RNA interference-mediated silencing of genes involved in the immune responses of the soybean pod borer *Leguminivora glycinivorella* (Lepidoptera: Olethreutidae) (#24130)

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RNA interference-mediated silencing of genes involved in the immune responses of the soybean pod borer *Leguminivora glycinivorella* (Lepidoptera: Olethreutidae)

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RNA interference (RNAi) technology may be useful for developing new crop protection strategies against the soybean pod borer (SPB; *Leguminivora glycinivorella*), which is a critical soybean pest in northeastern Asia. Immune-related genes were recently identified as potential RNAi targets for controlling insects. However, little is known about the immune-related genes or mechanism underlying their expression in the SPB. In this study, we completed a transcriptome-wide analysis of immune-related genes. We identified 41 genes associated with *L. glycinivorella* microbial recognition proteins, immune-related effectors, or signalling molecules of immune response pathways (e.g., Toll and immune deficiency pathways). Eleven of these genes were selected for a dsRNA artificial feeding assay. The down-regulated expression of *LgToll-5a*, *LgPGRP-LB2a*, and *Lgitype-1* resulted in relatively high larval mortality rates and abnormal development.Our data may be useful as a comprehensive genetic resource for immune-related *L. glycinivorella* genes, and may contribute to the elucidation of the mechanism regulating innate immunity in Lepidoptera species. Furthermore, three *L. glycinivorella* genes were identified as potential RNAi targets, which may be relevant for the development of RNAi-mediated methods to control SPB infestations.

Running title: immune-related genes *of Leguminivora glycinivorella*

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Abstract

RNA interference (RNAi) technology may be useful for developing new crop protection strategies against the soybean pod borer (SPB; *Leguminivora glycinivorella*), which is a critical soybean pest in northeastern Asia. Immune-related genes were recently identified as potential RNAi targets for controlling insects. However, little is known about the immune-related genes or mechanism underlying their expression in the SPB. In this study, we completed a transcriptome-wide analysis of immune-related genes. We identified 41 genes associated with *L. glycinivorella* microbial recognition proteins, immune-related effectors, or signalling molecules of immune response pathways (e.g., Toll and immune deficiency pathways). Eleven of these genes were selected for a dsRNA artificial feeding assay. The down-regulated expression of *LgToll-5a*, *LgPGRP-LB2a*, and *Lgitype-1* resulted in relatively high larval mortality rates and abnormal development.Our data may be useful as a comprehensive genetic resource for immune-related *L. glycinivorella* genes, and may contribute to the elucidation of the mechanism regulating innate immunity in Lepidoptera species. Furthermore, three *L. glycinivorella* genes were identified as potential RNAi targets, which may be relevant for the development of RNAi-mediated methods to control SPB infestations.

Keywords: *Leguminivora glycinivorella*, immune-related gene, RNA interference, double-stranded RNA (dsRNA), artificial feeding assay

INTRODUCTION

Leguminivora glycinivorella (Mats.) obraztsov [i.e., soybean pod borer (SPB)] belongs to the order Lepidoptera and family Olethreutidae. The SPB is the major economic pest of soybean in northeastern Asia (Zhao et al., 2008; Meng et al., 2017a). The larvae use the immature beans as a food source until they develop into mature larvae, resulting in soybean yield losses of up to 40% (Meng et al., 2017b). Insecticides have been used to control SPB infestations over the past three decades. However, larvae within soybean pods under a closed canopy are often not exposed to the applied insecticides. Additionally, because of a lack of effective SPB-resistant germplasm, conventional breeding has not resulted in the production of new SPB-resistant cultivars. Therefore, the SPB remains a considerable pest responsible for substantial soybean yield losses (Wang et al., 2014; Song et al., 2015). Consequently, soybean breeders and growers are interested in developing new strategies for controlling SPB infestations, with RNA interference (RNAi) representing a promising option (Khajuria et al., 2015; Fishilevich et al., 2016).

RNA interference involves the degradation of specific endogenous mRNAs by homologous double-stranded RNA (dsRNA) (Fire et al., 1998). Depending on the function of the targeted gene, RNAi can inhibit insect growth or result in death (Joga et al., 2016; Christiaens et al., 2014). RNA interference is conserved in nearly all eukaryotic organisms and recent studies have indicated that feeding insect pests dsRNA molecules may be useful

for protecting agriculturally important crops (i.e., feeding RNAi or plant-mediated RNAi) (Mao & Zeng., 2014; Ulrich et al., 2015). The effectiveness of the RNAi technique for controlling pests depends on whether appropriate candidate genes are targeted because RNAi efficacy and transmission of the RNAi signal varies among genes (Huvenne & Smagghe., 2010). To date, applying RNAi technology to control Lepidoptera insects (i.e., moths and butterflies) has been problematic (Shukla et al., 2016). ~~However, the available information regarding the highly efficient of RNAi to lepidopterans has recently increased~~, ~~and~~ RNAi is particularly successful when targeting genes involved in immune responses (Terenius et al., 2011).

Insects such as *Drosophila melanogaster* and *Bombyx mori* have a vigorous innate immune system with which to defend against microbial infections (Bao et al., 2013). Peptidoglycan recognition proteins (PGRPs) are important pattern recognition receptors (PRRs) that detect peptidoglycan (PGN) in the cell wall of gram-negative and gram-positive bacteria, activate the Toll or immune deficiency (IMD)/JNK pathways, or induce proteolytic cascades that generate antimicrobial peptides (Gao et al., 2015; Chen et al., 2014), which are critical for defending against invading pathogens and for protecting insects against infections (Tamura et al., 2011). However, little is known about SPB immune-related genes or the associated immune responses.

We generated SPB transcriptome datasets based on Illumina sequencing. These datasets were used to identify many genes associated with microbe recognition, immune-related signalling, and defence effectors. Furthermore, RNAi was applied to study the effects of silencing immune-related genes on first instar larvae. A feeding assay involving an artificial diet supplemented with dsRNA was used to identify candidate target genes for controlling the SPB by RNAi.

MATERIALS AND METHODS

Insect rearing

Leguminivora glycinivorella eggs collected from a naturally infested soybean field at the experimental station of Northeast Agricultural University in Harbin, China were hatched at 26 °C. The resulting larvae were reared on an artificial diet prepared in our laboratory (Meng et al., 2017a). Adult moths were fed a 5% honey solution, and were allowed to oviposit on young bean pods. The first instar larvae were selected and subjected to artificial diet feeding experiments.

Illumina sequencing

The DN50 plants (provided by the Key Laboratory of Soybean Biology of the Chinese Education Ministry, Harbin, China) were grown in a greenhouse at 24 ± 1 °C with 60% relative humidity under a 16-h light/8-h dark cycle[3]. At the R5 soybean stage (fully developed pods), three replicates of 50 first-instar larvae were reared on soybean pods of DN50 plants.

The larvae were collected 3 days later. Total RNA was extracted from pooled larvae using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA library constructed as described by Chen et al were sequenced using the Illumina HiSeq 2000 system (Chen et al., 2014). The unigenes from three samples were combined to create the SPB unigene database. All raw transcriptome data have been deposited in the NIH Short Read Archive (accession number SRR5985986, SRR5985987, SRR5985984, SRR5985985, SRR5985988, SRR5985989).

Could not find any of these listed in NCBI, databases, THUS must include weblink to the NIH SRA data website, here for reader access! Also, authors should have submitted the mRNA transcript sequences, and should have Accession numbers for each assembled sequence and predicted protein, in addition to current stated short archive data sequences which could not be located.

Identifi

A list of immune-related genes was compiled based on the available relevant literature (Table S2), while homologous *B. mori* and *D. melanogaster* genes in the GenBank database were identified. The tBLASTn tool was used to complete sequence similarity searches of the SPB transcriptome database (Tamura et al., 2011; Guan & Mariuzza, 2007).

Phylogenetic and domain analyses

Amino acid sequences were aligned with the Multiple Alignment program clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and phylogenetic tree was constructed in MEGA 5 based on neighbour-joining method with 1000 bootstrap replicates (Xu et al., 2012). The architecture of protein domains was analysed using the SMART program (http://smart.embl-heidelberg.de/).

dsRNA synthesis

We synthesised dsRNAs using the T7 RiboMAX Express Large Scale RNA Production System (Promega, Madison, WI, USA). The T7 RNA polymerase promoter sequence was added to each end of DNA templates during PCR amplifications. All primers containing the T7 RNA polymerase promoter were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table S3). For the negative control, the GFP (green fluorescent protein) gene was amplified from the PCAMBIA1302 vector as templates for GFP dsRNA synthesis. The template DNA and single-stranded RNA were eliminated from the transcription reaction by DNase I and RNase A, respectively. The prepared dsRNAs were purified by a phenol/chloroform extraction followed by an ammonium acetate precipitation. The dsRNAs were ultimately suspended in ultrapure water and quantified using the Nano Drop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Soybean pod borer feeding bioassay

The first instar larvae were fed an artificial diet containing dsRNA (10 μg/g) for specific target genes as described by Meng et al. (2017) (Meng et al., 2017a). Control larvae were treated with the same concentration of *GFP* dsRNA. The feeding bioassay was completed in triplicate with 50 larvae per treatment or control. Three biological replicates were used for each treatment. The larvae were reared for 15 days at 26 °C under a 16-h light/8-h dark cycle with 65% relative humidity. The dsRNA-supplemented artificial diet was refreshed every 3

days. Body weight, mortality, and phenotypic abnormalities were recorded every 3 days. The resulting data underwent a one-way analysis of variance to compare the effects of the dsRNA treatment on larval mortality and body weight between treated and control larvae (Student's *t*-test, n = 3).

Quantitative real-time polymerase chain reaction

Two surviving larvae were randomly collected at each time point of per biological replicates of each treatment from 0 to 15 days after larvae were fed the artificial diet containing the dsRNA for target genes. Total RNA was extracted from pooled larvae using the RNApure Tissue Kit (DNase I) (CWBIO, Beijing, China). Additionally, primer sets were synthesised (Table S4). The extracted RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) to remove any contaminating genomic DNA prior to being used as the template for first-strand cDNA synthesis with the TIANScript RT Kit (Tiangen, Beijing, China). The efficiencies of the qRT-PCR primer pairs were greater than 90% (Table S4). The SPB *β-actin* expression levels were used to normalize the Ct values obtained for each gene, and the qRT-PCR was completed with three biological replicates, each of which comprised two technical replicates. Significant differences in the data for the control and treatment groups were determined based on a one-way analysis of variance (Student's *t*-test, n =3).

RESULTS

Identification of Leguminivora glycinivorella *immune-related genes*

Amino acid sequences encoded by *D. melanogaster*, *B. mori*, and *Manduca sexta* (Lepidoptera) immune-related genes were used to search *L. glycinivorella* transcriptome sequences. The 41 putative *L. glycinivorella* immune-related genes that were identified were functionally classified into three groups, namely microbial recognition, immune signalling, and immune effector molecules (Table S1).

Microbial recognition molecules

The PGRPs recognize conserved molecular patterns present in pathogens, but absent in the host, including PGNs, which are essential cell wall components of almost all bacteria. The PGRPs are encoded by a highly conserved gene family in insects, and are generally classified as one of two types (i.e., short or long) (Dziarski & Gupta, 2006; Yang et al., 2017). After specific ligands are detected, microbial recognition molecules activate or modulate various immune response pathways. We identified eight *L. glycinivorella* PGRPs, with an equal number of short and long forms, with similarities to *D. melanogaster* PGRP-SC, PGRP-SD, and PGRP-LB (Fig. 1). Five (i.e., LgPGRP-SC1a, LgPGRP-SC1b, LgPGRP-SD1b, LgPGRP-LB1, and LgPGRP-LB2b) of the eight identified PGRPs were predicted to be secreted proteins (based on the presence of putative signal peptides) that function as amidases. We observed that LgPGRP-LB lacks a putative signal peptide, but consists of a transmembrane region and amidase domain, suggesting that it serves as a transmembrane

PGN receptor. In contrast, we revealed that LgPGRP-SD1a and LgPGRP-LB2a carry only the PGRP domain, implying they are intracellular proteins (Table S1).

The gram-negative bacteria-binding proteins (GNBPs) and β-1,3-glucan recognition proteins (βGRPs) belong to a subfamily of PRRs, and have a strong affinity for the β-1,3-glucan of fungi and for the lipopolysaccharide of gram-negative bacteria, but not for the PGN of gram-positive bacteria. Of the three GNBPs produced by *D. melanogaster* (i.e., GNBP1, GNBP2, and GNBP3), GNBP1 interacts with PGRP-SA to form a hydrolytic complex that activates the Toll pathway in response to gram-positive bacteria, while GNBP3 is required for detecting fungi and activating the Toll pathway (Hughes, 2012; Rao et al., 2017). We identified one *GNBP* gene and one *βGRP* gene in the *L. glycinivorella* transcriptome datasets. The neighbour-joining phylogenetic analysis indicated that *LgGNBP3* is a homologue of *DmGNBP3* (Fig. S1). A comparison between the deduced amino acid sequences and the *D. melanogaste*r GNBP sequences indicated that LgGNBP3 contains a putative N-terminal β-1,3-glucan-recognition domain (CBM39) and a C-terminal glucanase-like domain (glycosyl hydrolase family 16), suggesting LgGNBP3 may be able to bind to fungal β-1,3-glucan.

Immune signalling molecules

We also identified genes associated with the Toll and IMD pathways, which are the

major signalling pathways that mediate the innate immunity of insects. The Toll pathway regulates the production of antimicrobial peptides in response to infections by fungi or gram-positive bacteria with lysine-type PGNs in their cell walls (Roh et al., 2009). The Toll receptor, which is responsible for the signal transduction associated with the Toll pathway, is vital for insect innate immune responses and embryo development (Takeda & Akira, 2004; Benton et al., 2016). In this study, we identified 10 genes encoding Toll receptors in the *L. glycinivorella* transcriptome datasets. The TIR domain is highly conserved in insect Toll families. To investigate the orthologous relationships among these genes, we constructed a phylogenetic tree based on an alignment of the TIR domains from all *L. glycinivorella* and *D. melanogaster* Toll proteins. The Toll receptors analysed in this study formed five major clusters, namely Toll5, Toll-6, Toll-7, Toll-8, and Toll-9 (Fig. 2A). Based on the phylogenetic tree, the *L. glycinivorella Toll* genes were designated as *Toll-5a*, *Toll-5b*, *Toll-6a*, *Toll-6b*, *Toll-6c*, *Toll-7a*, *Toll-7b*, *Toll-8*, *Toll-9a*, and *Toll-9b*. All 10 predicted proteins contain the extracellular LRR domain as well as the transmembrane and cytoplasmic TIR domains (Fig. 2B). We also identified sequences matching the intracellular components, ECSIT and Tollip, which affect the Toll signalling pathway (Table S1)

The IMD pathway is mainly activated by gram-negative bacterial infections. Additionally, IMD signal transduction is reportedly mediated by IMD, fas-associated death

domain protein (FADD), death-related ced-3/Nedd2-like caspase (Dredd), inhibitor of apoptosis protein 2 (IAP2), transforming growth factor β-activated kinase (TAK1), TAK1-binding 2 (Tab2), ubiquitin conjugating 13 (Ubc13), and an inhibitor of nuclease factor B kinase subunits b and g (IKKb and IKKg)(Bao et al., 2013; Myllymäki et al., 2014). Although we did not detect *L. glycinivorella* orthologues of *TAK1*, *Tab2*, *Ubc13*, *IKKb*, and *IKKg*, we identified sequences that were homologous to *FADD*, *Dredd*, and *IAP2* (Table S1).

Immune-related effector genes

The PGRPs and βGRPs detect PGNs and β-1,3-glucans, which activates a clip-domain serine protease (CLIP) cascade that converts prophenoloxidase to active phenoloxidase, leading to the melanisation responses involved in eliminating pathogens(Monwan et al., 2017; Li et al., 2016). We identified two *CLIP* genes (*LgSnake-1* and *LgSnake-2*) in the *L. glycinivorella* transcriptome datasets. The deduced amino acid sequences contain a clip domain at the N-terminus and a serine protease domain at the C-terminus (Fig. S2A). Serine protease inhibitors (i.e., serpins) negatively regulate prophenoloxidase activation, which prevents the excessive activation of the CLIP cascade. In this study, we identified three serpin genes in the *L. glycinivorella* transcriptome datasets (i.e., *Lgserpin1*, *Lgserpin2*, and *Lgserpin3*). Their deduced amino acid sequences consist of a putative signal peptide sequence and a core serpin domain, suggesting they are secreted proteins (Fig. S2B).

Immune response effector genes

Antibacterial peptides are immune response effectors whose production is induced by immune challenges and are important for defence responses against insects. Diverse antibacterial peptide genes have been identified in many insect species, including genes encoding defensins, reeler, and lysozyme (Imler & Bulet, 2005; Bao et al., 2011). In this study, we identified one defensin gene in the *L. glycinivorella* transcriptome datasets (i.e., *Lgdefensin1*). The encoded amino acid sequence consists of a putative signal peptide sequence and a core Knot1 domain (Table S2). We also identified seven chicken (C-type) lysozymes and two invertebrate (I-type) lysozymes in the *L. glycinivorella* transcriptome (Fig. S3). The C-type lysozymes are bacteriolytic enzymes that hydrolyse the β (1-4) bonds between N-acetylglucosamine and N-acetylmuramic acid in the PGN of prokaryotic cell walls. We observed that with the exception of the C-type 3 protein, the predicted *L. glycinivorella* C-type proteins include an N-terminal signal peptide sequence (Table S1). Additionally, we detected eight conserved cysteine residues in the *L. glycinivorella* C-type lysozymes (Fig. S3A) as well as 12 conserved cysteine residues in the deduced *L. glycinivorella* I-type lysozyme sequences (Fig. S3B). These cysteine residues possibly form intramolecular disulfide bonds to enhance stability and resistance against proteolytic degradation.

Potential RNA interference targets identified in an artificial feeding assay and effects of

double-stranded RNA on soybean pod borer development and mortality

Eleven genes representing the immune-related SPB genes were selected and analysed to identify potential new RNAi targets useful for controlling the SPB. We synthesised the corresponding dsRNAs *in vitro* and mixed them in an artificial diet. The mortality rates 3-days after larvae were fed an artificial diet containing 10 μg/g dsRNA for *LgToll-5a*, *LgSerpin2*, *LgPGRP-LB*, *LgPGRP-LB2b*, or *LgChaoptin* were 42.86–92%. These mortality rates were significantly higher than those of control larvae treated with PBS or *GFP* dsRNA. Additionally, mortality rates were even higher at day 15. In contrast, the artificial diets containing dsRNA targeting *Lgitype-1*, *LgToll-5b*, *LgToll-9b*, *LgToll-6b*, *LgToll-7b*, or *LgPGRP-LB2a* did not have any considerable effects on larval mortality (Fig. 3). Moreover, we detected three main phenotypic differences among the surviving larvae after 15 days of feeding. First, the weight of the larvae fed *LgToll-5b* dsRNA increased more slowly than that of larvae fed *GFP* dsRNA, and was ultimately lower after 15 days of feeding (Fig. 4). Additionally, the cuticles of larval bodies were black (Fig. 5). Second, larvae treated with *Lgitype-1* dsRNA were more sensitive to pathogens, with approximately 50% of the larvae infected by bacteria even in the absence of an artificial pathogen inoculation. Third, larvae fed dsRNA targeting *LgPGRP-LB* or *LgPGRP-LB2a* underwent early pupation, with pupation

rates of 25 and 50%, respectively. The remaining larvae developed abnormally with stunted and twisted bodies (Fig. 5).

To investigate how larval mortality and abnormal development are correlated with the relative expression levels of specific target genes, we completed a quantitative real-time polymerase chain reaction (qRT-PCR) using total RNA extracted from the surviving larvae at different time points after feeding on the artificial diet. We observed that the *LgPGRP-LB2b*, *LgToll-5b*, *LgToll-7b*, *LgToll-9b*, and *LgItype-1* expression levels decreased significantly in larvae 3 days after being treated with the respective dsRNA, while the expression levels of the remaining genes decreased significantly after 6 or 9 days ($p < 0.01$; Student's *t*-test, $n = 3$) (Fig. 6). These results imply that the increased mortality and abnormal development of larvae fed dsRNA were due to the down-regulated expression of specific target genes. Moreover, we propose that unigenes *LgToll-5a*, *LgPGRP-LB2a*, and *Lgitype-1* may represent good RNAi targets for controlling the SPB.

DISCUSSION

Insects possess an efficient innate immune system that protects insects from microorganisms helps insects overcome abiotic stresses (Hillyer, 2015; Parsons& Foley, 2016). The immune-related genes have recently garnered interest among entomologists (Gendrin et al., 2017). In this study, 41 genes were identified in the *L. glycinivorella* transcriptome, including genes encoding components of the conserved immune signalling

pathways (i.e., Toll and IMD pathways) as well as pathogen recognition and immune response effectors. Most of these genes include conserved sequences that exist in orthologous *D. melanogaster* and *B. mori* genes (Table S1). However, immune-related gene families have expanded or contracted in different taxa. For example, the *PGRP* gene families in *D. melanogaster*, *B. mori*, and *L. glycinivorella* include 13, 12, and 8 members, respectively (Hillyer, 2015; Yang et al., 2015). Along with the differences in the sequences of immune-related genes, we observed that the encoded proteins exhibit diverse activities. For example, four of the *L. glycinivorella* PGRPs are closely related to each other and form an independent cluster with *D. melanogaster* PGRP LB (Fig. 1). Two of them contain a putative signal peptide and a conserved Ami_2 domain, whereas the others lack a signal peptide (Table S1). Furthermore, silencing *LgPGRP-LB* and *LgPGRP-LB2a* induced early pupation and abnormal larval development, while silencing *LgPGRP-LB2b* had no significant effect on larval development (Fig. 5). Previous studies revealed that PGRP-LB is a catalytic amidase that can degrade PGN and regulate host immune responses to infectious microorganisms by down-regulating the IMD pathway (Zaidman-Rémy et al., 2006; Troll et al., 2009), which protects the beneficial microbes in insects and prevents host-inflicted damage during development (Hashimoto et al., 2007). In the Tsetse fly (Diptera: Glossinidae), silencing *PGRP-LB* by RNAi decreases host fecundity because of the associated cost of activating the

host immune response (Wang & Aksoy, 2012).

L. glycinivorella is a univoltine insect. The mature larvae make cocoons in the soil and enter diapause during the winter and pupate in mid-July, for a diapause period of about 10 months[2]. In our study, *LgPGRP-LB* and *LgPGRP-LB2a* were silenced by RNAi, which broke the diapause and caused mature larvae to pupate. This termination of diapause may be due to an immune response that is initiated to prevent host-inflicted damage. Nevertheless, further research is needed to confirm that LgPGRP-LB influences the activation of host immune responses.

The Toll pathway is not only critical for innate immunity against bacteria, it also affects embryonic development, olfactory neuron processes, and TNF-induced JNK-dependent cell death in *D. melanogaster* (Yang et al., 2015; Valanne et al., 2011; Wu Valanne et al., 2015). Knocking down the *fusilli* and *cactin* genes, which are part of the Toll pathway, is lethal for the red flour beetle (*Tribolium castaneum*). Meanwhile, the silencing of *cactin* is 100% lethal at all developmental stages (i.e., larva to adult). Additionally, the knockdown of *pelle* and *dorsal* prevents eggs from hatching in the next generation (Bingsohn et al., 2017). In our study, an artificial diet containing dsRNA for *LgToll-5a* was 93% lethal to larvae 3 days after feeding. Furthermore, knocking down *LgToll-5b* decreased body weight and prevented old cuticles from separating from larval bodies. These results suggest that

LgToll-5a and LgToll-5b influence larval development.

Lysozymes are widely distributed immune effectors that exhibit muramidase activity against the PGNs in the bacterial cell wall to induce cell lysis (Zhou et al., 2017). In our study, the knockdown of LgI-type-1 resulted in bacterial infections of larvae. The LgI-type-1 gene encodes a destabilase domain, which is associated with isopeptidase and antibacterial activities. Meanwhile, the pI of LgI-type-1 is 7.93 (Table 2). Researchers have proposed that I-type lysozymes with a high pI influence immunity (Kurdyumov et al., 2015; Xue et al., 2004). Thus, LgI-type-1 exhibits isopeptidase activity and contributes to *L. glycinivorella* immunity.

CONCLUSION

We identified 41 genes associated with *L. glycinivorella* microbial recognition proteins, immune-related effectors, or signalling molecules of immune response pathways (e.g., Toll and immune deficiency pathways). This would be useful as a comprehensive genetic resource for immune-related *L. glycinivorella* genes, and may contribute to the elucidation of the mechanism regulating innate immunity in Lepidoptera species. In addition, the in vivo functions of 11 genes were analysed in RNAi experiments, which indicated that three genes may be appropriate RNAi targets for controlling the SPB. The observations described herein may be useful for future analyses of the mechanisms underlying the *L. glycinivorella* immune response pathways and for developing RNAi-mediated methods to control SPB infestations.

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Figure 1

Phylogenetic relationships among PGRPs from *Leguminivora glycinivorella* and *Drosophila melanogaster*

The phylogenetic tree was constructed using MEGA5.0 with a neighbour-joining approach. The bootstrap values (1000 replicates) are provided next to the branches. The first two letters of each PGRP name indicates the species (Dm, D. melanogaste*r*; Lg, *L. glycinivorella*).

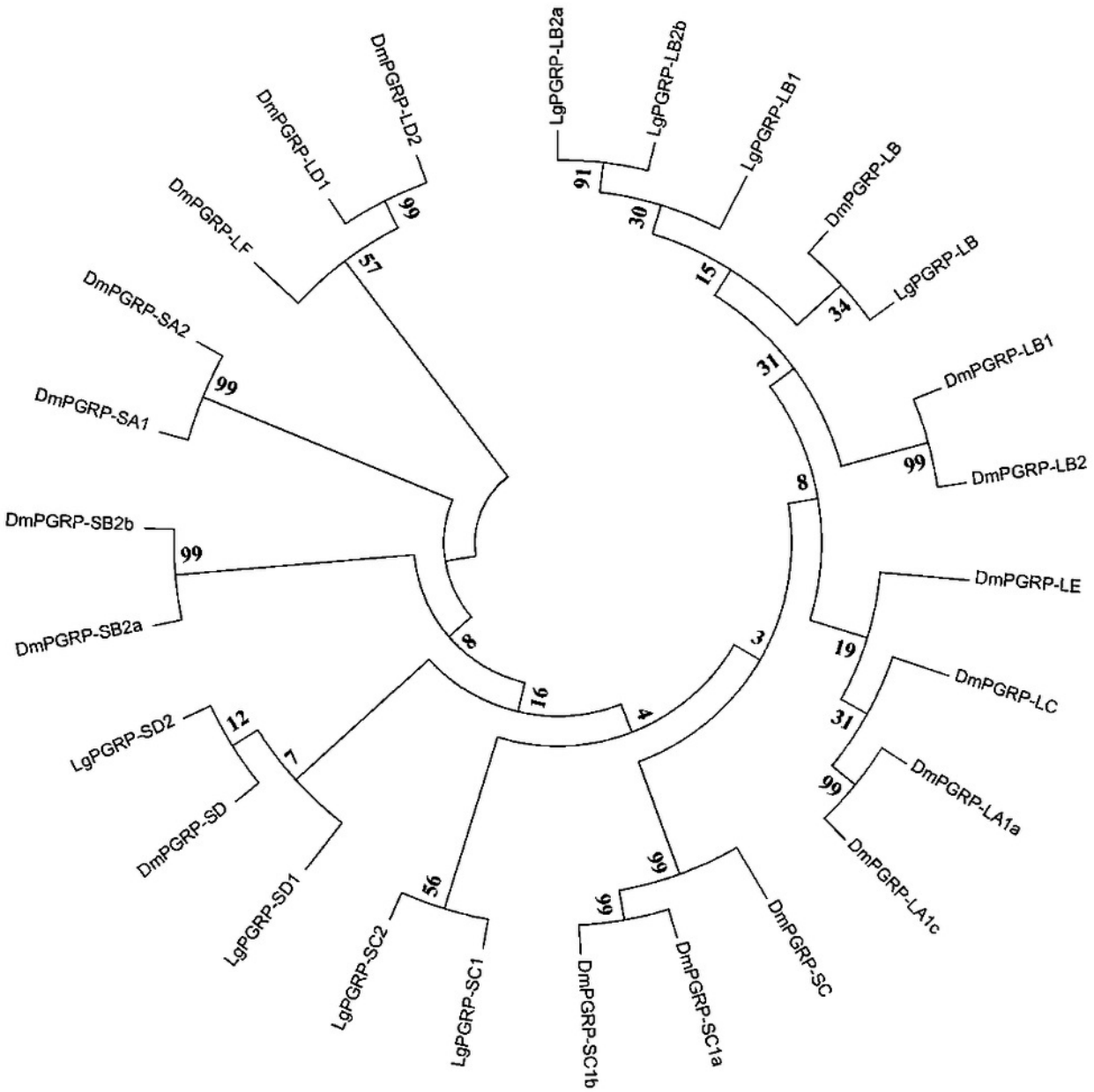
LgPGRP-LB2a
LgPGRP-LB2b
LgPGRP-LB1
DmPGRP-LB
LgPGRP-LB
DmPGRP-LB1
DmPGRP-LB2
DmPGRP-LE
DmPGRP-LC
DmPGRP-LA1a
DmPGRP-LA1c
DmPGRP-SC
DmPGRP-SC1a
DmPGRP-SC1b
LgPGRP-SC1
LgPGRP-SC2
LgPGRP-SD1
DmPGRP-SD
LgPGRP-SD2
DmPGRP-SB2a
DmPGRP-SB2b
DmPGRP-SA1
DmPGRP-SA2
DmPGRP-LF
DmPGRP-LD1
DmPGRP-LD2
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Figure 2

The analysis of Toll receptors of the *L. glycinivorella*

(A) Phylogenetic relationships among Toll receptors from *Leguminivora glycinivorella* and *Drosophila melanogaster*. The phylogenetic tree was constructed using MEGA5.0 with a neighbour-joining approach. The bootstrap values (1000 replicates) are provided next to the branches. Lg, L. glycinivorella; Dm, D. melanogaster. (B) Predicted domains of the L. *glycinivorella* Toll receptors. The domain organization was predicted using the SMART program (http://smart.embl.de/). The extracellular leucine-rich repeats (LRRs) are presented as rectangles and the LRR C-terminal domain is indicated with small ellipses. The intracytoplasmic TIR domains are presented as big ellipses and signal peptide are shown with red rectangles

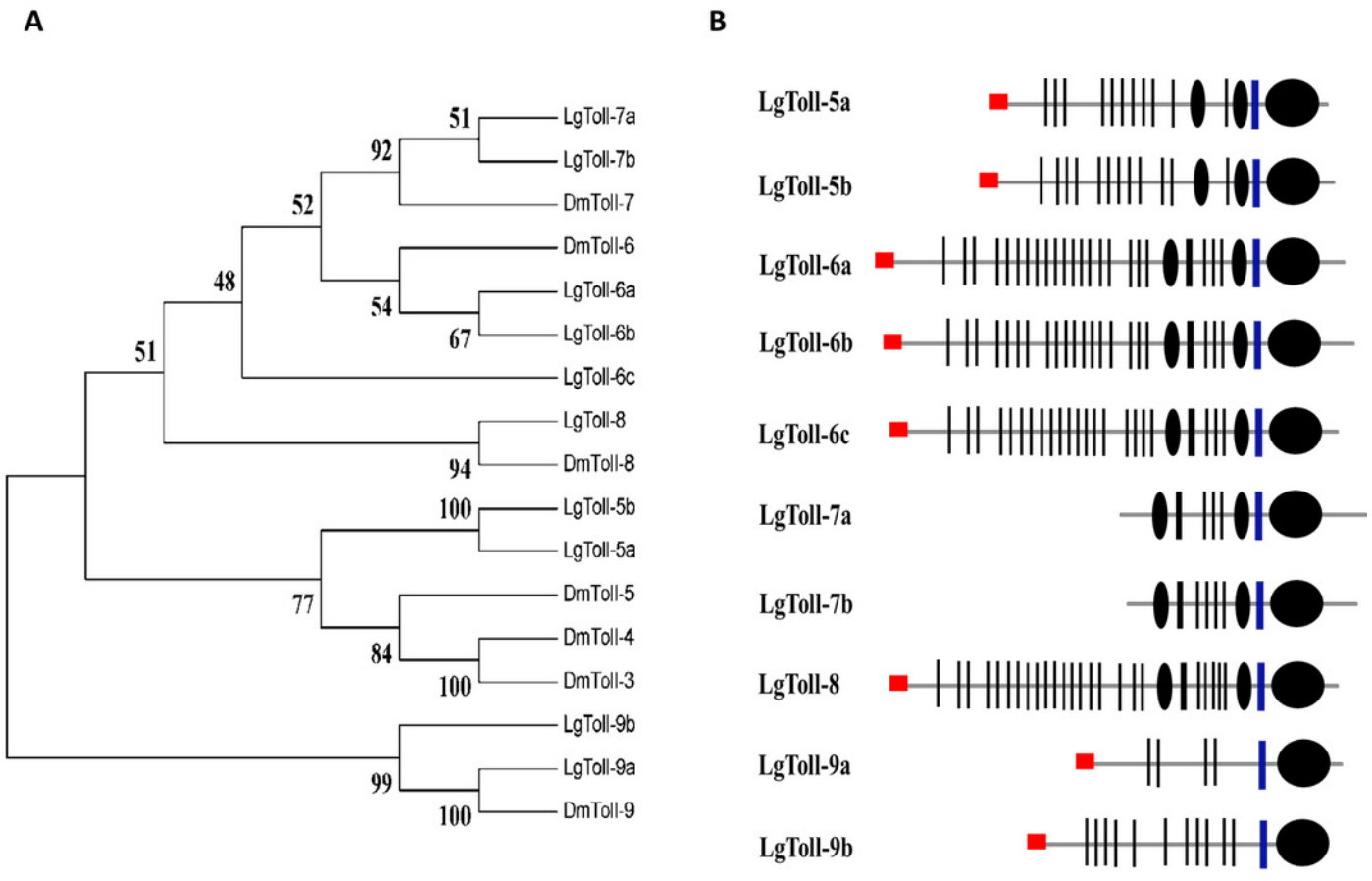

Figure 3

Mortality of larvae fed an artificial diet supplemented with dsRNA (10 μg/g) for 11 candidate RNA interference target genes.

*p < 0.05, **p < 0.01 (Student's t-test, n = 3).

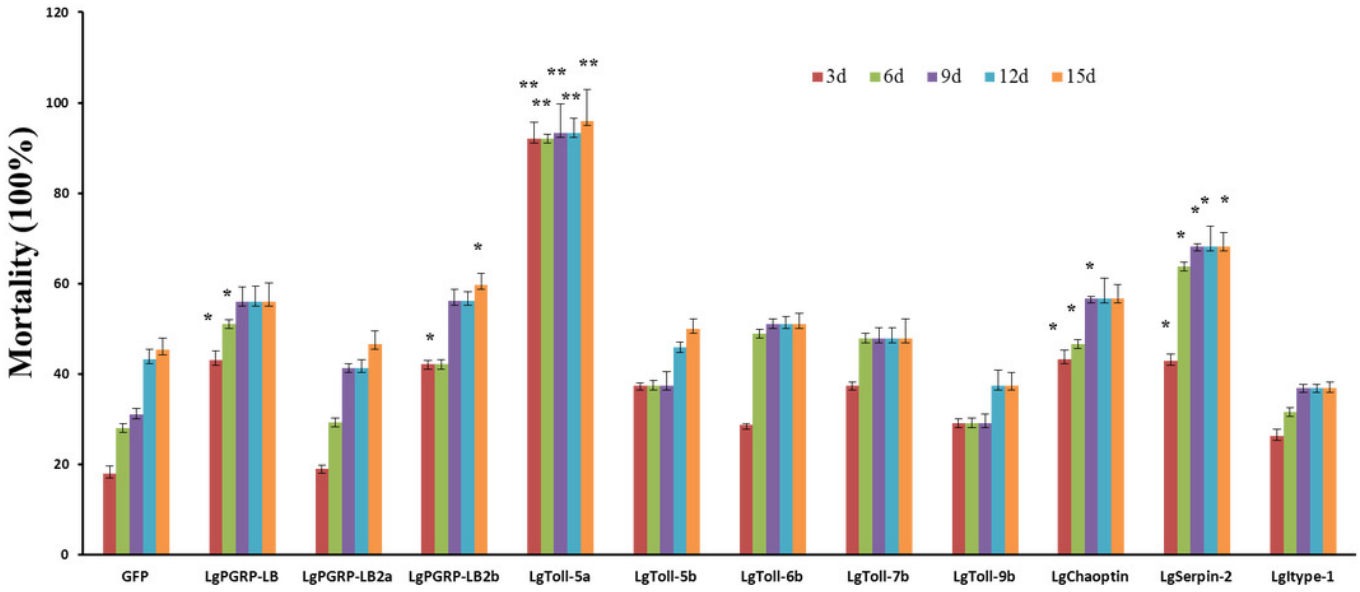

Figure 4

Body weight of larvae fed an artificial diet supplemented with dsRNA (10 μg/g) for 11 candidate RNA interference target genes.

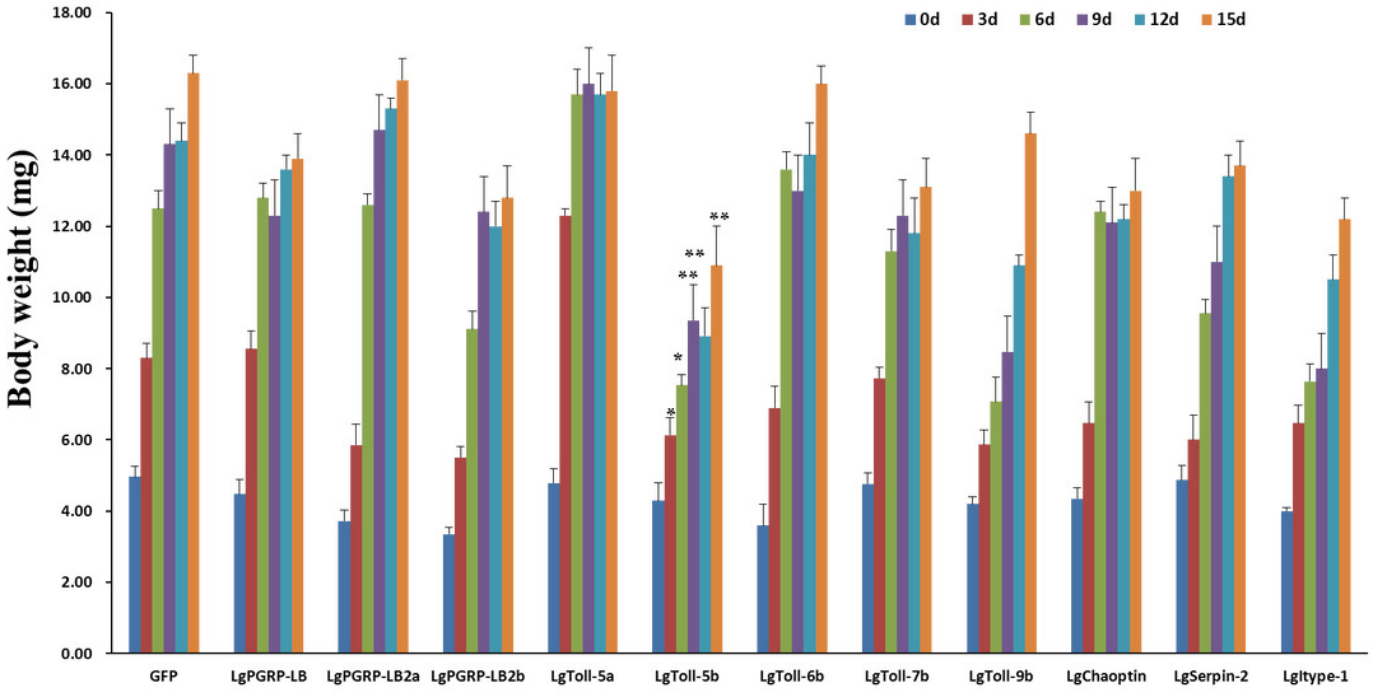

Figure 5

Images of larvae fed an artificial diet supplemented with dsRNA (10 μg/g) for 11 candidate RNA interference target genes for 15 days.

Of the larvae fed an artificial diet containing dsRNA for LgToll-5a, only one survived after 15 days.

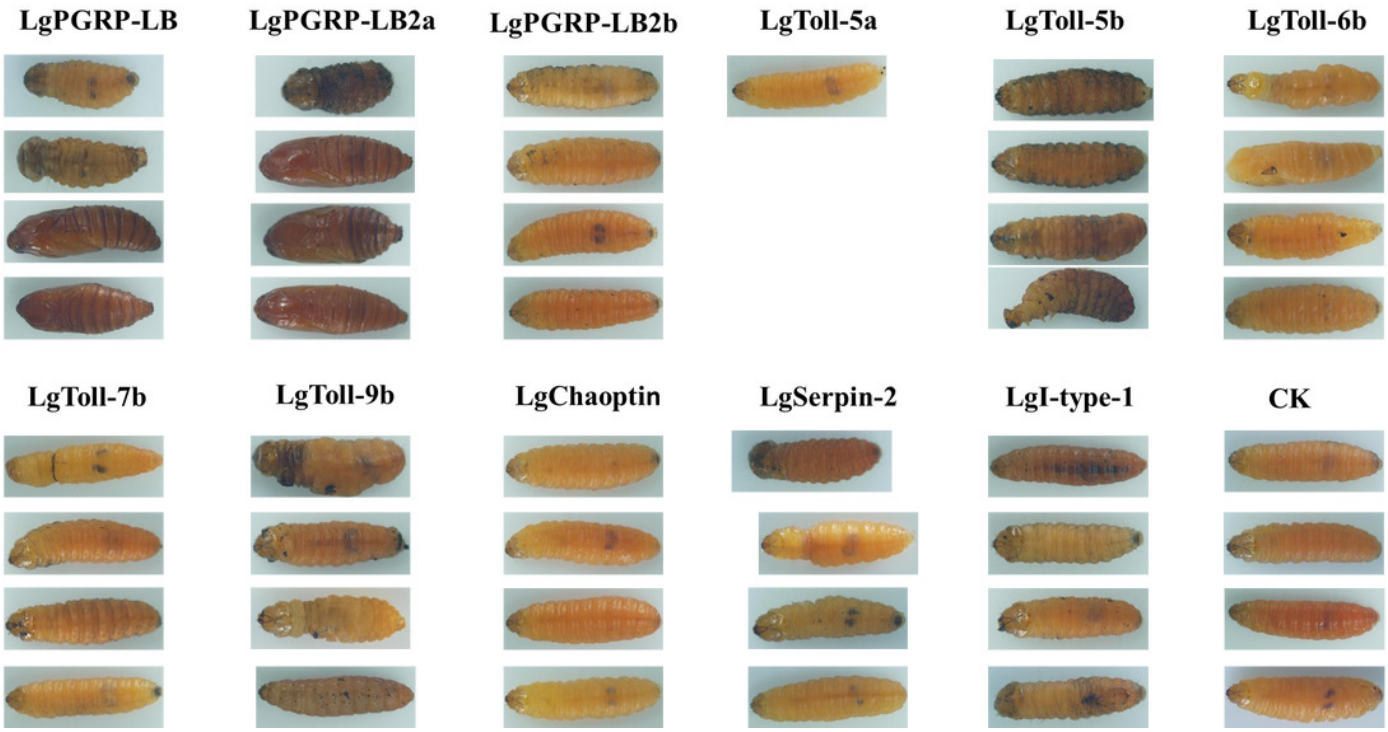

Figure 6

Relative expression levels of 11 candidate RNA interference target genes at different time points after larvae were fed an artificial diet containing dsRNA (10 μg/g).

Quantitative real-time polymerase chain reactions were completed using total RNA extracted from surviving larvae. *p < 0.05, **p < 0.01 (Student's t-test, n = 3).