitogen-activated protein kinase pathways are well known to play pivotal roles in regulating immune responses in mammals (Dong et al. 2002, Zhang and Dong 2005). This pathway was also reported to act as the key regulator to start the defense process against *Bacillus thuringiensis* (Bt) Crystal (Cry) 5B toxin in *Caenorhabditis elegans* (Huffman et al. 2004, Kao et al. 2011). RNA silencing of p38 accelerates virus replication and viral gene transcription in *Litopenaeus vannamei*; the results indicated that p38 MAPK may play a key role in response to virus (Shi et al. 2014). In insects, p38 MAPK has since been implicated in insect defense against Bt Cry toxins (Cancino-Rodezno et al. 2010). Meanwhile, p38 and JNK pathways participated in *Drosophila* host defense against pathogenic bacteria and fungi (Bond and Foley 2009, Chen et al. 2010). Recently, it has been found that infection of *Galleria mellonella* with *B. thuringiensis* led to activation of p38 MAPK and JNK (Wojda et al. 2014).

The *Chilo suppressalis* (Walker) is one of the most important lepidopteran pests in Asia that cause great economic losses in rice production (Goto et al. 2001, Zhu et al. 2007, Hou et al. 2009, Xu et al. 2011). Chemical pesticides are the primary strategy for pest control, which lead to pest resistance and environmental pollution (Zibaee et al. 2009). Integrated pest management through pathogenic bacteria may be the correct selection and requires understanding the molecular mechanisms of susceptibility and resistance to pathogen (Chouvenc et al. 2009). For this reason, we test the hypothesis that MAPK signaling pathways regulate defense responses to bacterial infection in *C. suppressalis*; the results showed that bacteria could induce the up-regulation of the MAPK genes postinjection and the protein phosphorylation was activated by bacteria. Hence, our results suggest that MAPK pathway is involved in response to bacteria in *C. suppressalis*.

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Table 1. Specific primers used in the experiments

Primer name	Orientation	Primer sequence (5′-3′)
EF-1-F	Sense	TGAACCCCCATACAGCGAATCC
EF-1-R	Antisense	TCTCCGTGCCAACCAGAAATAGG
P38-F	Sense	TCATTTACGCGGTTTTGAGTGCAA
P38-R	Antisense	TGCAAACTGTCGATCGCTGAA
JNK-F	Sense	GGCAGCTACGATACTGGCAT
JNK-R	Antisense	ACTCCCGATGTTTCTGCGTT
ERK1-F	Sense	TGCCTGCCTATATTTCGGCAACTT
ERK1-R	Antisense	CCGGTGGAAGGGTGAGGTTC
ERK2-F	Sense	CTGCGTCTGTACGGGAGTGTC
ERK2-R	Antisense	CGAACAGTTATTTGCCACCAGAAGT

Materials and Methods

Insect Rearing

Chilo suppressalis larvae were collected in Dawu County, Hubei province, China, in 2012 and maintained in a laboratory for 4 yr. Larvae were reared on an artificial diet (Han et al. 2012); the lab conditions were maintained at 28 ± 1 °C, >80% relative humidity, and a photoperiod of 16:8 (L:D) h.

Real-Time Quantitative PCR

To examine the effect of bacteria on MAPK activation, the gram-positive bacteria *S. aureus*, *B. thuringiensis*, and gram negative bacterium *E. coli* were used in this study. Bacteria were incubated overnight in LB media in a shaking incubator at 37 °C and 180 rpm. Bacterial cultures were then centrifuged (10,000 rpm, 4 °C) for 5 min and the pellets were resuspended in PBS (phosphate-buffered saline) buffer. Bacterial density in the PBS buffer was adjusted to obtain an optical density (OD) of 1.0, and 2 µl bacterial suspension was injected to the third-instar larvae by microsyringe. PBS buffer was used as control. Five larvae from each treatment were sampled at 0, 3, 6, 12, and 24 h postinjection. Total RNA was extracted from the larvae and cDNA was synthesized using the PrimeScript RT reagent kit with gDNA eraser (perfect real time; Takara, Dalian, China) according to the manufacturer's instructions. Gene-specific primer (Table 1) was designed by NCBI profile Server (http://www.ncbi.nlm.nih.gov/tools/primer-blast) for qRT-PCR. The *C. suppressalis* elongation factor-1 (*EF-1*) gene was used as the internal reference (Hui et al. 2011, Zhu et al. 2016). The following standard qPCR protocol was used: denaturing at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 59 °C for 30 s. Real-time quantitative PCR was performed in triplicates for each sample using the SYBR Premix Ex Taq (TaKaRa, Dalian, China) and a Bio-rad Detection iQ2 System. Melting curve analysis from 55 to 95 °C was performed to determine the specificity of qPCR primers. To determine the efficiency of the qRT-PCR primers, a fivefold dilution series of the third-instar larvae cDNA corresponding to 1 µg total RNA was used to produce a standard curve (cDNA concentration vs. Ct), with efficiencies calculated from the slope using linear regression. The corresponding qRT-PCR efficiencies were calculated according to the equation: $E = (10^{[-1/slope]} - 1) \times 100$ (Pfaffl 2001, Radonic et al. 2004; Table 2).

Western Blot Assays

Total protein was extracted from *C. suppressalis* larvae by collecting insects in lysis buffer (300 mM mannital, 5 mM EGTA, 17 mM Tris [pH 7.5], and 1 mM phenylmethanesulfonyl fluoride) from five larvae with different treatment of bacteria at 0, 3, 6, 12, 24 h. Protein concentration was determined by the Bradford method using bovine serum albumin (Bradford 1976) as the standard protein. Protein samples were boiled for 5 min, and 10 µg supernatant was applied to each lane for 8% Sodium dodecyl sulfate-polyacrylamide gelelectrophoresis SDS-PAGE and then transferred to polyvinylidine difluoride (PVDF) membranes (Bio-Rad, California, USA). For western blots, we used anti-phospho-p38, -ERK, and -JNK as the primary antibodies at a 1:1,000 dilution according to the manufacturer's instructions (Cell Signaling Technology, Danvers, USA); meanwhile, total -p38, -JNK, and -ERK antibodies were used to detect the total protein, respectively. Goat anti-rabbit lgG horseradish peroxidase (HRP)-linked antibody was used as the second antibody at a 1:5,000 dilution, followed by enhanced chemiluminescent ECL chemiluminescence detection kit (Fermentas, Thermo Fisher Scientific, Waltham, USA) and exposed to film.

Table 2. Primer specifications for optimized qRT-PCR amplification of *C. suppressalis*

Gene name	Efficiency	R^2	Slope
CsEF1	99.8%	0.992	−3.326
Csp38	100.9%	0.994	−3.300
CsJNK	103.9%	0.995	−3.235
CsERK1	99.9%	0.992	−3.325
CsERK2	103.5%	0.980	−3.242

Data Analysis

Quantitative expression data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). Means and variances of treatments were analyzed in one-way ANOVA by SPSS program for windows (SPSS, Chicago, IL).

Results and Discussion

Effect of Bacteria on *C. suppressalis* p38 MAPK Pathway Activation

Mitogen-activated protein kinase is an important component of signaling pathways within cells for its role in transmitting stress signals (Mizutani et al. 2003a,b; Fujiwara and Denlinger 2007; Gorovits and Czosnek 2008; Li et al. 2012; Moshe et al. 2012). Extensive studies revealed that p38 MAPK signaling has a critical role in the cellular immune response to bacteria (Hoefen and Berk 2002, Chen et al. 2010). In the present paper, the level of gene expressions and phosphorylation status were detected after bacterial infection in *C. suppressalis* larvae. As shown in Fig. 1A, infection of *E. coli* resulted in up-regulation of *Csp38* gene at 12 h to approximately twofold and 24 h to fivefold compared to PBS (control). Up-regulation of *Csp38* gene to 2.6-fold was also found at 24 h after *S. aureus* infection. Moreover, the protein of total p38 and phospho-p38 signals were monitored by western blot in similar treatments; the results suggest that the p38 protein phosphorylation was elevated at 12 h by *B. thuringiensis* treatment (Fig. 2A). In agreement with other studies, our results showed that p38 pathway may represent a specific response to pore-forming toxin produced by bacterium *B. thuringiensis* in nematode and insect (Huffman et al. 2004, Cancino-Rodezno et al. 2010, Kao et al. 2011); these results are also similar to other report, in which p38 MAPK could be induced by α-toxin and hemolysin produced by *S. aureus* and *E. coli*, respectively (Kloft et al. 2009, Yan et al. 2013).

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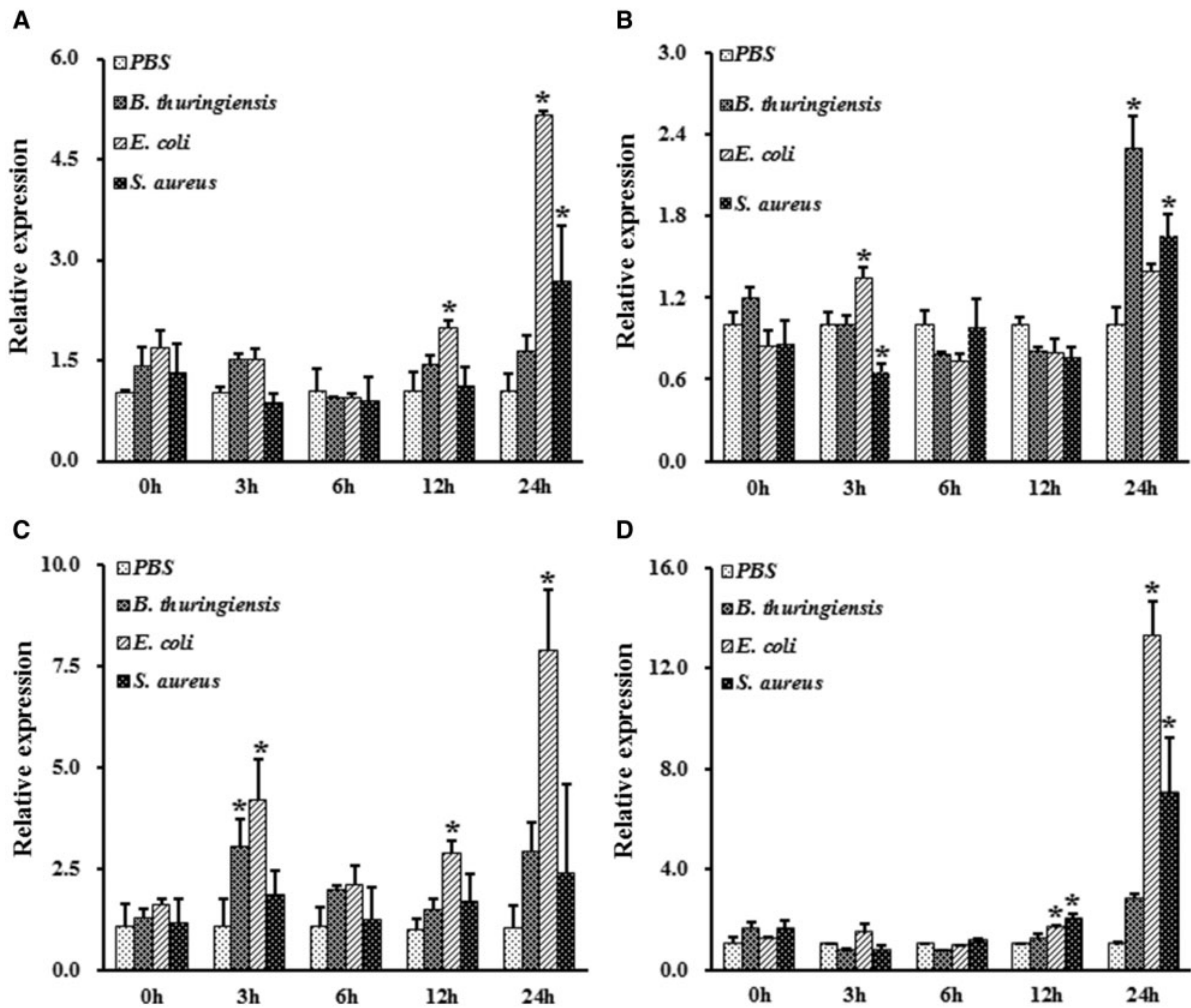

Fig. 1. Effects of bacterial treatment on *C. suppressalis* MAPKs. (**A**) Relative estimates of *Csp38* transcripts from qRT-PCR of *C. suppressalis* larvae treated with bacteria for 0, 3, 6, 12, and 24 h. (**B**) Relative estimates of *CsJNK* transcripts from qRT-PCR of *C. suppressalis* larvae treated with bacteria for 0, 3, 6, 12, and 24 h. (**C**) Relative estimates of *CsERK1* transcripts from qRT-PCR of *C. suppressalis* larvae treated with bacteria for 0, 3, 6, 12, and 24 h. (**D**) Relative estimates of *CsERK2* transcripts from qRT-PCR of *C. suppressalis* larvae treated with bacteria for 0, 3, 6, 12, and 24 h. Relative amounts of genes transcript were normalized to the expression of *EF1*. Each symbol and vertical bar represents the mean ± SE ($n = 3$). Asterisks indicate significant differences ($P < 0.05$; ANOVA).

Responses of *C. suppressalis* JNK Pathway to Bacteria Challenge

Following the bacterial infection treatment, the *CsJNK* transcript level was increased at 24 h posttreatment with *B. thuringiensis* (2.3-fold) and *S. aureus* (1.6-fold) infection. Slight alteration of the *CsJNK* expression was observed after the *E. coli* treatment at 3 h (Fig. 1B). To further confirm whether JNK are involved in the antibacterial responses in *C. suppressalis*, western blot analysis was performed by using anti-total JNK and anti-pho JNK to monitor the level of protein phosphorylation alterative in JNK. The results indicated that an increase in phosphorylation of JNK was observed in 3 h after infected with *B. thuringiensis*; moreover, increasing level was found at 3 and 24 h after being treated by *S. aureus* (Fig. 2B). Our results suggest that the level of gene expression and protein phosphorylation of JNK was altered by infection with *S. aureus* and *B. thuringiensis*. Hence, JNK may play an important role in host defense response to bacteria. Actually, previous studies have indicated that JNK is involved in response to bacteria and virus (Sluss et al. 1996, Katsuma et al. 2007).

Analysis of *C. suppressalis* ERK1/2 Pathways in Response to *S. aureus*, *B. thuringiensis*, and *E. coli* Immune Stimulations

Current studies suggested that ERK plays a key role in response to environmental stresses (Fujiwara and Denlinger 2007). Here, real-time PCR was performed to assess the expression levels of *CsERK1* and *CsERK2* in response to three bacteria. The results revealed that the expression level of *CsERK1* was up-regulated at 3 h (4.2-fold), 12 h (2.8-fold), and 24 h (7.9-fold) after *E. coli* infection (Fig. 1C), and increased after 3 h when injected by *B. thuringiensis* to approximately threefold compared to control. *CsERK2* was induced by *E.coli* and *S. aureus* at 12 h and 24 h pi (Fig. 1D). Phosphorylation alterative was also tested in response to bacteria, as shown in Fig.

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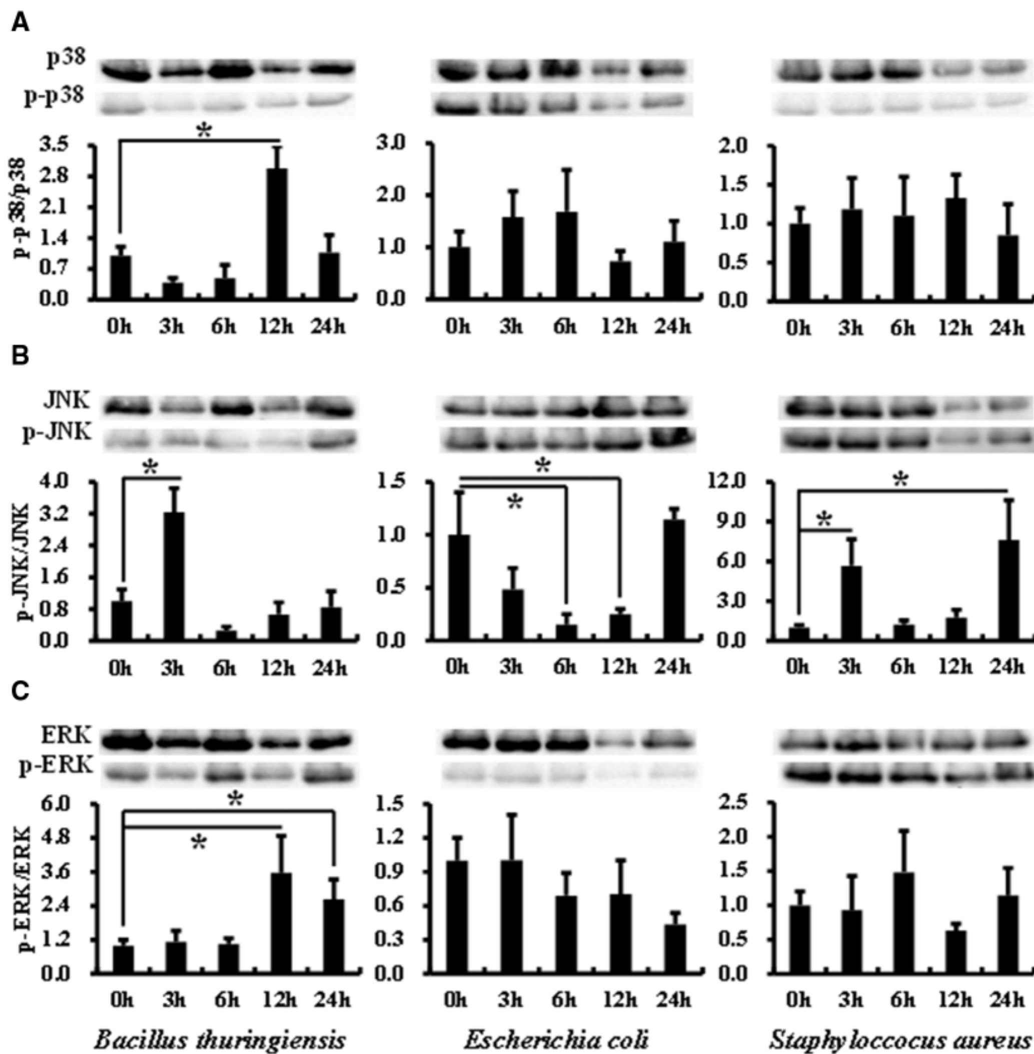

Fig. 2. Phosphorylation level of MAPKs in *C. suppressalis* analyzed by western blot. (**A**) Phosphorylation level of P38 in *C. suppressalis* larvae treated with bacteria at 0, 3, 6, 12, and 24 h postinfection. (**B**) Phosphorylation level of JNK in *C. suppressalis* larvae treated with bacteria at 0, 3, 6, 12, and 24 h postinjection. (**C**) Phosphorylation level of ERK in *C. suppressalis* larvae at 0, 3, 6, 12, and 24 h postinjection. Asterisks indicate significant differences ($P < 0.05$; ANOVA).

2C; the protein phosphorylation level of ERK was elevated at 12 h and 24 h by *B. thuringiensis* treatment. The results indicated that ERK has involvement in response to bacterial infection (Figs. 1 and 2). It has also been reported that ERK plays a critical role in response to microorganism in previous studies (Katsuma at al. 2007, Li et al. 2013). Our data, together with other works, support the ERK involvement in response to bacteria.

In previous studies, it has been reported that the entomopathogenic fungi can physiologically induce both cell-mediated immune responses (such as changes in hemocyte numbers and nodule formation) and humoral immunity, where activation of phenoloxidase cascade is responsible for fighting against toxin derived from entomopathogen (Zibaee and Malagoli 2014). Actually, these defensive responses might also be regulated by genetic signals in insects, for example, in *Drosophila melanogaster*, Toll cascade is triggered by microorganism-activated Spatzle-processing enzyme to produce antimicrobial peptides (Jang et al. 2006). However, in *C. suppressalis*, further research is required to determine the role and mechanism of MAPK pathway defending against bacteria.

In conclusion, our results suggest that *Cs*p38, *Cs*JNK, and *Cs*ERK1/2 are involved in response to bacterium *B. thuringiensis*, *S. aureus*, and *E. coli* infection. These evidences support that the MAPKs pathway play critical roles in *C. suppressalis* immunity. This study can gain insight into insect immune response to different bacteria, but the mechanism of *C. suppressalis* MAPK pathways regulation in infection remains unknown and needs deep research.

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