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Effect of RNA*i*-mediated Suppression of *PGRP-LC* Gene Expression on Mortality Rates in Brown Planthoppers (*Nilaparvata lugens*) Infected with Live Bacteria

Aki SAGISAKA, Ritsuko MURAKAMI*, Kenji WATANABE and Hiromitsu TANAKA

Institute of Plant Protection, National Agriculture and Food Research Organization, Tsukuba, Japan

Abstract

The brown planthopper (*Nilaparvata lugens*) is among the most destructive pests of rice in Asia. However, control of this pest is hampered because of the emergence of strains with resistance to standard chemical pesticides. Herein, we investigated the use of RNA interference (RNA*i*) to knock down genes in the immune system of *N. lugens* as a possible approach to the control of the pest. We suppressed the expression of the peptidoglycan recognition protein–*LC* gene and *Toll-1* gene by RNA*i*-mediated knockdown in *N. lugens* infected with live bacteria. Mortality rates were compared between insects with and without RNA*i*-mediated gene knockdown. We found that suppression of peptidoglycan recognition protein–*LC*, which encodes a factor in the immune deficiency pathway, resulted in a significantly increased mortality rate in insects infected with *Escherichia coli* (gram-negative bacteria) or *Micrococcus luteus* (gram-positive bacteria). Our results indicate that RNA*i* knockdown of genes in the immune deficiency pathway will be of value for identifying candidate genes that can be used to control this pest species.

Discipline: Biotechnology
Additional key words: IMD pathway, pest control, *Toll-1*

Introduction

The brown planthopper (*Nilaparvata lugens*) is distributed worldwide and is a destructive insect pest of rice (*Oryza sativa*). Control of this pest has become increasingly difficult following the emergence of strains with resistance to standard chemical pesticides used in East and Southeast Asia (Matsumura et al. 2008). Although the use of entomopathogenic microorganisms has been suggested for the control of *N. lugens* instead of chemical pesticides (Rombach et al. 1986, Qian et al. 2021), in practice, the effects of these microorganisms are mild and their application is difficult compared to chemical pesticides. We therefore evaluated the use of RNA interference (RNA*i*) gene knockdown technology as an alternative approach for the development of new control methods. Herein, we investigated the targeting of genes in the innate immune system of *N. lugens* to identify candidates that might be of value in the control of this pest species.

Unlike mammals, insects do not have an acquired immunity system. However, each insect species has a unique innate immune system that acts against infection by microorganisms (Zhou et al. 2019). In the *Drosophila* innate immune system, an immune deficiency (IMD) signaling pathway recognizes invading gram-negative and gram-positive bacteria (Ma & Kanost 2000, Ochiai & Ashida 2000); in addition, the Toll pathway recognizes invading fungi and gram-positive bacteria (Valanne et al. 2011). The peptidoglycan recognition protein (PGRP)-LC of the IMD pathway binds to peptidoglycans of gram-negative bacteria in *Drosophila* and *Bombyx mori* (Gottar et al. 2002) and activates an IMD signaling cascade that produces antimicrobial peptides (Choe et al. 2002, Takehana et al. 2002). By contrast, the aphid species *Acyrthosiphon pisum* does not possess an IMD pathway (International Aphid Genomics 2010).

A transcriptome analysis of *N. lugens* identified signal transduction molecules, immune effectors,

*Corresponding author: mritsuko@affrc.go.jp
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modulation molecules, and pattern recognition molecules in the immune pathways (Bao et al. 2013). Antimicrobial peptides such as lugensin A, lugensin B, defensin A, and defensin B have also been reported (Liu et al. 2015). However, the relationship between the immune pathways and mortality rates after infection with live bacteria has not yet been directly examined. *PGRP-LC* is present in the IMD pathway of *N. lugens* (Bao et al. 2013, Liu et al. 2015). In the present study, we compared mortality rates in *N. lugens* after RNA*i*-mediated knockdown of expression of *PGRP-LC* and infection with live *Escherichia coli* (gram-negative bacteria) or *Micrococcus luteus* (gram-positive bacteria), and the mortality rate was higher in insects with gene knockdown than in untreated insects. These results suggest that genes in the IMD pathway are valuable targets for the control of *N. lugens* by RNA*i* knockdown.

Materials and methods

1. Brown planthoppers

A laboratory population of *N. lugens* was maintained on rice seedlings at 27°C under a 16 h light/8 h dark photoperiod. The laboratory population was derived from insects originally collected from Shimane Prefecture in Japan in 1987.

2. RNA*i*-mediated gene silencing

For gene silencing by RNA*i*, cDNA fragments of *PGRP-LC* (Accession No. KC355212; Fig. 1) in the IMD pathway and *Toll-1* (KC355234; Fig. 2) in the Toll pathway were amplified by PCR using KOD-Plus (Toyobo, Osaka). T7 RNA polymerase promotor sequences were introduced into the 5′-ends of both forward and reverse primers of each target gene (Table 1). *EGFP* was used as a control. *ds*RNAs were synthesized from cDNAs using a MEGAscript RNA*i* Kit (Ambion, Tokyo), and 50 ng of the synthesized *ds*RNA was injected into third-stage nymphs using a glass capillary. The planthoppers were maintained on rice seedlings for 2 days after injection.

Fig. 1. Comparison of *PGRP-LC* sequences in *Nilaparvata lugens*, *Laodelphax striatellus*, and *Sogatella furcifera*
Bold letters show matched nucleic sequences in *Nilaparvata lugens* (Accession No. KC355212), *Laodelphax striatellus* (KU866527), and *Sogatella furcifera* (MW323547), and underlined letters indicate primer sequences used for the synthesis of *ds*RNA for RNA*i* (Table 1) and for RT-qPCR of *PGRP-LC* expression (Table 2). Numbers show nucleotide positions of *N. lugens PGRP-LC*.

Fig. 2. Comparison of *Toll-1* sequences in *Nilaparvata lugens*, *Laodelphax striatellus*, and *Sogatella furcifera*
Bold letters show matched nucleic sequences in *Nilaparvata lugens* (Accession No. XM039444589), *Laodelphax striatellus* (MV048393), and *Sogatella furcifera* (MW048389), and underlined letters show primer sequences used for the synthesis of *ds*RNA for RNA*i* (Table 1) and for RT-qPCR of *Toll-1* expression (Table 2). Numbers show nucleotide positions of *N. lugens Toll-1*.

Table 1. Primer sequences for synthesis of *ds*RNAs

Target gene	Primer name	Sequence
PGRP-LC	PGRP-LC-F	5′-TAATACGACTCACTATAGGGAAGTGTTCAATATTTCCAATG-3′
	PGRP-LC-R	5′-TAATACGACTCACTATAGGGCTAAAGTTGTTGCGACC-3′
Toll-1	Toll-1-F	5′-TAATACGACTCACTATAGGGGTTGACTGACATCAGTAGA-3′
	Toll-1-R	5′-TAATACGACTCACTATAGGGGCTTGTTACGAGAAGCC-3′
EGFP	EGFP-F	5′-TAATACGACTCACTATAGGGAGCTGACCCTGAAGTTCA-3′
	EGFP-R	5′-TAATACGACTCACTATAGGGTCCATGCCGAGAGTGATC-3′

A T7 RNA polymerase promotor sequence (underlined) was introduced into the 5′-ends of each target gene primer.

The expression of the target genes was quantified after extracting total RNA from the body of individual *N. lugens* using ISOGEN II (NIPPON GENE, Tokyo) according to the manufacturer's instructions. First-strand cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (ABI, Tokyo) with random hexamers. Gene expression was measured by quantitative reverse transcription PCR (RT-qPCR) with the appropriate primers (Table 2) using LightCycler 480 and SYBR Green I Master Ver. 13 (NIPPON GENE). The relative expression levels of the target genes were normalized against *N. lugens* 60S ribosomal protein L32 (XM022350197).

3. Bacterial infection

E. coli K12 strain JM109 and *M. luteus* were cultured in LB medium at 37°C and 30°C, respectively. The cells were collected and washed with PBS (pH 7.3) and diluted to 1×10^9 cells/mL with PBS. Bacterial solution (30 nL) was injected into the body of individual *N. lugens* 2 days after the injection of *ds*RNAs, and the insects were maintained on rice seedlings for 1-3 days. In each test, 10 planthoppers were used and five replicate experiments were performed.

4. Statistical analyses

Mortality in insects was determined, and statistical analyses were performed using R version 4.0.2 (R Core Team 2020).

Table 2. Primer sequences for RT-qPCR

Target gene	Primer name	Sequence
PGRP-LC	qPGRP-LC-F	5′-CAACGGTTACATATACGAG-3′
	qPGRP-LC-R	5′-GCCTGTAAATGTACCTA-3′
Toll-1	qToll-1-F	5′-AAGGTCACGCATCTCTA-3′
	qToll-1-R	5′-CAAATTGTTATTGGTCAAATC-3′
Ribosomal protein[1)]	qRP-F	5′-GCTCAGGACAAACTGGCGTAAACC-3′
	qRP-R	5′-GCTTCCATAACCAATAGAGGGCAT-3′

[1)] *Nilaparvata lugens* 60S ribosomal protein L32 (Accession No. XM_022350197)

Results

1. RNA*i*-mediated gene knockdown

Previous studies have demonstrated that the use of double-strand RNAs (*ds*RNAs) of 400-600 bp nucleotide lengths is effective for RNA*i*-mediated knockdown of target genes in insects (Davis et al. 2018, Vogel et al. 2019). We designed primers for RNA*i* (Table 1, Figs. 1, 2) that yielded *ds*RNA products for *PGRP-LC* and *Toll-1* of 467 and 509 bp, respectively. As a control, *ds*RNA of *EGFP* of 580 bp was used. Fifty ng of each *ds*RNA was injected into *N. lugens*.

The expression levels of *PGRP-LC* and *Toll-1* were quantified by real-time PCR, and the designed primers yielded products of approximately 50 and 102 bp, respectively. The relative levels of expression of the target genes were quantitated against the level of expression of ribosomal protein L32. The RT-qPCR product of this ribosomal protein was 90 bp in size. When *ds*RNA of *PGRP-LC* was injected into *N. lugens*, the relative mRNA amounts (RmRAs) of *PGRP-LC* gene expression against the ribosomal protein were in the range of 0.02-0.07 (Fig. 3). However, when *ds*RNA of *EGFP* was injected, the RmRAs of *PGRP-LC* were in the range of 0.16-0.3. The RmRAs of *PGRP-LC* were significantly decreased by RNA*i* treatment ($P = 9.0E{-}05$, Student's *t*-test). When *ds*RNA of *Toll-1* was injected, RmRAs of 0.4-0.6 were obtained. When *ds*RNA of *EGFP* gene was injected, the RmRAs of *Toll-1* were in the range of 1.0-1.9. The RmRAs of *Toll-1* were significantly decreased by RNA*i* treatment ($P = 7.6E{-}07$). Therefore, the expression of both genes was suppressed by the RNA*i* methods used here.

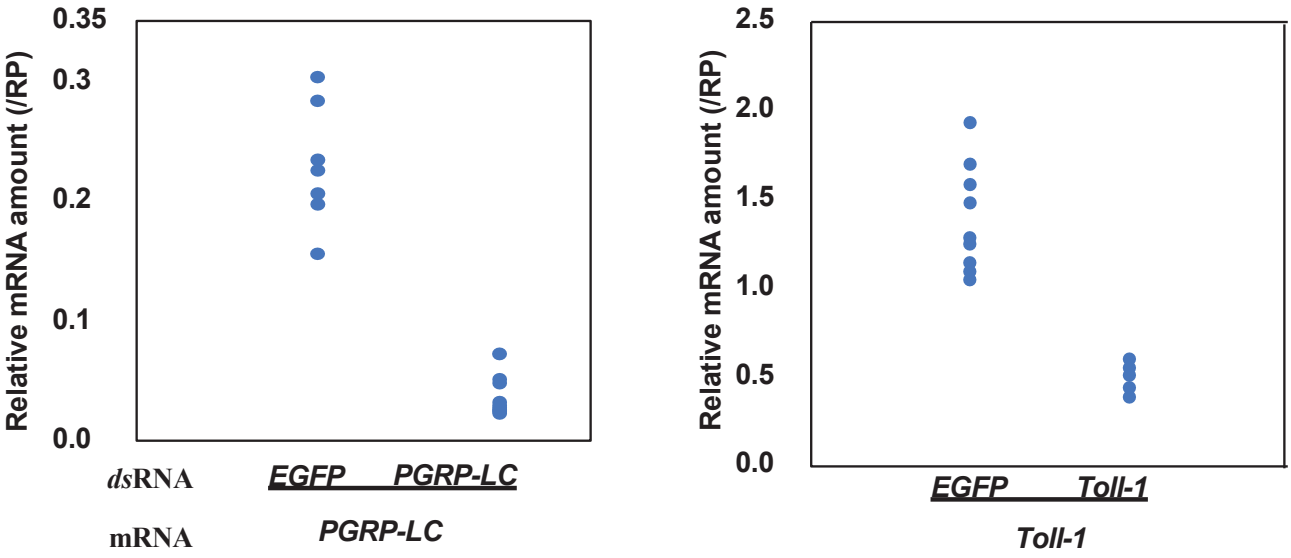

Fig. 3. RNA*i*-mediated knockdown of *PGRP-LC* and *Toll-1* gene expression in *Nilaparvata lugens*
mRNA levels of target genes (*PGRP-LC*, left; *Toll-1*, right) were examined at 48 h after *ds*RNA injection. Transcription levels of each target gene were quantified by quantitative reverse transcription PCR and normalized against expression of ribosomal protein 49 of *N. lugens* 60S ribosomal protein L32. RNA*i*-mediated suppression of *EGFP* was used as a control.

2. Mortality

At *N. lugens* without bacterial inoculation, there were no significant mortalities of the insects injected with *ds*RNA of *PGRP-LC* and *ds*RNA of *Toll-1* and that of *ds*RNA of *EGFP*. Changes to the mortality rates in *N. lugens* after the suppression of *PGRP-LC* or *Toll-1* expression were assessed after injecting *E. coli* or *M. luteus* (Fig. 4). At 1 day after injection of live bacteria, the mortality rate after suppression of *PGRP-LC* expression increased: after injection of *M. luteus*, 55%-77% of the insects died; after injection of *E. coli*, 80%-88% died. Insects with suppression of *PGRP-LC* expression showed a higher mortality rate than that of control insects after injection of *M. luteus* ($P < 0.01$, χ^2 test) or *E. coli* ($P < 0.05$). Therefore, *PGRP-LC* in *N. lugens* might be involved with immunity responses against bacterial infection. By contrast, the mortality rate after suppression of *Toll-1* expression did not differ significantly from that of the control insects with suppression of *EGFP* expression. The results indicate that *Toll-1* expression might not be involved with immunity responses against bacterial infection.

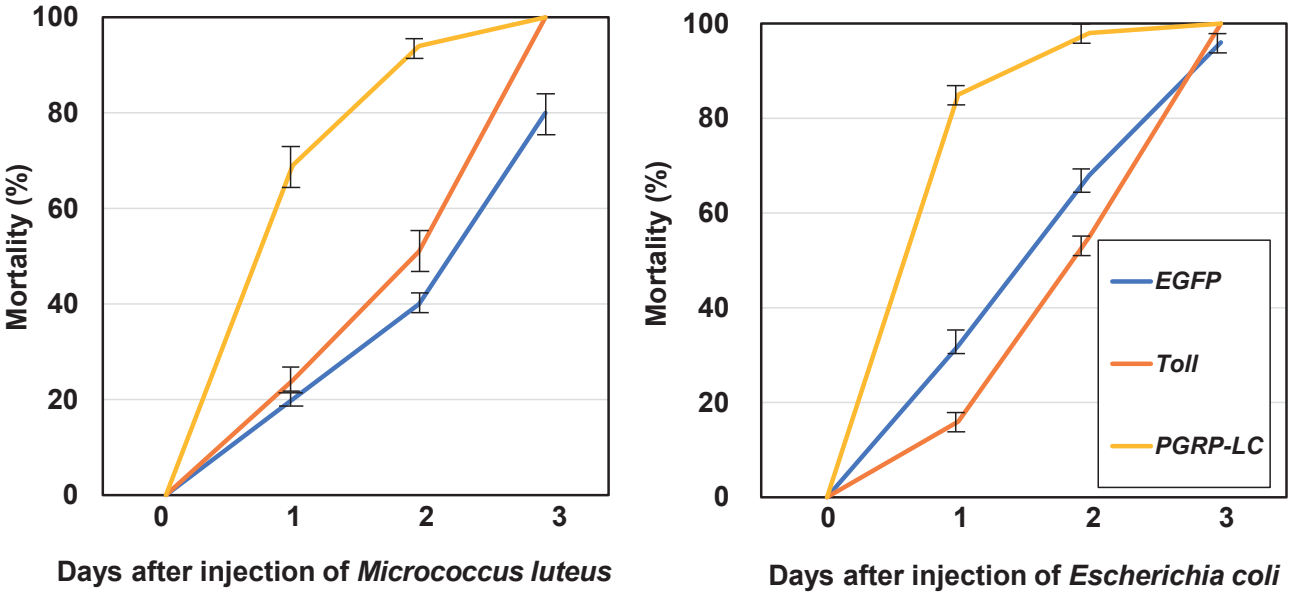

Fig. 4. Effect of knockdown of target genes (*PGRP-LC* and *Toll-1*) on immune responses in *Nilaparvata lugens* to bacterial infection (left, gram-positive bacteria: *Micrococcus luteus*; right, gram-negative bacteria: *Escherichia coli*)
Third-stage nymphs were injected with *ds*RNA against the target gene and were then injected with a bacterial suspension. Data shown as mean ± SE.

Discussion

Our investigation showed that *PGRP-LC* is a strong candidate for involvement in immunity responses against infection with live bacteria. Previous studies have analyzed the activities of antimicrobial peptides following injection of heat-killed *E. coli* (Zhou et al. 2019) or heat-killed gram-positive or gram-negative bacteria into *N. lugens* (Bao et al. 2013). The latter study measured the levels of expression of *PGRP-LC* and *Toll-1* genes. However, neither of these studies examined mortality rates in *N. lugens* after RNAi-mediated suppression of immune response genes and exposure to living pathogens. Additionally, they did not examine the relationship between immune gene expression and mortality in *N. lugens*. In the present study, we chose to use live bacteria as these provide conditions closer to the actual situation for *N. lugens* in the field. Our study demonstrated that *PGRP-LC*, which is involved in a signal transduction pathway, is important in the immune response of *N. lugens* to infection with live bacteria. RNA*i*-mediated suppression of *PGRP-LC* expression increased the mortality rate in *N. lugens* following infection with live bacteria. In addition to *PGRP-LC*, knockdown of *Relish*, a gene in the IMG pathway, increases the mortality rate in *N. lugens*. In addition to *E. coli* or *M. luteus*, injections of other bacteria increased mortalities of *N. lugens* (data not shown). *E. coli* and *M. luteus* are opportunistic

bacteria for *N. lugens* and, like many other bacterial species, can survive in the field. *N. lugens* is exposed to a wide range of infective bacteria under natural conditions. Because bacterial infections tend to develop more rapidly than fungal infections, suppression of genes in the IMD pathway might be a more useful approach than suppression of those in the Toll pathway for the control of *N. lugens*.

In *Drosophila*, the Toll pathway is activated by both fungal (Lemaitre et al. 1996) and gram-positive bacterial infections (Valanne et al. 2011). *PGRP-SA* and *PGRP-SD* are required for activating the Toll pathway in response to gram-positive bacteria (Gobert et al. 2003). *N. lugens* has at least six *Toll*-like genes, and expression of *Toll-1* has been shown to be stimulated by injection of heat-killed *E. coli* in fifth-instar nymphs (Bao et al. 2013). In the present study, the mortality rate in *N. lugens* after the suppression of *Toll-1* expression did not differ from that of the *EGFP* control. Although *D. melanogaster* has 13 *PGRP* genes, only *PGRP-LB* and *PGRP-LC* are present in *N. lugens* (Bao et al. 2013). Thus, immune-related genes in *N. lugens* differ from those in *D. melanogaster*. Immune-related genes vary among insect species. When particular genes are absent, their functions are generally compensated by other genes (Bao et al. 2013, International Aphid Genomics 2010). Therefore, gene functions in the Toll pathway in *N. lugens* might differ from those in *D. melanogaster*. The level of expression of particular genes might not necessarily be directly related to viability in *N. lugens*. The results from the analyses of mortality might differ from those on gene expression levels. Therefore, our results on mortality rates in *N. lugens* after the suppression of *Toll-1* expression may not contradict those of previously reported studies.

An attempt to control *N. lugens* by a feeding-based approach to achieve RNA*i*mediated suppression of the trehalose phosphate synthase gene reported a peak of mortality of 60% at 10 days after ingestion (Chen et al. 2010). Overall, however, feeding-based RNA*i* of the trehalose phosphate synthase gene yielded relatively poor control of *N. lugens*, and it took a long time for the RNA*i* treatment to take effect. For this reason, we chose an injection-based method for RNA*i*-mediated suppression of immune system genes in the present study. Injection with *ds*RNA of *PGRP-LC* increased mortality in *N. lugens*; however, for practical reasons, feeding-based methods will be of more value for control of *N. lugens* in the field. The nucleotide sequences of the component proteins of the IMD pathway of insects vary among species (Figs. 1, 2). Therefore, control of specific insect species could be achieved using insect immunity gene sequences. The use of an RNA*i*-based approach for the knockdown of immune system components for control of *N. lugens* could decrease the use of pesticides and diminish the likelihood of development of pesticide-resistant strains. More studies are clearly needed to explore the practicality of the use of RNA*i* knockdown systems for pest control. However, the approach here demonstrates the feasibility of using immune effectors against bacterial invasion via RNA*i*-mediated suppression of selected genes and shows that knockdown of immunity in insects could be useful for the control of pests.

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References

Bao, Y.-Y. et al. (2013) The genome- and transcription-wide analysis of innate immunity in the brown planthopper, *Nilaparvata lugens*. *BMC Genom.*, **14**, 160.

Chen, J. et al. (2010) Feeding-based RNA interference of a trehalose phosphate synthase gene in the brown planthopper, *Nilaparvata lugens*. *Insect Mol. Biol.*, **19**, 777-786.

Choe, K.-M. et al. (2002) Requirement for a peptidoglycan recognition protein (*PGRP*) in *Relish* activation and antibacterial immune responses in *Drosophila*. *Science*, **296**, 359-362.

Davis, V. C. et al. (2018) Knockdown of RNA interference pathway genes impacts the fitness of western corn rootworm. *Sci. Rep.*, **8**, 7858.

Gobert, V. et al. (2003) Dual activation of the *Drosophila* Toll pathway by two pattern recognition receptors. *Science*, **302**, 2126-2130.

Gottar, M. et al. (2002) The *Drosophila* immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. *Nature*, **416**, 640-644.

International Aphid Genomics, C. (2010) Genome sequence of the pea aphid *Acyrthosiphon pisum*. *PLoS Biol.*, **8**, e1000313.

Lemaitre, B. et al. (1996) The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell*, **86**, 973-983.

Liu, W. et al. (2015) Immune signaling pathways activated in response to different pathogenic microorganisms in *Bombyx mori*. *Mol. Immunol.*, **65**, 391-397.

Ma, C. C. & Kanost, M. R. (2000) A beta 1,3-glucan recognition protein from an insect, *Manduca sexta*, agglutinates microorganisms and activates the phenoloxidase cascade. *J. Biol. Chem.*, **275**, 7505-7514.

Matsumura, M. et al. (2008) Species-specific insecticide resistance to imidacloprid and fipronil in the rice planthoppers *Nilaparvata lugens* and *Sogatella furcifera* in East and South-east Asia. *Pest Manag. Sci.*, **64**, 1115-1121.

Ochiai, M. & Ashida, M. (2000) A pattern-recognition protein for beta-1,3-glucan-the biding domain and the cDNA

cloning of beta-1,3-glucan recognition protein from the silkworm, *Bombyx mori*. *J. Biol. Chem*., **275**, 4995-5002.

Qian, Z. et al. (2021) Sustainable control of the rice pest, *Nilaparvata ligens*, using the entomopathogenic fungus *Isaria javanica*. *Pest Manag. Sci*., **77**, 1452-1464.

R Core Team (2020) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rombach, M. C. et al. (1986) Infection of rice brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae), by field application of entomopathogenic Hyphomycetes (Deuteromycotina). *Environ. Entomol*., **15**, 1070-1073.

Takehana, A. (2002) Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in *Drosophila* larvae. *Proc. Natl. Acad. Sci. U.S.A*., **99**, 13705-13710.

Valanne, S. et al. (2011) The Drosophila Toll signaling pathway. *J. Immunol*., **186**, 649-656.

Vogel, E. et al. (2019) RNA interference in insects: Protecting beneficials and controlling pests. *Front. Physiol*., **11**, https://doi.org/10.3389/fphys.2018.01912.

Zhou, X. et al. (2019) Identification of novel antimicrobial peptides from rice planthopper, *Nilaparvata lugens*. *Insect Biochem. Mol. Biol*., **113**, 103215.