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

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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 (RNAi) 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,

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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 RNAi-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 RNAi 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. RNAi-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.

		12	1 PGRP-LC-F
N.	lugens	(getgaacagttggataaactgcctgaaaaaattatcagcaatggcgatttcatttgccaaactcagggac <u>aagtgttcaatatttccaatg</u> ggaagatacctacaaattcAccgctcata
L.	striatellus	i	AGTGAACAATTGGATAAACTGCCGGAGAAAATAATCAGCAACGGTGATTATGTTTGTCAA ACACAGGGACAAGTGTTCAATATTTCCAATGGAAAGATTCCCACCGCTTCACCCTTGAT
s.	furcifera		
		24	11
N .	lugens		$aggattgaaacgaaagatcagaaaaaacgccccaagatttttcaaaagagagcgtggtgttcgcaagcatagccgtttgcttcatcctg{tatccaca-gtcatctatcttcccaaattttccacaatttttcaaaagagagcgtggtgttcgcaagcatagccgtttgcttcatcctgttatccaca-gtcatctatcttctccaaatttttcaaaagagagcgtggtgttcgcaagcatagccgtttgcttcatcctgttatcctgtattccacaagagagggtggtgttcgcaagcatagccgtttgcttcatcctgttatcctgtattccacaaggagggtgttcgcaaggaggtggtgttcgcaaggagggtggtttgcttcatcctgttattcctgtattccacaaggagggtggtgttcgcaaggagggtggtgttcgcaaggagggtggtgttcgcaaggagggtggtttgcttcatcctgttattcctgttattccacaaggagggtggtgttcgcaaggagggtggtgttcgcaaggagggtggtgttcgcaaggagggtggtgttcgcaaggagggtggtttggttcgcaaggagggtggtgttcgcaaggagggtggtgttcgcaaggagggtggtgttcgcaaggagggtggtgttcgcagggtggtgttcgcaaggagggtggtgttcgcaaggagggtggtggtgttcgcaaggagggtggtggtgttcgcaaggagggggtggtggtggtggtggtggtggtggtgttcgcagggtggtggtggtggtggtggtggtggtggtggtggtgg$
			CGCATTGAGACGAGGGATGAAAAAACGCCCAAGATATTCCAAAAGAGATTCGTAGTCTATGCAAGCATAGCTGTCTGCTTCATCCTA TTA TC AC G- GT C AT C TA TC T GC T AAAA T
			ATAACTCACGCGTTATTTA-CTATTGTGAT
	:	361	
Ν.	lugens		TGGTATTCACAGCAAGTCCAGCTTCTCAGGAAGTTACAACAAAGATGAGAAG-GAGGAGATCTCCCTCCGTGCATCGTCCCAACTGGGTGGCCCAACCTCCTCTA
L.	striatellus		CGGAATTCATGCCAA ATCGAATGATGGAAGATATTCAGGAACTTACAACAA CAA AGATGAATAC-GAGGA GATCTATC ATCATTTTGT CTCTCAGGCAAAGCTGG CTCGC CCCC CCTT CCT CCC CCC CCT CCC C
s.	furcifera		TTTTACGCTGTGACTTTATCGGAATCTGAACAAATTGGTGCTAGAACTATTACTGCAGACGTTTGCCCTGTTGTATTACCTCGGGCGTTCTGGGGTGCT-AACCCCCCC
	4	471	L
N.	lugens		$\mathbf{AAC} \texttt{CCGGCTCCAG} \mathbf{AC} \mathbf{CAAAC} \mathbf{AAC} \mathbf{CAAC} \mathbf{AC} \mathbf{CG} \mathbf{CGA} \mathbf{AAT} \mathbf{CCG} \mathbf{CC} CC$
L.	striatellus		$\mathbf{AA} \texttt{G} \texttt{G} \texttt{G} \texttt{G} \texttt{A} \texttt{C} \texttt{C} \texttt{C} \texttt{A} \texttt{C} \texttt{C} \texttt{C} \texttt{G} \texttt{A} \texttt{A} \texttt{T} \texttt{C} \texttt{C} \texttt{C} \texttt{C} \texttt{C} \texttt{C} \texttt{C} C$
s.	furcifera		AACAACAACGCAAACTTAAACTTCCACTTCCTGAAGTGATAATTCACCATACATA
		66	i8 qPGRP-LC-F PGRP-LC-R
N .	lugens		agataggt agggg ggggg gggggggggggggggggg
L.	striatellus		AGATCGGAAGTGGAAATTTCGATGACATAGCCTACAACTTCCTGGTGGACGCGACGGGTACGTGTACGAGGGCCGAGGCTGGGACACAGTGTCCGCCAGTGTCCAGGGACGCAACAAC
s.	furcifera		ACCAGATTACGAAAGGCTGGGACGATATTGGCTACAATTTGTTATTTGTGGCGATGAAAATATCTATGAAGGGCGAGGATGGTATAATATTGGAGCGCATGCAACCACTCATAATAGTA
		8	08 qPGRP-LC-R
N.	lugens		$\underline{\texttt{TTAG}} \texttt{TGTCGGCATTGCTCTO} \underline{\texttt{ATAGGTACATTTACAGC}} ACCGCACCGTCCGAACTGCAAATGTTGGCCCTGTCGCGATTCATCCGCGAAGGAGTCGACTACGGCCGGATAGCTGCCGACTGCCGAAGGAGTCGACTACGGCCGGATAGCTGCCGAAGGAGTCGACTACGGCCGGATAGCTGCCGAAGGAGTCGACTGCGACTGCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTCGACTGCGAAGGAGTGCGACTGCGAAGGAGTGGAGTGCGAAGGAGTGCGAAGGAGTGCGAAGGAGTGCGAAGGAGTGCGAAGGAGGAGTGCGACTGCGAAGGAGTGCGAAGGAGGAGGAGTGCGAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG$
L.	striatellus		TCAGCGTCAGCATCGCTCTGATCGGAACATTCACTGCGACCGCACCGTCCGACCGCCACGCACG
s.	furcifera		GGAGTATTGGCATTGCCTTCATCGGGGATTATACAGATGTTGCACCATCATCCA-ATATGTTGGAATTAGCTAAACACCTTATAGCTTGTGGATTAGAGAAAAATATCATAAT

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*.

		711 Toll-1-F
N .	lugens	CTGTTGBACCTTTGGACGAACCAG <u>TTGACTGACATCAGTAGA</u> CTGTGTCACGAGCACACCCGACCTCGAGTCGCCCGACTGAGTCACGACACGTCTCACGACACCCAACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCCGACCCGACCCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCGACCCCGACCCGACCCGACCCGACCCGACCCCGACCCGACCCCGACCCCGACCCCGACCCCGACCCCGACCCCGACCCCCC
L.	striatellus	CTGTTGAACCTGTGGAGTAATCAGCTGACATGATAGACTGTTTACAAGCACTCCAAACCTGGAATCGCTGGACTGAGTCATACAGCTTTGGCCACCCTGCGCGCGC
s.	furcifera	CTGTTGAATTTGTGGAGTAACCAGTTGACCGGCATCACCAGGGCGTGTTCACCAGTACCACGAACCTAGAGTCGCTCGACTCGAGTCACAACAGTTTGGGCACACGGCCCCGATCTTTTCGCCGGCCTCGGCAACTTGGGCACGCCCGAACTGGCCCCGACCTCGGCCCGACCTCGGCCCGACCTCGGCCCGACCTCGGCCCGACCTCGGCCCGACCTCGGCCCGACCTCGGCCCGACCTCGGCCCCGACCTCGGCCCCGACCTCGGCCCCGACCTCGGCCCGACCTCGGCCCGACCTCGGCCCGACCTCGGCCCGACCTCGGCCCGACCTCGGCCCGACCTCGGCCCGGCCCGGCCGG
		******** * ***** ** *** **** ***** *****
		861
N .	lugens	ANTTTGCGGGAAATGAGTTTTCAACACGCCACAAAATCTGTTTTTGGACAACCTCAAGCTGAAAATGTTCACAATGGTCAATCAGGGCCACTCTGAAGACAATCCGCCACTCTGGCCAATATCTGGAATATCTGCAATTCGTCAG
L.	striatellus	ANTITICICGGGGAATGAGTITGTGACACCGCCCAAAATCTGTCCTCGACAACCCCAAGGTGAAAATGTTCACCATGGTCAATCAGGGCACTCTGAAAACAATCCCCTCGCAGGTTGGGCGAATTTGGAGGATTTGGAGGTTGTGCAG
s.	furcifera	aatttgtcgggcaatgagttgtctccccagaatctgttccccagcaatctgtccctagtgtcaatggtcaatgggtacatggtcaatggtccatggtccatggtccatggtcaatggttctcccttcccatgttcccatggtacatggttca

	1	011
N .	lugens	atcascasatcscagatatcttccccasatatcscastcscastcscastcscascatcatcatcatcatcatcatcatcatcatcatcatcatc
L.	striatellus	atcastcsatastcscagatatctscactegecaaggatgststctggaattccacttggatcatggtcaaggatcatggcaggaagtgaaaaaatatcagg
s.	furcifera	ATCAAATCTCAGATCACCTCTCTCCCCCAAGGACGTGTTCTGGAATTCGACTTCGATTGCCAGGATTATGCTTAATGAAAATCAGCTGAATGACGAAGGACAATGTCGAAGGACAATGTCGAAGGACAAATGATATTAAGC
		**** *** ***** * *** * ***** ***** *****
	1	161 Toll-1-R
N .	lugens	TCCAACCAGATAACTTCGTTGCCAGATGAATTATTCAAGTACAATAAAAAGTTGACTGGATTGAAGGCTTCTCGTAACAAGCTGAATTTCTATCTGCAGACTTCTTGAGGGGCGCTGTACGACTTGAATGGATTGAATGACATGAGTTTCAAT
L.	striatellus	TCTAACCAGATCACTGCTTTGCCCGATGAATTATTCAAGTACAATAAAAAGCTGGATGGA
s.	furcifera	TCAAACCAGTTAGCTGAGCTGCCCGATGAATTGTTCAAGTACAAATAAAAAACTGACCGGGTTGAGAGCTTCTCGTAACAAGCTGAAAATTTCTGTCAGGGGCCTGTATGACTAGCATGCAT
		*** ***** * ** **** ******** **********
		2212
		qToll-1-F qToll-1-R
N .	lugens	argegettereg <u>argeteregareter</u> ttegeeteerrekenentetertrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeerrekeer
L.	striatellus	aagggattcaagaaagtcactcacctcactcgccaacaacatctcccccaccacccac
s.	furcifera	AAGGATTCAAGAAGGTGACACAGCTCTACTTGGCCAACAATTTCTCGTCCATCAATATCACGCAGCTTTCGCCGAATATAACGTTTATCGATCTGAATAACAATAATTTGACCCAC
		***** **** *** ** ** ******************

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	s for synthesis	of dsRNAs
rabit 1.	i i i inici sequence.	, ioi synthesis	01 40 101 11 10 10

Target gene	Primer name	Sequence
PGRP-LC	PGRP-LC-F	5'- <u>TAATACGACTCACTATAGGG</u> AAGTGTTCAATATTTCCAATG-3'
	PGRP-LC-R	5'-TAATACGACTCACTATAGGGCTAAAGTTGTTGCGACC-3'
Toll-1	Toll-1-F	5'-TAATACGACTCACTATAGGGGGTTGACTGACATCAGTAGA-3'
	Toll-1-R	5'-TAATACGACTCACTATAGGGGGCTTGTTACGAGAAGCC-3'
EGFP	EGFP-F	5'-TAATACGACTCACTATAGGGAGCTGACCCTGAAGTTCA-3'
	EGFP-R	5'- <u>TAATACGACTCACTATAGGG</u> TCCATGCCGAGAGTGATC-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 $1 \times 10^{\circ}$ 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).

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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'

Table 2. Primer sequences for RT-qPCR

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

Results

1. RNAi-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 dsRNA 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 dsRNA 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 dsRNA of Toll-1 was injected, RmRAs of 0.4-0.6 were obtained. When dsRNA 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 dsRNA of PGRP-LC and dsRNA of Toll-1 and that of dsRNA 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

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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 RNAimediated 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 RNAi of the trehalose phosphate synthase gene yielded relatively poor control of N. lugens, and it took a long time for the RNAi treatment to take effect. For this reason, we chose an injection-based method for RNAi-mediated suppression of immune system genes in the present study. Injection with dsRNA 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 RNAi-based approach for the

knockdown of immune system components for control of N. lugens could decrease the use of pesticides and development diminish the likelihood of of pesticide-resistant strains. More studies are clearly needed to explore the practicality of the use of RNAi knockdown systems for pest control. However, the approach here demonstrates the feasibility of using immune effectors against bacterial invasion via RNAi-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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