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Phospholipase A_2 Inhibitors in Bacterial Culture Broth Enhance Pathogenicity of a Fungus *Nomuraea rileyi*

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An entomopathogenic fungus, *Nomuraea rileyi*, was isolated and its identity was confirmed by its internal transcribed spacer DNA sequence. The isolated *N. rileyi* exhibited a specific pathogenicity to lepidopteran species. This study was focused on enhancing the fungal pathogenicity by using immunosuppressive agents. In response to infection of *N. rileyi*, *Spodoptera exigua* larvae significantly induced catalytic activity of phospholipase A_2 (PLA_2) in three immune-associated tissues, namely hemocytes, fat body, and hemolymph plasma. Furthermore, the infected *S. exigua* larvae induced transcription of several antimicrobial peptide (AMP) genes. Two entomopathogenic bacteria, *Xenorhabdus nematophila* (Xn) and *Photorhabdus temperata* subsp. *temperata* (Ptt), possessed specific PLA_2-inhibitory activities and their culture broths significantly inhibited the enzyme activities in hemocytes, fat body, and plasma of *S. exigua*. In addition, the bacterial metabolites inhibited transcription of AMP genes in *S. exigua* that would normally respond to the immune challenge by *N. rileyi*. The immunosuppressive effect of Xn or Ptt bacterial broth resulted in significant enhancement of the fungal pathogenicity against late instar larvae of *S. exigua* and *Plutella xylostella*. The effect of such a mixture was confirmed by field assay against two lepidopteran species. These results suggest that the bacterial and fungal mixture can be applied to develop a novel biopesticide to control lepidopteran species.

Keywords: *Nomuraea rileyi*, *Xenorhabdus nematophila*, *Photorhabdus temperata temperata*, *Spodoptera exigua*, immune, eicosanoids

Introduction

Entomopathogenic fungi (EPF) have been recognized as potent biological agents for control of various insect pests (Shah and Pell, 2003). There are several products currently commercially available, including products based on *Beauveria bassiana*, *Metarhizium anisopliae*, *Paecillomyces farinosus*, *Lecanicillium lecanii*, and *Nomuraea rileyi*. Once fungal conidia germinate on the target insect integument by penetrating the cuticle, the entering hyphae multiply by budding and septa formation in the hemocoel (Kumar *et al.*, 1997). Ramification of hyphal bodies in the hemocoel causes mummification and finally kills the insects.

An EPF, *N. rileyi*, is well known to cause a green muscardine in sericulture and inhibits molting of *Bombyx mori* larvae (Kawakami, 1972; Qin *et al.*, 2009). It is a cosmopolitan species and has been isolated from a variety of lepidopteran insect hosts (Ignoffo, 1981). However, the restricted spray conditions needed for its application and its relatively slow pathogenesis prevent its wide spread use to control insect pests. To be an effective biological control agent, *N. rileyi* has to stably germinate on insect cuticle and subsequently overcome insect defense immune responses (Boucias *et al.*, 1982; Pendland and Boucias, 1985, 2000). Recent efforts to increase efficiency of fungal germination of *N. rileyi* have been focused on selection of strains having high levels of chitin-degrading enzymes and development of penetration-stimulating agents (Supakdamrongkul *et al.*, 2010; Noda *et al.*, 2011). In contrast, little research is underway to develop a technique to suppress antifungal immune responses of the host.

Insect immune responses consist of cellular and humoral factors that are innately programmed to be expressed after nonself recognition of specific pathogen molecular patterns (Lemaitre and Hoffmann, 2007; Strand, 2008). Usually, the nonself recognition signal propagates to nearby immune effectors, such as hemocytes and fat body, by immune mediators (Gillespie *et al.*, 1997). Eicosanoids play a crucial role in mediating immune signals in response to various microbial infections (Stanley, 2005). Eicosanoids are synthesized from phospholipids by catalytic activity of phospholipase A_2 (PLA_2), which is activated by immune challenge (Tunaz *et al.*, 2003; Shrestha and Kim, 2010). Thus, eicosanoid biosynthesis inhibitors can suppress immune responses, which would enhance the potency of microbial pathogens (Tunaz, 2006; Park and Kim, 2011).

Two entomopathogenic bacteria, *Xenorhabdus nematophila* (Xn) and *Photorhabdus temperata* subsp. *temperata* (Ptt), are known to synthesize and secrete specific PLA_2 inhibitors, which suppress immune responses of infected insects (Kim *et al.*, 2005; Kim and Kim, 2011). This study was focused on enhancing the fungal pathogenicity of *N. rileyi* recently collected under field conditions. To this end, immune responses against *N. rileyi* were analyzed in a natural host, *Spodoptera exigua*. Then, the bacterial metabolites of Xn and Ptt were applied to suppress the antifungal immune responses of *S. exigua*. Finally, the fungal and bacterial mixture was applied to field populations of two lepidopteran pests, *S.*

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exigua and *Plutella xylostella*.

Materials and Methods

Culture of insects and bacteria

The beet armyworm, *S. exigua*, used in this study originated from a field population infesting Welsh onion (*Allium cepa*) in Andong, Korea. The larvae were reared on an artificial diet (Goh *et al*., 1990) at 25°C and the adults were fed 10% sucrose. Larvae of the diamondback moth, *Plutella xylostella*, were reared on cabbage. Both bacterial species, Xn and Ptt, were cultured in Luria-Bertani (LB) broth at 28°C on a shaking incubator at 200 rpm for 12 h. The samples were frozen at -70°C and dried at a pressure of 2×10^{-4} Torr for 96 h using a freeze-drier (PVTFD, Ilshin Biobase, Korea). The freeze-dried samples contained 3.6×10^{11} CFU/mg for Xn and 8.7×10^{10} CFU/mg for Ptt. The dried samples were kept at 4°C until use. For field bioassay (described below) the bacterial concentration was given as ppm of the freeze-dried sample.

Culturing *N. rileyi*

N. rileyi was isolated from infected *S. exigua* in a Welsh onion field. For immunological assay, the fungal hyphae and spores were cultured and purified. *N. rileyi* was cultured on solid potato dextrose agar (PDA) medium (5% potato extract, 0.5% dextrose, 1.7% agar) at 25°C for 7 days. The cultured fungal colonies were resuspended with phosphate-buffered saline (PBS, 50 mM phosphate, 0.7% NaCl, pH 7.0). The hypha and spore mixture was cleaned using a filter paper (pore size=20 μm) and the resulting flow-through solution was used for immune-challenge. For field assay, *N. rileyi* was cultured in liquid medium (5% potato extract, 0.5% dextrose) for 168 h at 25°C with 200 rpm. The cultured fungi were collected by centrifugation and the resulting pellet was freeze-dried as described above. The freeze dried sample contained 3.2×10^{8} blastospores/mg. Fungal concentration was described as the number of spores (or blastospores) per ml for immune assay or by ppm of the freeze-dried fungal sample for field assay.

DNA extraction and sequencing of the internal transcribed spacer (ITS) of *N. rileyi*

After collection of growing fungi from the PDA medium, the mixture of hyphae and spores were ground in 400 μl saline solution (0.8% NaCl containing 0.0125% Tween 20). Ten microliter of proteinase K (0.5 mg/ml) was added to the fungal extract and incubated for 10 min at 50°C. The digested solution was subjected to phenol extraction to get rid of proteins and then ethanol precipitation to purify DNA. The ITS region was amplified with universal primers (5′-TTG ATT ACG TCC CTG CCC TTT-3′ and 5′-TTT CAC TCG CCG TTA CTA AGG-3′). The PCR used 35 cycles consisting of 1 min at 94°C, 1 min at 46°C, and 2 min at 72°C. The resulting PCR product was cloned into pCR2.1 vector (Invitrogen, USA). The insert was bidirectionally sequenced using the dideoxy method (Macrogen, Korea).

Enzyme assay of PLA_2

PLA_2 activity was measured by spectrofluorometry using a pyrene-labeled phospholipid [1-hexadecanoyl-2-(1-pyrene-decanoyl)-*sn*-glycerol-3-phosphatidylcholine] as a substrate in the presence of bovine serum albumin (BSA) (Radvanyi *et al*., 1989). The fluorometric phospholipid was dissolved in ethanol to prepare a 10 mM stock solution, and 10% BSA was prepared in sterilized distilled water. The reaction mixture (2 ml) was prepared in a cuvette by sequentially adding 1,946 μl of 50 mM Tris-HCl buffer (pH 7.0), 20 μl of 10% of BSA, 12 μl of 1 M $CaCl_2$, and 20 μl of enzyme extract. The reaction was initiated by addition of 2 μl of 10 mM pyrene substrate and subsequently fluorescence intensity was monitored with an Aminco Bowmen Series 2 luminescence spectrometer (FA257, Spectronic Instruments, USA) using excitation and emission wavelengths of 345 and 398 nm, respectively. The specific enzyme activity was calculated in pmol/min according to the method of Radvanyi *et al.* (1989). To check inhibition of its activity, 2 μl of test chemical was added to the reaction mixture.

RT-PCR of antimicrobial peptide (AMP) genes

Total RNAs were extracted from the treated larvae using Trizol reagent (Invitrogen) followed by isopropanol precipitation. The resulting RNA pellet was washed with 70% ethanol and resuspended in diethylpyrocarbonate-treated water. First strand cDNA was synthesized with 1 μl of the extracted total RNA using RT-Premix containing oligo-dT (5′-CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GC TTT TTT TTT TTT TTT T-3′, Intron, Korea). AMP genes were amplified with gene-specific primers: Toll

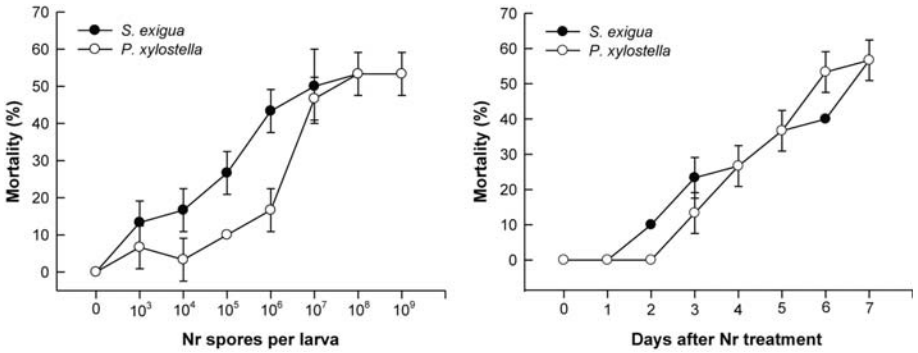

Fig. 1. Pathogenicity of *N. rileyi* (Nr). Dose response and time course of Nr pathogenesis against two lepidopteran species, *P. xylostella* and *S. exigua* at the final instar stage. Each treatment was replicated three times. Each replication consisted of 10 larvae. Error bars represent standard deviations.

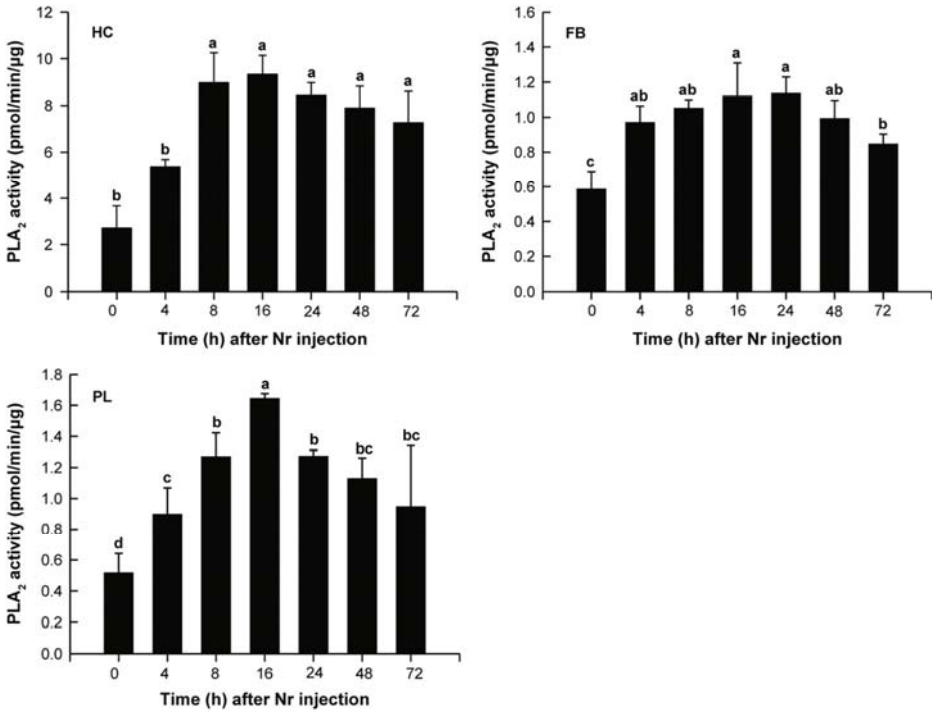

Fig. 2. Induction of enzyme activities of different PLA_2s extracted from hemocytes (HC), plasma (PL), and the fat body (FB). An entomopathogenic fungus, *N. rileyi*, was injected into the hemocoel of fifth instar *S. exigua* in a dose of 2.5×10^5 spores and incubated for different periods at 25°C. Each measurement consisted of three independent replications. Different letters above standard deviation bars indicate significant difference among means at Type I error =0.05 (LSD test).

receptor (*SeToll*, 5′-GAG TGC GAC TGT ACA ATG G-3′ and 5′-GGT CGC ATC CAT CGG TAT TC-3′), apolipophorin III (*SeApoLp III*, 5′-ATG GTC GCC AAG TTG TTC GTG-3′ and 5′-CTC CTG CGC GGT GTT CTG CA-3′), transferrin I (*SeTf I*, 5′-GTC CCT CTC TGT CCT GAA GG-3′ and 5′-CAG AAA CAC GAA GAA AGA TGG-3′), transferrin II (*SeTf II*, 5′-GAT GTT CTG GCG CAG CTG TC-3′ and 5′-CCG GCT GAA CGC AAA CAC AG-3′), attacin A (*SeAttA*, 5′-GCT TTC CTC TCC AGG AAT ATG-3′ and 5′-CCT TAG AGT AAA TCC AGT GG-3′), attacin B (*SeAttB*, 5′-TCC CGA ATG TGC CCA ACT TC-3′G and 5′-GAA AGA TCT GCC GAA AGT AAG-3′), gloverin (*SeGlv*, 5′-CGT GGA CAT CTT CAG GGC C-3′ and 5′-GTC GTG TTC AAT GCC ACC G-3′), hemolin (*SeHem*, 5′-AAG ACC AGG GCG AGT ACA AG-3′ and 5′-AGC GAC ATG AAC CAA GGT TTC-3′), and gallerimycin (*SeGal*, 5′-TCA GTC ATG AAG GCT TGC GTA-3′ and 5′-TCG CAC ACA TTG GCA TCC ATT G-3′). PCR was performed with 35 cycles of denaturation (94°C, 1 min), annealing (50°C, 1 min), and extension (72°C, 1 min).

Laboratory bioassay

Bioassay of *N. rileyi* pathogenicity to the last instar larvae used a leaf-dipping method. Cabbage leaves were cut into 1×1 cm squares and soaked in different concentrations of a mixture treatment of *N. rileyi* with bacterial metabolites of benzylideneacetone (BZA), Xn or Ptt. BZA was purchased from Sigma-Aldrich Korea (Korea). The treated leaves were fed to the last instar larvae for 24 h and then fresh nontreated leaves were supplied until larval death. Each pathogen treatment was replicated three times with 10 larvae per replicate. Mortality was determined every 24 h after treatment.

Field bioassay

Cabbage fields infested with *S. exigua* or *Plutella xylostella*

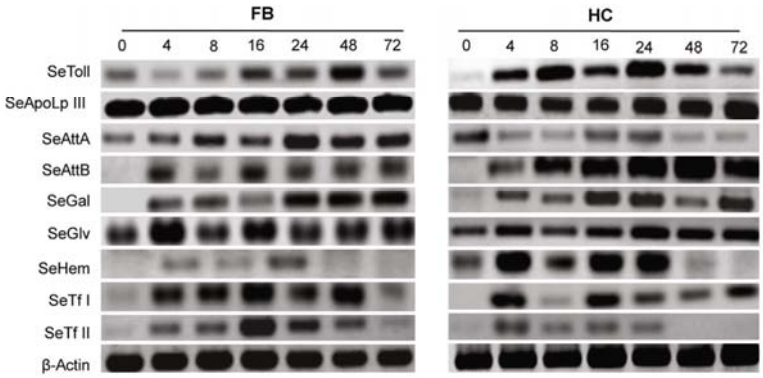

Fig. 3. Suppression of antimicrobial peptide (AMP) gene expression in the fat body (FB) and hemocytes (HC) of 5th instar *S. exigua* in response to infection by *N. rileyi* (Nr, 2.4×10^5 spores/ larva). The transcription of AMP genes was analyzed by semi-quantitative RT-PCR. Analyzed AMPs include Toll receptor (*Toll*), apolipophorin III (*ApoLp III*), attacin (*Att*), gallerimycin (*Gal*), gloverin (*Glv*), hemolin (*Hem*), and transferrin (*Tf*). Expression of β-actin was analyzed to confirm the integrity of cDNA preparation.

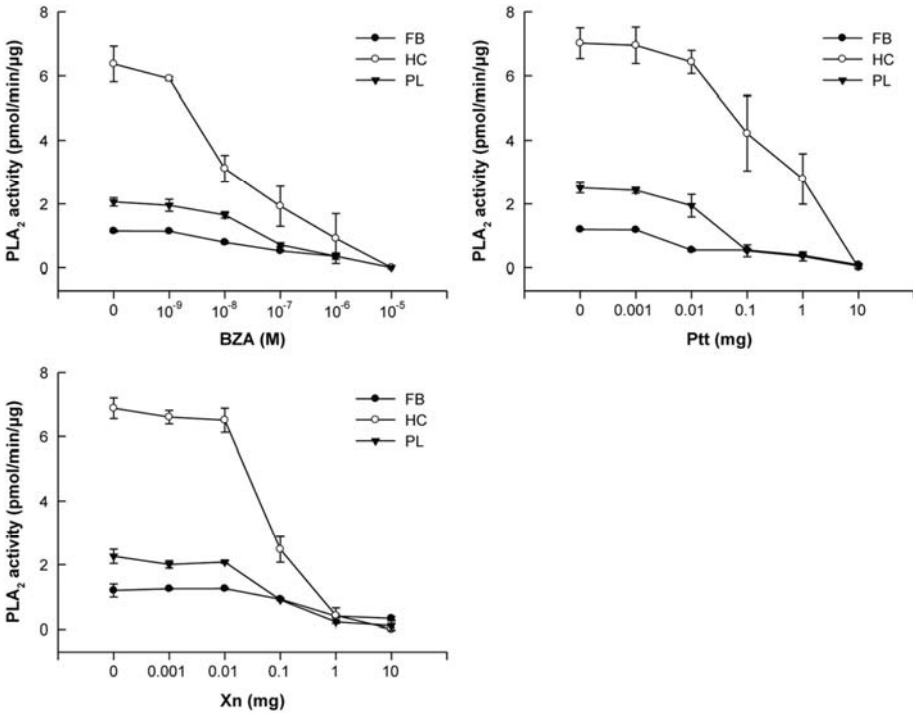

Fig. 4. Inhibitory effects of benzylideneacetone (BZA) and bacterial metabolites on phospholipase A_2 (PLA_2) activities of different tissues isolated from 5th instar *S. exigua*. A reaction mixture (2 ml) consisted of 40 μl enzyme extract, 2 μl pyrene substrate, 4 μl test chemical, 20 μl BSA, and 1,934 μl PBS. Test chemicals were BZA, bacterial metabolites of *X. nematophila* (Xn) or *Photorhabdus temperata* subsp. *temperata* (Ptt). Each measurement was replicated three times. Different letters above standard deviation bars indicate significant difference among means at Type I error=0.05 (LSD test).

were selected in Andong, Korea. Five treatments consisted of untreated, *N. rileyi* (1,000 ppm), *N. rileyi* (1,000 ppm) + BZA (1,000 ppm), *N. rileyi* (1,000 ppm) + Xn (1,000 ppm), *N. rileyi* (1,000 ppm) + Ptt (1,000 ppm). Experimental units in each treatment were assigned by a randomized block design with three replications. Each unit consisted of three cabbages initially infested with at least 60 larvae (> 3rd instar for *P. xylostella* or > 4th instar for *S. exigua*). After counting the starting population, the test solutions were sprayed with ≈ 100 ml volume per experimental unit. Living larvae were counted five days after spraying. Control efficacy was estimated by relative mortality using comparison to that of untreated plots.

Statistical analysis

All laboratory studies were performed in three independent replicates and plotted by mean±standard deviation using Sigma Plot (Systat Software, Inc., USA). Treatment means and variances were analyzed by one-way ANOVA by PROC GLM of the SAS program (SAS Institute, 1989). The field assay treatments were analyzed by two-way ANOVA. All means were compared by least squared difference (LSD) tests at Type I error=0.05.

Results

Field collection of *N. rileyi*

Larvae of *S. exigua* infected with a fungal pathogen were collected in the field. Cadavers were covered with green-colored hyphae and conidia. Hemocoel of the infected larvae was filled with mycelia. After culture of the fungus in PDA medium, its total DNA was extracted and the ITS region was sequenced (GenBank accession no.=FJ824809). The ITS re-

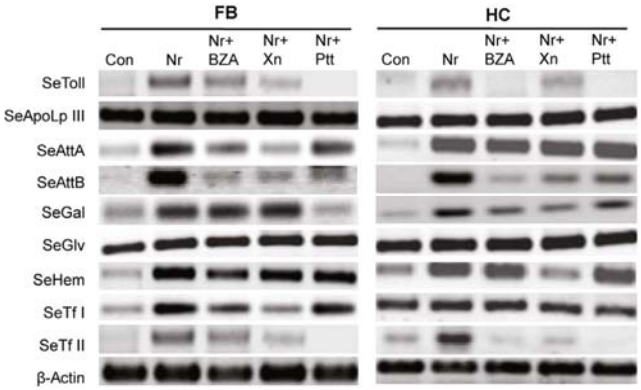

Fig. 5. Inhibitory effects of benzylideneacetone (BZA) and bacterial metabolites on transcription of antimicrobial peptides (AMP) in response to infection by *N. rileyi* (Nr). Nr was injected into larvae of *S. exigua* at 2.5×10^5 spores per larva with or without test chemicals. BZA injection was 10 μg. Injection of bacterial metabolites of *X. nematophila* (Xn) or *Photorhabdus temperata* subsp. *temperata* (Ptt) was 100 ng. At 8 h after the fungal treatment, the hemocytes (HC) and fat body (FB) were isolated and subjected to RT-PCR. Analyzed AMPs include Toll receptor (*Toll*), apolipophorin III (*ApoLpIII*), attacin (*Att*), gallerimycin (*Gal*), gloverin (*Glv*), hemolin (*Hem*), and transferrin (*Tf*). Expression of β-actin was analyzed to confirm the integrity of cDNA preparation.

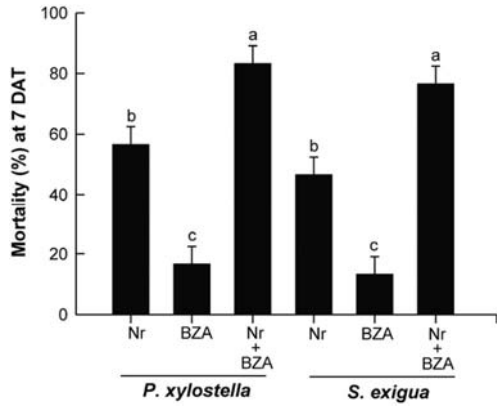

Fig. 6. Synergistic pathogenic effects of *N. rileyi* (Nr) with benzylideneacetone (BZA) on mortalities of 4th instar *P. xylostella* and 5th instar *S. exigua* larvae. Fungal treatment used a leaf-dipping bioassay method. Mortality was measured at 7 days after treatment (DAT). Each treatment was replicated three times. Each replication consisted of 10 larvae. Different letters above standard deviation bars indicate significant difference among means at Type I error=0.05 (LSD test).

gion included nucleotide sequences of a partial 18S rRNA, spacer, and a partial 5.8S rRNA. The ITS nucleotide sequence was highly matched to that of *N. rileyi* (Identity= 100%; Match score=257 bits; E value=3e-164).

Pathogenicity of the fungal isolate was specific to lepidopteran species because it did not show any pathogenicity against coleopteran (*Tribolium castaneum*) and dipteran (*Liriomyza trifolii*) species (data not shown). The pathogenicity of the fungal isolate was dependent on concentration when used against *S. exigua* and *P. xylostella* larvae (Fig. 1). However, it showed a relatively slow mortality rate and did not exceed 60% in both larval species. Based on the ITS sequence and pathogenic symptoms, the collected fungus was confirmed to be *N. rileyi*.

N. rileyi induces both cellular and humoral immune responses of *S. exigua*

A previous report on a cellular immune response against *B. bassiana* showed that hemocyte nodulation was dependent on PLA_2 activity (Park and Kim, 2011). In this study, PLA_2 activities induced in response to infection of *S. exigua* by *N. rileyi* were analyzed in three different immune-associated tissues, hemocytes, fat body, and plasma (Fig. 2). In all three tissues, the fungal infection increased their PLA_2 activities. The increased level of PLA_2 activity was maintained for at least three days in the tissues, with a slight decrease 24 h post-infection.

In response to fungal infection, the Toll receptor gene was highly expressed in both hemocytes and fat body (Fig. 3). The fungal infection also induced expression of AMP genes. With the exception of *apolipophorin III* and *gloverin*, all the measured AMP genes had highly induced transcription in hemocytes and the fat body.

Bacterial metabolites inhibit immune responses of *S. exigua* against *N. rileyi*

BZA is a metabolite of Xn (Ji *et al*., 2005). Our current study shows that Ptt also produces BZA and secretes it into culture broth (Seo *et al*., 2010). BZA significantly inhibited catalytic activities of PLA_2 from the hemocyte, fat body, and plasma (Fig. 4). As expected, Xn or Ptt bacterial culture broth also inhibited PLA_2 in a dose-dependent manner.

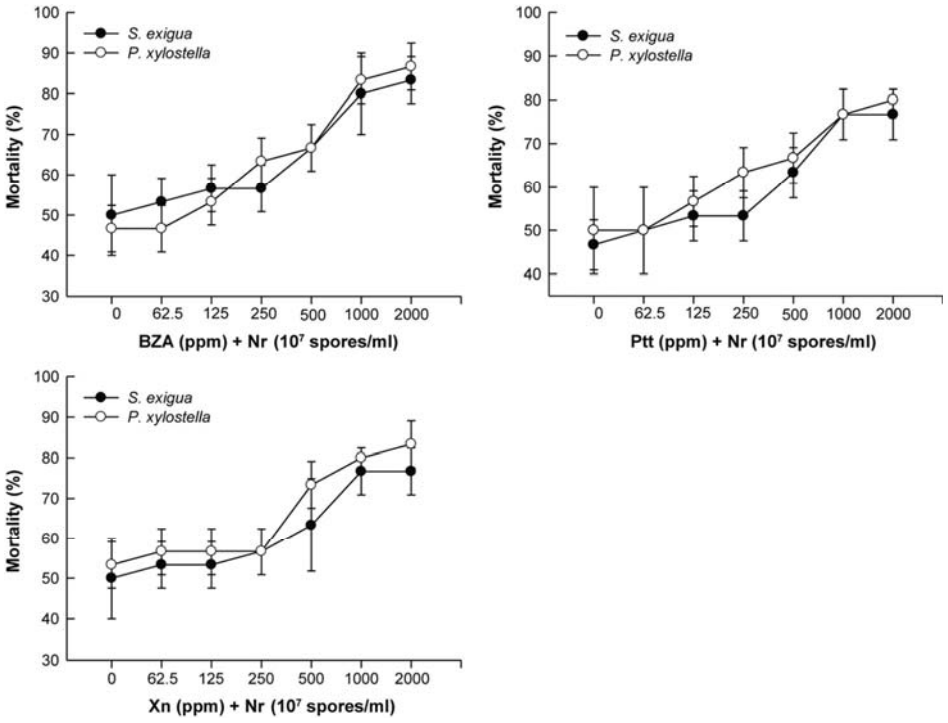

Fig. 7. Effect of different doses of PLA_2 inhibitors on pathogenicity of *N. rileyi* (Nr) to 4th instar *P. xylostella* and 5th instar *S. exigua*. Fungal treatment used a leaf-dipping bioassay method. Mortality was measured at 7 days after treatment (DAT). Each treatment was replicated three times. Each replication consisted of 10 larvae. Different letters above standard deviation bars indicate significant difference among means at Type I error =0.05 (LSD test).

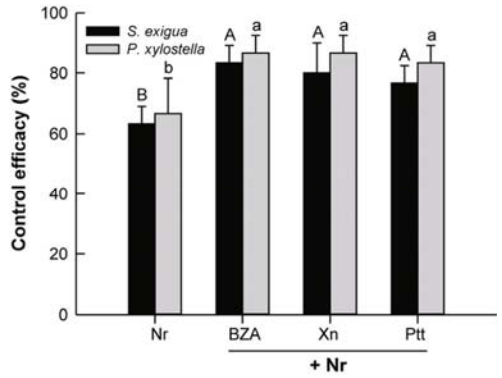

Fig. 8. Field assay of a treatment of *N. rileyi* (Nr) together with bacterial metabolites, either benzylideneacetone (BZA), or used media of *X. nematophila* (Xn) or *Photorhabdus temperata* subsp. *temperata* (Ptt). Nr was applied at a concentration of 10^7 blastospores/ml. Benzylideneacetone (BZA) was used at 1,000 ppm. Freeze-dried culture media of Xn and Ptt respectively were also used at 1,000 ppm. Initial average population of each treatment were 63.3, 83.3, 80.0, and 76.6 individuals in Nr, BZA+Nr, Xn+Nr, Ptt+Nr treatments, respectively. Survival numbers were counted at five days after treatment. Insect control efficacy was reported as a relative mortality based on comparison to untreated plots. Each treatment was replicated three times. Different letters above standard deviation bars in each species indicate significant difference among means at Type I error=0.05.

Transcription of the Toll receptor gene was inhibited by BZA in both the fat body and hemocyte (Fig. 5). BZA also inhibited expression of some AMP genes that were inducible through infection by *N. rileyi*. Transcription of *Attacin B* and *transferring II* was inhibited by BZA in the hemocytes and fat body. Transcription of these two AMPs was also inhibited by both bacterial culture broths. Ptt culture broth especially inhibited *gallerimycin* in the fat body.

Enhancement of *N. rileyi* pathogenicity by bacterial metabolites

The fact that bacterial metabolites suppress the antifungal responses of *S. exigua* suggests that it might be able to enhance pathogenicity of *N. rileyi.* When BZA was applied along with *N. rileyi*, it significantly increased the fungal pathogenicity in the last instar larvae of both *S. exigua* and *P. xylostella* (Fig. 6). With an increase of the bacterial metabolite amounts, pathogenicity of *N. rileyi* significantly increased for larvae of both species (Fig. 7). Based on these dose-mortality responses, we chose 1,000 ppm of bacterial metabolites to be mixed with *N. rileyi* (1,000 ppm). This mixture was applied to field populations of *S. exigua* and *P. xylostella* (Fig. 8). Compared to treatment of *N. rileyi alone*, the mixture treatment significantly enhanced the treatment efficacies against these two lepidopteran species.

Discussion

Insects effectively defend against various microbial pathogens with both cellular and humoral immune factors. Thus immunosuppressive agents may enhance the efficacies of microbial pesticides. This hypothesis was tested in this study using Xn and Ptt bacterial metabolites to enhance pathogenicity of the fungus *N. rileyi*. First, a Lepidoptera-specific EPF was collected from the field and confirmed to be *N. rileyi* by pathogenicity test and DNA sequence analysis. However, its treatment efficacy was not enough for application to control insects, though it showed a specific pathogenicity against lepidopteran species. Second, in response to *N. rileyi*, immune responses were induced in *S. exigua* (a natural host). Third, bacterial metabolites (=used culture broth) of Xn and Ptt could suppress the antifungal immune responses. Fourth, in a laboratory bioassay, treatments using *N. rileyi* together with the bacterial metabolites significantly increased the fungal pathogenicity. The increase of fungal pathogenicity was dependent on the amount of bacterial metabolites. Finally, the bacterial and fungal mixture was applied to field populations of two lepidopteran species. This treatment showed a significant increase in efficacy, compared to fungal treatment alone.

N. rileyi was specific to lepidopteran insects, but its pathogenicity was relatively slow and not enough to effectively control late instar larvae of *S. exigua* and *P. xylostella*. In general, a major obstacle for fungal pathogenicity is having the right conditions for spore attachment and germination on the target insect cuticle (Kosir *et al.*, 1991). The life cycle of *N. rileyi* begins with attachment of conidia to insect integument followed by cuticle penetration. Insect epicuticle contains phenol-stabilized protein covered by a waxy layer containing fatty acids, lipids, and sterols (Anderson, 1979; Pedrini *et al.*, 2007). *N. rileyi* conidia produce cuticle-degrading enzymes, such as chitinase, proteinase, and lipase during the penetration process (Boucias *et al.*, 1982). A host insect-derived factor stimulates the penetration process of *N. rileyi* and this factor was identified as D-erythro-C_{14}-sphingosine (Noda *et al.*, 2010a, 2010b). Later, the penetration-stimulatory factor was applied as an adjuvant for the fungal pesticide by shortening the initial colonization period of the fungi on the target insect (Noda *et al.*, 2011). Another obstacle *N. rileyi* being an effective biological agent is the insect immune response after it enters the hemocoel. To understand the antifungal responses, we needed to analyze the immune responses against *N. rileyi* infection.

Infection by *N. rileyi* induced the immune responses of *S. exigua*. PLA_2s were activated in three immune-associated tissues. Activated PLA_2s catalyze arachidonic acid (AA) release from phospholipids at the *sn-2* position (Burke and Dennis, 2009). AA is then oxidized by cyclooxygenase or lipoxygenase and converted into various eicosanoids, which then mediate both cellular and humoral immune responses (Stanley, 2011). These eicosanoids mediate immune signals in response to fungal infections of *B. bassiana* (Lord *et al.*, 2002) and *M. anisopliae* (Dean *et al.*, 2002). Furthermore, hemocyte nodule formation in response to fungal infection is highly correlated with increase of PLA_2 activity in *S. exigua* (Shrestha and Kim, 2009a; Park and Kim, 2011). Increased PLA_2 activity induces eicosanoid biosynthesis and resulting prostaglandins activate phenoloxidase, which is a key enzyme for the nodulation response, by inducing oenocytoid cell lysis (Shrestha and Kim, 2008). Thus, the activated PLA_2s can be explained in terms of an antifungal response to the infection by *N. rileyi*.

Expression of the *Toll* receptor gene was induced in *S. exigua* by the *N. rileyi* infection. Toll and Imd immune signal pathways are well known in *Drosophila* (Lemaitre *et al*., 1995; Michel *et al*., 2001; Tanji *et al*., 2007). Upon infection by Gram-positive bacteria (Lys-type peptidoglycan) or fungi, the Toll pathway is activated by prior proteolytic activation of proSpätzle in the plasma and its subsequent binding to the Toll receptor leads to an intracellular signaling cascade, which results in degradation of Cactus (an inhibitor of kB) and allows NF-kB (Dif or Dorsal) to migrate into the nucleus to induce expression of AMP genes (Hoffmann, 2003). On the other hand, the Imd pathway is activated by the challenge of Gram-negative bacteria (diaminopimelate-type peptidoglycan), in which cooperative activity of PGRP-LC and PGRP-LE recognition receptors activates an unidentified transmembrane receptor; subsequently, an adaptor protein, Imd, associates with FADD via its death domain (Leclerc and Reichhart, 2004). Thereafter, a sequential activation of TAK1, Dredd, and the IKK complex cleaves inactive NF-kB (Relish), which migrates into the nucleus to induce specific AMPs (Tanji and Ip, 2005). In *S. exigua*, both Toll and Imd signal components have been identified and have been proposed to function in immune signaling pathways like those of *Drosophila* (Hwang, 2011). In this study, infection by *N. rileyi* significantly induced Toll receptor expression of *S. exigua*. Expression induction of Toll receptor in response to fungal infection was also reported in *Bombyx mori* (Wu *et al*., 2010). Indeed, transcription of several AMP genes of *S. exigua* was up-regulated in response to infection by *N. rileyi*. Though it remains unanswered whether these AMP gene transcriptions are under control of Toll signaling, these AMPs may play crucial roles in defending against growth of *N. rileyi*. In addition to AMPs, *B. mori* larvae induced other genes, such as several stress proteins, alcohol dehydrogenase, and catalase, in response to *B. bassiana* (Chengxiang *et al*., 2011). This suggests that the antifungal response of insects against EPF recruits multiple factors. This study suggests that Toll and subsequent AMP expressions are associated with the antifungal response of *S. exigua* against *N. rileyi*.

Bacterial metabolites of Xn and Ptt significantly suppressed the antifungal responses of *S. exigua* against *N. rileyi*. Especially, BZA (a common bacterial metabolite of Xn and Ptt (Kwon and Kim, 2008; Seo *et al*., 2010) significantly inhibited PLA_2 activation in all three immune-associated tissues and suppressed induction of some AMPs. The inhibitory activity of BZA against expression of AMP genes can be explained by its specific inhibition to PLA_2 activity (Shrestha *et al*., 2010; Hwang, 2011). Eicosanoids are implicated in the activation of humoral immune responses (Morishima *et al*., 1997; Yajima *et al*., 2003). In *S. exigua*, different prostaglandins and leukotriene activate expression of specific AMP genes, but dexamethasone (a specific PLA_2 inhibitor) inhibits their expression (Shrestha and Kim, 2009b). Thus, the inhibitory activity of the bacterial metabolites in this study may inhibit expression of AMP genes.

These immunosuppressive activities of bacterial metabolites enhanced pathogenicity of *N. rileyi* to both lepidopteran species under laboratory and field conditions. Thus the enhanced pathogenicity from the bacterial metabolites can be explained by their immunosuppressive activity in target insects. These results demonstrate that insect immune responses play crucial roles in defending against fungal infection and that immunosuppression enhances fungal pathogenicity.

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References

Anderson, S.O. 1979. Biochemistry of insect cuticle. *Annu. Rev. Entomol.* **24**, 29–61.

Boucias, D.G., Schoborg, E.A., and Allen, G.E. 1982. The relative susceptibility of six noctuid species to infection by *Nomuraea rileyi* isolated from *Anticarsia gemmatalis*. *J. Invertebr. Pathol.* **39**, 238–240.

Burke, J.E. and Dennis, E.A. 2009. Phospholipase A_2 structure/function, mechanism and signaling. *J. Lipid Res.* **50**, S237–S242.

Chengxiang, H., Guangxing, Q., Ting, L., Xinglin, M., Rui, Z., Pan, Z., Zhongyuan, S., and Xijie, G. 2011. Differential gene expression in silkworm in response to *Beauveria bassiana* infection. *Gene* **484**, 35–41.

Dean, P., Gadsden, J.C., Richards, E.H., Edwards, J.P., Charnley, A.K., and Reynolds, S.E. 2002. Modulation by eicosanoid biosynthesis inhibitors of immune responses by the insect *Manduca sexta* to the pathogenic fungus *Metarhizium anisopliae*. *J. Invertebr. Pathol.* **79**, 93–101.

Gillespie, J.P., Kanost, M.R., and Trenczek, T. 1997. Biological mediators of insect immunity. *Annu. Rev. Entomol.* **42**, 611–643.

Goh, H.G., Lee, S.G., Lee, B.P., Choi, K.M., and Kim, J.H. 1990. Simple mass-rearing of beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), on an artificial diet. *Kor. J. Appl. Entomol.* **29**, 180–183.

Hoffmann, J.A. 2003. The immune response of *Drosophila*. *Nature* **426**, 33–38.

Hwang, J. 2011. Transcriptional control of humoral immune responses in the beet armyworm, *Spodoptera exigua*. MS thesis. Andong National University, Andong, Republic of Korea.

Ignoffo, C.M. 1981. The fungus *Nomuraea rileyi* as a microbial insecticide: fungi, pp. 513–538. *In* Burges, H.D. (ed.), Microbial Control of Pests and Plant Disease 1970-1980, Academic Press, London, UK.

Ji, D., Yi, Y., Kang, G.H., Choi, Y.H., Kim, P., Baek, N.I., and Kim, Y. 2005. Identification of an antibacterial compound, benzylideneacetone, from *Xenorhabdus nematophila* against major plant-pathogenic bacteria. *FEMS Microbiol. Lett.* **239**, 241–248.

Kawakami, K. 1972. Relationship between the pathogenic patches on the integument and the fungal reproduction in the muscardine-inoculated larvae of the silkworm, *Bombyx mori* L. *J. Seric. Sci. Jpn.* **41**, 144–149.

Kim, Y., Ji, D., Cho, S., and Park, Y. 2005. Two groups of entomopathogenic bacteria, *Photorhabdus* and *Xenorhabdus,* share an inhibitory action against phospholipase A_2 to induce host immunodepression. *Arch. Insect Biochem. Physiol.* **59**, 230–244.

Kim, J. and Kim, Y. 2011. Three metabolites from an entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibit larval development of *Spodoptera exigua* (Lepidoptera: Noctuidae) by inhibiting a digestive enzyme, phospholipase A_2. *Insect Sci.* **18**, 282–288.

Kosir, J.M., MacPherson, J.M., and Khachatourians, G.G. 1991. Genomic analysis of a virulent and a less virulent strain of the entomopathogenic fungus, *Beauveria bassiana* using restriction fragment length polymorphisms. *Can. J. Microbiol.* **37**, 534–541.

Kumar, V., Singh, G.P., Kumar, V., Babu, A.M., and Datta, R.K. 1997. SEM study on the invasion of *Nomuraea rileyi* (Farlow) on silkworm, *Bombyx mori* Linn. causing green muscardine. *Mycopathologia* **138**, 141–144.

Kwon, B. and Kim, Y. 2008. Benzylideneacetone, an immunosuppressant, enhances virulence of *Bacillus thuringiensis* against beet armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* **101**, 36–41.

Leclerc, V. and Reichhart, J.M. 2004. The immune response of *Drosophila melanogaster*. *Immunol. Rev.* **198**, 58–71.

Lemaitre, B. and Hoffmann, J. 2007. The host defense of *Drosophila melanogaster*. *Annu. Rev. Immunol.* **25**, 697–743.

Lemaitre, B., Meister, M., Govind, S., Georgel, P., Steward, R., Reichhart, J.M., and Hoffmann, J.A. 1995. Functional analysis and regulation of nuclear import of dorsal during the immune response in *Drosophila*. *EMBO J.* **14**, 536–545.

Lord, J.C., Anderson, S., and Stanley, D.W. 2002. Eicosanoids mediate *Manduca sexta* cellular response to the fungal pathogen *Beauveria bassiana*: a role for lipoxygenase pathway. *Arch. Insect Biochem. Physiol.* **51**, 46–54.

Michel, T., Reichhart, J.M., Hoffman, J.A., and Royet, J. 2001. *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature* **414**, 756–759.

Morishima, I., Yamano, Y., Inoue, K., and Matsuo, N. 1997. Eicosanoids mediate induction of immune genes in the fat body of the silkworm, *Bombyx mori*. *FEBS Lett.* **419**, 83–86.

Noda, T., Meguri, T., Iimure, K., Ono, M., and Araki, T. 2011. Potential of D-*erythro*-C_{14}-sphinosine as an adjuvant for a fungal pesticide of *Nomuraea rileyi*. *Biosci. Biotechnol. Biochem.* **75**, 373–375.

Noda, T., Ono, M., Iimure, K., and Araki, T. 2010a. Isolation of a bioactive substance from the silkworm (*Bombyx mori* Linnaeus) that accelerates the germination of the entomopathogenic fungus *Nomuraea rileyi* (Farlow) Samson. *Biosci. Biotechnol. Biochem.* **74**, 563–568.

Noda, T., Ono, M., Iimure, K., and Araki, T. 2010b. Characterization of a germination-accelerating factor from the silkworm (*Bombyx mori* Linnaeus) of entomopathogenic fungus *Nomuraea rileyi* (Farlow) Samson. *Biosci. Biotechnol. Biochem.* **74**, 1226–1230.

Park, J. and Kim, Y. 2011. Benzylideneacetone suppresses both cellular and humoral immune responses of *Spodoptera exigua* and enhances fungal pathogenicity. *J. Asia Pac. Entomol.* **14**, 423–427.

Pedrini, N., Crespo, R., and Juárez, M.P. 2007. Biochemistry of insect epicuticle degradation by entomopathogenic fungi. *Comp. Biochem. Physiol. C* **146**, 124–137.

Pendland, J.C. and Boucias, D.G. 1985. Hemagglutinin activity in the hemolymph of *Anticarsia gemmatalis* larvae infected with the fungus *Nomuraea rileyi*. *Dev. Comp. Immunol.* **9**, 21–30.

Pendland, J.C. and Boucias, D.G. 2000. Comparative analysis of the binding of antibodies prepared against the insect *Spodoptera exigua* and against the mycopathogen *Nomuraea rileyi*. *J. Invertebr. Pathol.* **75**, 107–116.

Qin, L., Liu, X., Li, J., Chen, H., Yan, Q., Yang, Z.Y., Wang, L., and Chen, K. 2009. Protein profile of *Nomuraea rileyi* spore isolated from infected silkworm. *Curr. Microbiol.* **58**, 578–595.

Radvanyi, F., Jordan, L., Russo-Marie, F., and Bon, C. 1989. A sensitive and continuous fluorometric assay for phospholipase A_2 using pyrene-labeled phospholipids in the presence of serum albumin. *Anal. Biochem.* **177**, 103–109.

SAS Institute, Inc. 1989. SAS/STAT User's Guide, release 6.03 ed. SAS Institute, Cary, NC.

Seo, S., Jang, H., Kim, K., and Kim, Y. 2010. Comparative analysis of immunosuppressive metabolites synthesized by an entomopathogenic bacterium, *Photorhabdus temperata* ssp. *temperata*, to select economic bacterial culture media. *Kor. J. Appl. Entomol.* **49**, 409–416.

Shah, P.A. and Pell, J.K. 2003. Entomopathogenic fungi as biological control agents. *Appl. Microbiol. Biotechnol.* **61**, 413–423.

Shrestha, S. and Kim, Y. 2008. Eicosanoid mediates prophenoloxidase release from oenocytoids in the beet armyworm, *Spodoptera exigua*. *Insect Biochem. Mol. Biol.* **38**, 99–112.

Shrestha, S. and Kim, Y. 2009a. Oenocytoid cell lysis to release prophenoloxidase in induced by eicosanoid via protein kinase C. *J. Asia Pac. Entomol.* **12**, 301–305.

Shrestha, S. and Kim, Y. 2009b. Various eicosanoids modulate the cellular and humoral immune responses of the beet armyworm, *Spodoptera exigua*. *Biochem. Biophys. Biotech.* **73**, 2077–2084.

Shrestha, S. and Kim, Y. 2010. Activation of immune-associated phospholipase A_2 is functionally linked to Toll/Imd signal pathways in the red flour beetle, *Tribolium castaneum*. *Dev. Comp. Immunol.* **34**, 530–537.

Shrestha, S., Park, Y., Stanley, D., and Kim, Y. 2010. Genes encoding phospholipase A_2 mediate insect nodulation reactions to bacterial challenge. *J. Insect Physiol.* **56**, 324–332.

Stanley, D.W. 2005. Eicosanoids, pp. 307–339. *In* Gilbert, L.I., Iatrou, K., and Gill, S.S. (eds.), Comprehensive Insect Molecular Science, Vol. 4, Elsevier, Amsterdam, Netherlands.

Stanley, D.W. 2011. Eicosanoids: progress towards manipulating insect immunity. *J. Appl. Entomol.* **135**, 534–545.

Strand, M.R. 2008. Insect hemocytes and their role in immunity, pp. 25–47. *In* Beckage, N.E. (ed.), Insect Immunology, Academic Press, New York, N.Y., USA.

Supakdamrongkul, P., Bhumiratana, A., and Wiwat, C. 2010. Characterization of an extracellular lipase from the biocontrol fungus, *Nomuraea rileyi* MJ, and its toxicity toward *Spodoptera litura*. *J. Invertebr. Pathol.* **105**, 228–235.

Tanji, T., Hu, X., Weber, A.N., and Ip, Y.T. 2007. Toll and IMD pathway synergistically activate an innate immune response in *Drosophila melanogaster*. *Mol. Cell Biol.* **27**, 4578–4588.

Tanji, T. and Ip, Y.T. 2005. Regulators of the Toll and Imd pathways in the *Drosophila* innate immune response. *Trends Immunol.* **26**, 193–198.

Tunaz, H. 2006. Eicosanoid biosynthesis inhibitors influence mortality of *Pieris brassicae* larvae co-injected with fungal conidia. *Arch. Insect Biochem. Physiol.* **63**, 93–100.

Tunaz, H., Park, Y., Büyükgüzel, K., Bedick, J.C., Nor Aliza, A.R., and Stanley, D.W. 2003. Eicosanoids in insect immunity: bacterial infection stimulates hemocytic phospholipase A_2 activity in tobacco hornworms. *Arch. Insect Biochem. Physiol.* **52**, 1–6.

Wu, S., Zhang, X., Chen, X., Cao, P., Beerntsen, B.T., and Ling, E. 2010. BmToll9, an arthropod conservative Toll, is likely involved in the local gut immune response in the silkworm, *Bombyx mori*. *Dev. Comp. Immunol.* **34**, 93–96.

Yajima, M., Tanaka, M., Tanahashi, N., Kikuchi, H., Natori, S., Oshima, Y., and Kurata, S. 2003. A newly established *in vitro* culture using transgenic *Drosophila* reveals functional coupling between the phospholipase A_2-generated fatty acid cascade and lipopolysaccharide-dependent activation of the immune deficiency (imd) pathway in insect immunity. *Biochem. J.* **371**, 205–210.