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

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

Ruixue Ran [1], Tianyu Li [1], Xinxin Liu [1], HejiaNi[2], Wenbin Li [1,3], Fanli Meng [1,3] *

[1] Key Laboratory of Soybean Biology in the Chinese Ministry of Education, Northeast Agricultural University, Harbin 150030, China

[2] Colleges of Life Science, Northeast Agricultural University, Harbin, China, 150030

[3] Division of Soybean Breeding and Seeds, Soybean Research & Development Center, CARS (Key Laboratory of Biology and Genetics & Breeding for Soybean in Northeast China, Ministry of Agriculture), Northeast Agricultural University, Harbin 150030, China

Ruixue Ran: ranruixue@outlook.com

Tianyu Li: litianyu151243@outlook.com

Xinxin Liu: Liu_xinxin6@163.com

HejiaNi: nhjwinner@163.com

Wenbin Li: wenbinli@neau.edu.cn

Fanli Meng: mengfanli@neau.edu

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

Ruixue Ran and Tianyu Li contributed equally to this work

*Corresponding author:

Fanli Meng

Key Laboratory of Soybean Biology in the Chinese Ministry of Education, Northeast Agricultural University, Harbin, China, 150030

Email: mengfanli@neau.edu

Phone: (+86) 451-55191413

Fax: (+86) 451-55103336

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

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 have been recently identified as potential RNAi targets for controlling insects. However, little is known about these genes or mechanisms underlying their expression in the SPB. In this study, we completed a transcriptome-wide analysis of SPB immune-related genes. We identified 41 genes associated with SPB microbial recognition proteins, immune-related effectors or signalling molecules in immune response pathways (e.g., Toll and immune deficiency pathways). Eleven of these genes were selected for a double-stranded RNA artificial feeding assay. The down-regulated expression levels of *LgToll-5-1a* and *LgPGRP-LB2a* resulted in relatively high larval mortality rates and abnormal development. Our data represent a comprehensive genetic resource for immune-related SPB genes, and may contribute to the elucidation of the mechanism regulating innate immunity in Lepidoptera species. Furthermore, two immune-related SPB genes were identified as potential RNAi targets, which may be used in the development of RNAi-mediated SPB control methods.

INTRODUCTION

Leguminivora glycinivorella (Mats.) obraztsov [soybean pod borer (SPB)] belongs to the order Lepidoptera and family Olethreutidae. The SPB is the major pest of soybean in northeastern

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

Asia (Zhao et al., 2008; Meng et al., 2017a). The larvae use the immature beans as a food source until they reach maturity, 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 that are under a closed canopy are often not exposed to the applied insecticides. Because of the lack of effective SPB-resistant germplasm, conventional breeding has not resulted in the production of new SPB-resistant cultivars. Therefore, the SPB remains a major pest and is 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).

RNAi involves the degradation of specific endogenous mRNAs by homologous double-stranded RNAs (dsRNAs) (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). RNAi is conserved in nearly all eukaryotic organisms, and feeding insect pests dsRNA molecules may be useful in protecting agriculturally important crops (feeding RNAi or plant-mediated RNAi) (Mao & Zeng, 2014; Ulrich et al., 2015). However, Microinjection RNAi is particularly successful when targeting genes involved in immune responses (Terenius et al., 2011). The

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

effectiveness of the RNAi technique for controlling pests depends on whether appropriate candidate genes are targeted because RNAi efficacy and the RNAi signal transmission vary among genes (Huvenne & Smagghe, 2010). While, applying RNAi technology to control Lepidoptera insects (i.e., moths and butterflies) has been problematic (Shukla et al., 2016), the information available regarding its efficiency has recently increased.

Insects such as *Drosophila melanogaster* and *Bombyx mori* have a vigorous innate immune systems with which to defend against microbial infections (Myllymäki et al., 2014; Parsons et al., 2016; Yang et al., 2017; Chen et al., 2018). Peptidoglycan recognition proteins (PGRPs) are important pattern recognition receptors that detect peptidoglycan (PGN) in the cell walls of gram-negative and gram-positive bacteria. PGRPs 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). Antimicrobial peptides are critical for defending against invading pathogens and for protecting insects against infections (Gegner et al., 2018). However, little is known about SPB immune-related genes or the associated immune responses.

To identify the immune-related genes of SPB, 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

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

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& METHODS

Insect rearing

L. 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 T3 dsSpbP0 (double-stranded SBP ribosomal protein P0 RNAi transgenic soybean line) and wild-type '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 (Meng et al., 2017b). At the R5 soybean stage (fully developed pods), three replicates of 50 first-instar larvae were reared on soybean pods of DN50 and T3 dsSpbP0 plants. The larvae were collected after 3 d. cDNA library preparation and

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

Illumina sequencing were conducted by the Biomarker Technology Company (Beijing, China). Briefly, total RNA was extracted from six pooled larvae using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was synthesised using random hexamer-primers from purified Poly (A) mRNA. Second-strand cDNA was synthesised using DNA polymerase I and RNaseH, and then purified using a QiaQuick PCR extraction kit (Qiagen, Hilden, Germany). cDNA fragments of a suitable length (300–500 bp) were obtained by agarose gel electrophoresis and amplified byPCR to construct the final cDNA libraries for paired-end sequencing using the Illumina HiSeq 2000 system. The unigenes from six samples were combined to create the SPB unigene database (Chen et al., 2014; Meng et al., 2017c). All raw transcriptome data have been deposited in the NIH Short Read Archive (accession numbers SRR5985984–SRR5985989).

Identification of immune-related genes

A list of immune-related genes was compiled based on the available relevant literature (Table S1), and homologous *B. mori*, *D. melanogaster, Danaus plexippus* and *Papilio polytes* genes in the GenBank database were identified (Guan & Mariuzza, 2007; Xu et al., 2012). The tBLASTn algorithm-based tool was used to complete sequence similarity searches of the SPB transcriptome database (Boratyn et al., 2013). The 41 SPB immune-related genes were submitted to NCBI GenBank and their Accession numbers are shown in TableS1.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

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 the phylogenetic tree was constructed in MEGA 5 based on the neighbour-joining method with 1000 bootstrap replicates (Tamura et al., 2011; Sievers et al., 2011). The architecture of the 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 the DNA templates during PCR amplifications. The primers containing the T7 RNA polymerase promoter were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table S2). For the negative control, the green fluorescent protein (*GFP*) gene was amplified from the PCAMBIA1302 vector as a template 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 a Nano Drop 2000

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

spectrophotometer (Thermo Scientific, Waltham, MA, USA).

SPB feeding bioassay

The first-instar SPB larvae were fed an artificial diet containing dsRNA (10 μg/g) for specific target genes as described by 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 d 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 d. Body weight, mortality, and phenotypic abnormalities were recorded every 3 d.

Quantitative real-time PCR (*qRT-PCR)*

For every treatment, two surviving larvae were randomly collected at each time point from all of the biological replicates from 0 to 15 d after larvae were fed the artificial diet containing the target gene's dsRNA, frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from two pooled larvae using the RNApure Tissue Kit (DNase I) (CWBIO, Beijing, China). Additionally, primer sets were synthesised (Table S2). The extracted RNA samples were treated with DNase I (Invitrogen) to remove any contaminating genomic DNA. They were then being used as the template for first-strand cDNA synthesis with the TIANScript RT Kit (Tiangen,

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

Beijing, China). The qRT-PCR analysis was completed using the SYBR Green kit (Bio-Rad, Hercules, CA, USA) and a Roche LightCycler® 480 real-time PCR system (Roche, Basel, Switzerland). The efficiencies of the qRT-PCR primer pairs were greater than 90%. The qRT-PCR conditions were as follows: 95°C for 5 min; 40 cycles at 95°C for 30 s, 60 °C for 15 s, and 72°C for 45 s; 95°C for 1 min and 55°C for 1 min. At the end of each qRT-PCR experiment, a melt curve was generated to check for primer-dimer formation. The qRT-PCR analysis contained three biological replicates, each having three technical replicates (Meng et al., 2017c). Each target gene's qRT-PCR products were sequenced to confirm their identities. Relative expression levels were calculated by the following formula using LightCycler 480 Software v1.5.0: $R = 2^{-(\Delta Ct\ sample - \Delta Ct\ calibrator)}$, where Rrepresents the relative expression level, ΔCt sample is the average difference between the Ct of the gene and that of SBPß-actin in the expreimental sample (Meng et al., 2017b), and ΔCt calibrator is the average difference between the Ct of the gene and the e SBPß-actin in the calibrator. A representative sample was set as the calibrator (Bustin et al., 2009). The differences in Ct values between technical replicates was less than 0.5. The relative expression level of 0d for each target gene was set to 1.00. The relative expression level of other time point for each target gene compare to 0d value.

Statistical analyses

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

All of the data in this study are presented as mean ± SE. Significant differences were determined by one-way analysis of variance followed by least significant difference tests for mean comparisons. The statistical analysis was performed with SAS 9.21 software (SAS Institute, Cary, NC, USA). *P*-values were correct by Bonferroni.

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 SPB transcriptome sequences. The 41 identified putative SPB immune-related genes were functionally classified into three groups, 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 into two types (short and long) (Dziarski & Gupta, 2006; Yang et al., 2017). We identified eight SPB PGRPs, four short and four long types, with similarities to *D. melanogaster* PGRP-SC, PGRP-SD, and PGRP-LB (Fig. 1). Five (LgPGRP-SC1a, LgPGRP-SC1b, LgPGRP-SD1b, LgPGRP-LB1, and LgPGRP-LB2b) of the eight identified PGRPs were predicted to be secreted

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

proteins, based on the presence of putative signal peptides, that function as amidases. LgPGRP-LB lacks a putative signal peptide, but contains a transmembrane region and amidase domain, suggesting that it serves as a transmembrane PGN receptor. In contrast, LgPGRP-SD1a and LgPGRP-LB2a carry only the PGRP domain, implying that 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 pattern recognition receptors and have strong affinities for the lipopolysaccharide of gram-negative bacteria and the β-1,3-glucan of fungi, but not for the PGN of gram-positive bacteria. Of the three GNBPs produced by *D. melanogaster* (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 in the SPB transcriptome datasets. The neighbour-joining phylogenetic analysis indicated that *LgGNBP3* is an ortholog 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 that LgGNBP3 may bind to fungal

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

β-1,3-glucan.

Immune signalling molecules

After specific ligands are detected, microbial recognition molecules activate or modulate various immune response pathways. Here, we 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 SPB 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 SPB and *D. melanogaster* Toll proteins. The Toll receptors analysed in this study formed six major clusters, namely Toll-3, Toll-4, Toll-5, Toll-6, Toll-7 and Toll-8 (Fig. 2A). Based on the phylogenetic tree, the *L. glycinivorella Toll* genes were designated as *LgTLR-3*, *LgTLR-4, LgToll-5-1a, LgToll-5-1b, LgToll-6, TLR-6-1*, *TLR-6-2, LgToll-7, LgTLR-7* and *LgToll-8.* All 10 predicted proteins contain an extracellular LRR domain

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

as well as 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). Of these,we only 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, Amparyup & Tassanakajon, 2017; Li et al., 2016). We identified two *CLIP* genes (*LgSnake-1* and *LgSnake-2*) in the SPB transcriptome datasets. The deduced amino acid sequences each contain a clip domain at the N-terminus and a serine protease domain at the C-terminus (Fig. S2). Serine protease

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

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, *Lgserpin1*, *Lgserpin2*, and *Lgserpin3*, in the SPB transcriptome datasets. Their deduced amino acid sequences each contained a putative signal peptide sequence and a core serpin domain, suggesting that they are secreted proteins (Table S1).

Immune response effector genes

Antibacterial peptides are immune response effectors that are 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 two defensin genes, *Lgdefensin1* and *Lgdefensin2*, in the SPB transcriptome datasets. The encoded amino acid sequences each consisted of a putative signal peptide sequence and a core Knot1 domain (Table S1). We also identified seven chicken-type (C-type) lysozymes and two invertebrate-type (I-type) lysozymes in the *L* SPB transcriptome. 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. The predicted SPB C-type proteins， with the exception of the C-type 3 protein, each include an N-terminal signal peptide sequence (Table S1). Additionally,

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

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 SPB I-type lysozyme sequences (Fig. S3B). These cysteine residues may form intramolecular disulphide e bonds to enhance stability and resistance against proteolytic degradation.

Potential RNAi targets identified in an artificial feeding assay and effects of double-stranded RNA on soybean pod borer development and mortality

In total, 11genes 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 3d after larvae were fed artificial diets, which contained containing 10 μg/g dsRNA for *LgPGRP-LB*, *LgPGRP-LB2b*, *LgToll-5-1a, LgToll-5-1b, LgTLR-7, LgSerpin2* or *LgChaoptin* were 37%–92%. These mortality rates were significantly greater than those of control larvae treated with *GFP* dsRNA. Additionally, the final mortality rates of the larvae fed *LgPGRP-LB*, *LgPGRP-LB2b, LgToll-5-1a*, *LgSerpin2* and *LgChaoptin* dsRNA were even higher at 15 d. In contrast, the artificial diets containing dsRNA independently targeting *LgPGRP-LB2a, LgTLR-3* and *Lgitype-1* did not have any statistically significant effects on larval mortality (Fig. 3). Moreover, two main phenotypic differences were observed among the surviving larvae after 15 d of feeding. First, the

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

weights of the larvae fed *LgToll-5-1b* and *LgItype-1* dsRNA increased more gradually than those of larvae fed *GFP* dsRNA, and was ultimately lower after 15 d of feeding (Fig. 4). Additionally, the *LgToll-5-1b* RNAi treatment resulted partly black cuticles. Second, 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 performed qRT-PCR using total RNA extracted from the surviving larvae at different time points after feeding on artificial diets. The expression levels of the genes, except for those of *LgPGRP-LB2a*, *LgTLR-6-1 and LgChaoptin* decreased in larvae 3 d after being treated with the respective dsRNAs, while the expression levels of all of the genes decreased significantly after 6 or 9 d (Fig. 6). Thus, the increased mortality rates and abnormal development of larvae fed dsRNA may result from the down-regulated expression of specific target genes. Moreover, unigenes *LgToll-5a* and *LgPGRP-LB2a* may represent good RNAi targets for controlling the SPB.

DISCUSSION

Insects possess efficient innate immune system that protect them from microorganisms and

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

aid in abiotic stresses (Hillyer, 2015; Parsons& Foley, 2016). In this study, we identified 41 genes in the SPB transcriptome that encode components of conserved immune signalling pathways (Toll and IMD pathways) as well as pathogen recognition and immune response effectors. Most of these genes contained 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*, SPB have 13, 12, and 8 members, respectively (Hillyer, 2015; Yang et al., 2015). In addition to sequences differences among the immune-related genes, the encoded proteins exhibited diverse activities. For example, four of the SPB's 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, while the others lack a signal peptide (Table S1). Furthermore, silencing *LgPGRP-LB* and *Lg*PGRP-LB2a induced early pupation and abnormal larval development, while silencing *LgPGRP-LB2b* lead to significantly higher mortality rates at 3d and 6 d, indicating that *LgPGRP-LB2b* may be essential for early larval development (Fig. 5). 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； Gendrin et al., 2017), which protects the beneficial microbes in insects and

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

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).

The SPB is a univoltine insect. The mature larvae make cocoons in the soil, enter diapause during the winter and pupate in mid-July, resulting in f a diapause period of 10 months (Meng et al., 2017b). In our study, *LgPGRP-LB* and *LgPGRP-LB2a* were silenced by RNAi, which broke diapause and caused mature larvae to pupate. This termination of diapause result from an immune response that was initiated to prevent host-inflicted damage. Further research is needed to confirm that LgPGRP-LB influences the host immune responses' activation..

The Toll pathway is critical for innate immunity against bacteria and 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 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*), and the silencing *cactin* is 100% lethal at all developmental stages (from 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, a 10% knocked down of *LgToll-5-1a* lead to 92%larval mortality rate 3 d after feeding. While *LgToll-5-1a* is more critical for the first

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

instar larvae development. A 56% knocked down of *LgToll-5-1b* did not impact on the survival rate of larvae 3 d after feeding. Knocking down *LgToll-5b* took considerably longer (15 d) to have an impact on body weights, and it prevented old cuticles from separating from larval bodies. *LgToll-5-1b* may influence mid-to-late larval development. Thus, *LgToll-5* may be play critical roles on larval development, and it may function in immunity in adults. A future study will challenge *Toll-5-1a* or *LgToll-5-1b* RNAi-treated insects with pathogen infections to determine their roles, if any, in immunity.

Lysozymes are widely distributed immune effectors that exhibit muramidase activities against the PGNs in bacterial cell wall to induce cell lysis (Zhou et al., 2017). In our study, the LgI-type-1 gene encodes a destabilase domain, which is associated with isopeptidase and antibacterial activities. The pI of LgI-type-1 is 7.93 (Table S1). Researchers have proposed that I-type lysozymes with high pI values influence immunity (Kurdyumov et al., 2015; Xue et al., 2004). Thus, LgI-type-1 may have isopeptidase activity and play a role in the SPB's immune system

CONCLUSION

We identified 41 genes associated with SPB microbial recognition proteins, immune-related effectors, or signalling molecules of immune response pathways (e.g., Toll and immune

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

deficiency pathways). This will be useful as a comprehensive genetic resource for immune-related SPB genes and may help elucidate 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 SPB mmune response pathways and for developing RNAi-mediated methods to control SPB infestations.

ACKNOWLEDGEMENTS

We thank Professor Xiaoyun Wang (Northeast Agricultural University, China) for her technical support in culturing the SPB.

ADDITIONAL INFORMATIONS AND DECLARATIONS

Funding

This work was supported by the Foundation for University Key Teacher of the Department of Education of Heilongjiang Province (1253G008).

Competing interests

Authors have declared that no competing interests exist.

REFERENCES

Bao YY, Qu LY, Zhao D, Chen LB, Jin HY, Xu LM, Cheng JA, Zhang CX. 2013. The genome-and transcriptome-wide analysis of innate immunity in the brown planthopper, *Nilaparvata lugens.* BMC genomics 14:160 DOI 10.1186/1471-2164-14-160

1 Bao YY, Xue J, Wu WJ, Wang Y, Lv ZY, Zhang CX. 2011. An immune-induced reeler protein is
2 involved in the *Bombyx mori* melanization cascade. Insect Biochem Mol Biol. 41(9):696-706
3 DOI 10.1016/j.ibmb.2011.05.001
4 Benton MA, Pechmann M, Frey N, Stappert D, Conrads KH, Chen YT, Stamataki E, Pavlopoulos
5 A, Roth S. 2016. Toll Genes Have an Ancestral Role in *Axis Elongation*. Curr Biol.
6 26(12):1609-1615 DOI 10.1016/j.cub.2016.04.055.
7 Bingsohn L, Knorr E, Billion A, Narva KE, Vilcinskas A. 2017. Knockdown of genes in the Toll
8 pathway reveals new lethal RNA interference targets for insect pest control. Insect Mol Biol
9 26(1):92-102 DOI: 10.1111/imb.12273.
10 Boratyn GM, Camacho C, Cooper PS, Coulouris G, Fong A, Ma N, Madden TL, Matten WT,
11 McGinnis SD, Merezhuk Y, Raytselis Y, Sayers EW, Tao T, Ye J, Zaretskaya I. 2013. BLAST: a
12 more efficient report with usability improvements. Nucleic Acids Res.W29-33. doi:
13 10.1093/nar/gkt282.
14 Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl
15 MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The MIQE guidelines: minimum
16 information for publication of quantitative real-time PCR experiments. Clin Chem. (4):611-22.
17 doi: 10.1373/clinchem.2008.112797.
18 Chen EH, Wei DD, Shen GM, Yuan GR, Bai PP, Wang JJ. 2014. De novo characterization of the
19 Dialeurodes citri transcriptome: mining genes involved in stress resistance and simple sequence
20 repeats (SSRs) discovery. Insect Mol Biol. 23(1):52-66 (2014). DOI 10.1111/imb.12060
21 Chen K, Lu Z. 2018. Immune responses to bacterial and fungal infections in the silkworm,
22 *Bombyx mori. Dev Comp Immunol.* 83:3-11. doi: 10.1016/j.dci.2017.12.024.
23 Christiaens O, Swevers L, Smagghe G. 2014. DsRNA degradation in the pea aphid

(*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. Peptides 53:307-14 DOI 10.1016/j.peptides.2013.12.014

Dziarski R, Gupta D. 2006. The peptidoglycan recognition proteins (PGRPs). Genome Biol. 7(8):232 DOI 10.1186/gb-2006-7-8-232

Fishilevich E, Vélez AM, Khajuria C, Frey ML, Hamm RL, Wang H, Schulenberg GA, Bowling AJ, Pence HE, Gandra P, Arora K, Storer NP, Narva KE, Siegfried BD. 2016. Use of chromatin remodeling ATPases as RNAi targets for parental control of western corn rootworm *(Diabrotica virgifera virgifera*) and Neotropical brown stink bug (*Euschistus heros*). Insect Biochem Mol Biol 71:58-71 DOI 10.1016/j.ibmb.2016.02.004

Fire A., Xu S., Montgomery M. K., Kostas S. A., Driver S. E., Mello C. C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391(6669):806-811 DOI 10.1038/35888.

Gao Y, Tang T, Gu J, Sun L, Gao X, Ma X, Wang X, Liu F, Wang J. 2015. Down regulation of the *Musca domestica* peptidoglycan recognition protein SC (PGRP-SC) leads to overexpression of antimicrobial peptides and tardy pupation. Mol Immunol 67(2 Pt B):465-74 DOI 10.1016/j.molimm.2015.08.007.

Gendrin M, Turlure F, Rodgers FH, Cohuet A, Morlais I, Christophides GK. 2017. The Peptidoglycan Recognition Proteins PGRPLA and PGRPLB Regulate Anopheles Immunity to Bacteria and Affect Infection by Plasmodium. J Innate Immun 9(4):333-342 DOI 10.1159/000452797.

Gegner T, Schmidtberg H, Vogel H, Vilcinskas A. 2018. Population-specific expression of antimicrobial peptides conferring pathogen resistance in the invasive ladybird *Harmonia axyridis.* Sci Rep. 8(1):3600. doi: 10.1038/s41598-018-21781-4.

Guan R, Mariuzza RA. 2007. Peptidoglycan recognition proteins of the innate immune system. Trends Microbiol 15(3):127-34 DOI 10.1016/j.tim.2007.01.006

Hashimoto K, Mega K, Matsumoto Y, Bao Y, Yamano Y, Morishima I. 2007. Three peptidoglycan recognition protein (PGRP) genes encoding potential amidase from eri-silkworm, *Samia cynthia ricini.* Comp Biochem Physiol B Biochem Mol Biol 148(3):322-8 DOI 10.1016/j.cbpb.2007.06.011

Hillyer JF. 2015. Insect immunology and hematopoiesis. Dev Comp Immunol 58:102-18 DOI 10.1016/j.dci.2015

Hughes AL. 2012. Evolution of the βGRP/GNBP/β-1,3-glucanase family of insects. Immunogenetics. 64(7):549-58 DOI 10.1007/s00251-012-0610-8

Huvenne H, Smagghe G. 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. J Insect Physiol 56(3):227-35 DOI 10.1016/j.jinsphys.2009

Imler JL, Bulet P. 2005. Antimicrobial peptides in Drosophila: structures, activities and gene regulation. Chem Immunol Allergy.86:1-21 DOI 10.1159/000086648

Joga MR, Zotti MJ, Smagghe G, Christiaens O. 2016. RNAi Efficiency, Systemic Properties, and Novel Delivery Methods for Pest Insect Control: What We Know So Far. Front Physiol 7:553 DOI10.3389/fphys.2016.00553

Khajuria C, Vélez AM, Rangasamy M, Wang H, Fishilevich E, Frey ML, Carneiro NP, Gandra P, Narva KE, Siegfried BD. 2015. Parental RNA interference of genes involved in embryonic development of the western corn rootworm, Diabrotica virgifera virgifera LeConte. Insect Biochem Mol Biol 63:54-62 DOI 10.1016/j.ibmb.2015

Kurdyumov AS, Manuvera VA, Baskova IP, Lazarev VN. 2015. A comparison of the enzymatic

properties of three recombinant isoforms of thrombolytic and antibacterial protein--Destabilase-Lysozyme from medicinal leech. BMC Biochem 16:27 DOI: 10.1186/s12858-015-0056-3.Li J, Ma L, Lin Z, Zou Z, Lu Z. 2016. Serpin-5 regulates prophenoloxidase activation and antimicrobial peptide pathways in the silkworm, *Bombyx mori*. Insect Biochem Mol Biol. 73:27-37 DOI 10.1016/j.ibmb.2016.04.003

Mao J, Zeng F. 2014. Plant-mediated RNAi of a gap gene-enhanced tobacco tolerance against the Myzus persicae. Transgenic Res 23(1):145-52 DOI 10.1007/s11248-013-9739-y

Meng FL, Ran RX, Li Y, Li N, Li HZ, Wang ZK, Li WB. 2017a. RNAi-mediated knockdown of a serine protease gene (Spbtry1) from SPB (soybean pod borer) affects the growth and mortality of the pest. Florida Entomologist 100(3):607-615 DOI /10.1653/024.100.0319.

Meng FL,Yang MY, Li Y, Li TY ,Liu XX, Wang GY, Wang ZC, Jin XH, Li WB. 2017c. Functional analysis of RNA interference-related soybean pod borer (Lepidoptera) genes based on transcriptome sequences. Front. Physiol. DOI 10.3389/fphys.2018.00383Meng F, Li Y, Zang Z, Li N, Xue R, Cao Y, Li T, Zhou Q, Li W. 2017b. Expression of the Double-stranded RNA of the soybean pod borer *Leguminivora glycinivorella* (Lepidoptera: Tortricidae) Ribosomal Protein *P0* Gene Enhances the Resistance of Transgenic Soybean Plants. Pest Manag Sci. (12):2447-2455 DOI 10.1002/ps.4637

Monwan W, Amparyup P, Tassanakajon A. 2017. A snake-like serine proteinase (PmSnake) activates prophenoloxidase-activating system in black tiger shrimp *Penaeus monodon*. Dev Comp Immunol. 67:229-238 DOI 10.1016/j.dci.2016.09.016.

Myllymäki H, Valanne S, Rämet M. 2014. The Drosophila imd signaling pathway. J Immunol. 192(8):3455-62 DOI 10.4049/jimmunol.1303309

Parsons B, Foley E. 2016. Cellular immune defenses of Drosophila melanogaster. Dev Comp

Immunol 58:95-101 DOI 10.1016/j.dci.2015.12.019

Rao XJ, Zhan MY, Pan YM, Liu S, Yang PJ, Yang LL, Yu XQ. 2017. Immune functions of insect βGRPs and their potential application. Dev Comp Immunol. pii: S0145-305X(17)30524-4 DOI 10.1016/j.dci.2017.12.007

Roh KB, Kim CH, Lee H, Kwon HM, Park JW, Ryu JH, Kurokawa K, Ha NC, Lee WJ, Lemaitre B, Söderhäll K, Lee BL. 2009. Proteolytic cascade for the activation of the insect toll pathway induced by the fungal cell wall component. J Biol Chem. 284(29):19474-81 DOI 10.1074/jbc.M109.007419

Shukla JN, Kalsi M, Sethi A, Narva KE, Fishilevich E, Singh S, Mogilicherla K, Palli SR. 2016. Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. RNA Biol 13(7):656-69 DOI 10.1080/15476286.2016.1191728

Song LW, Wen XY, Zang LS, Ruan CC, Shi SS, Shao XW, Zhang F. 2015. Parasitism and Suitability of Different Egg Ages of the *Leguminivora glycinivorella* (Lepidoptera: Tortricidae) for Three Indigenous Trichogramma Species. J Econ Entomol 108(3):933-9 DOI 10.1093/jee/tov027

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol. 7:539. doi: 10.1038/msb.2011.75.

Takeda K, Akira S. 2004. Microbial recognition by Toll-like receptors. J Dermatol Sci. 34(2):73-82 DOI 10.1016/j.jdermsci.2003.10.002

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony

methods. Mol Biol Evol 28(10):2731-9 DOI 10.1093/molbev/msr121

Terenius O, Papanicolaou A, Garbutt JS, Eleftherianos I, Huvenne H, Kanginakudru S, Albrechtsen M, An C, Aymeric JL, Barthel A, Bebas P, Bitra K, Bravo A, Chevalier F, Collinge DP, Crava CM, de Maagd RA, Duvic B, Erlandson M, Faye I, Felföldi G, Fujiwara H, Futahashi R, Gandhe AS, Gatehouse HS, Gatehouse LN, Giebultowicz JM, Gómez I, Grimmelikhuijzen CJ, Groot AT, Hauser F, Heckel DG, Hegedus DD, Hrycaj S, Huang L, Hull JJ, Iatrou K, Iga M, Kanost MR, Kotwica J, Li C, Li J, Liu J, Lundmark M, Matsumoto S, Meyering-Vos M, Millichap PJ, Monteiro A, Mrinal N, Niimi T, Nowara D, Ohnishi A, Oostra V, Ozaki K, Papakonstantinou M, Popadic A, Rajam MV, Saenko S, Simpson RM, Soberón M, Strand MR, Tomita S, Toprak U, Wang P, Wee CW, Whyard S, Zhang W, Nagaraju J, Ffrench-Constant RH, Herrero S, Gordon K, Swevers L, Smagghe G. 2011. RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. J Insect Physiol 57(2):231-45 DOI 10.1016/j.jinsphys.2010.11.006.

Troll JV, Adin DM, Wier AM, Paquette N, Silverman N, Goldman WE, Stadermann FJ, Stabb EV, McFall-Ngai MJ. 2009. Peptidoglycan induces loss of a nuclear peptidoglycan recognition protein during host tissue development in a beneficial animal-bacterial symbiosis. Cell Microbiol 11(7):1114-27 DOI 10.1111/j.1462-5822.2009.01315.x

Ulrich J, Dao VA, Majumdar U, Schmitt-Engel C, Schwirz J, Schultheis D, Ströhlein N, Troelenberg N, Grossmann D, Richter T, Dönitz J, Gerischer L, Leboulle G, Vilcinskas A, Stanke M, Bucher G. 2015. Large scale RNAi screen in Tribolium reveals novel target genes for pest control and the proteasome as prime target. BMC Genomics 16:674 DOI 10.1186/s12864-015-1880-y.

Valanne S, Wang JH, Rämet M. 2011. The Drosophila Toll signaling pathway. J Immunol

186(2):649-56 DOI: 10.4049/jimmunol.1303309

Wang L, Yang S, Han L, Fan D, Zhao K. 2014. Phenotypic plasticity of HSP70s gene expression during diapause: signs of evolutionary responses to cold stress among Soybean Pod Borer populations (*Leguminivora glycinivorella*) in Northeast of China. PLoS One 9(10):e109465(2014) DOI 10.1371/journal.pone.0109465

Wang J, Aksoy S. 2012. PGRP-LB is a maternally transmitted immune milk protein that influences symbiosis and parasitism in tsetse's offspring. Proc Natl Acad Sci U S A. 109(26):10552-7 DOI10.1073/pnas.1116431109

Wu C, Chen C, Dai J, Zhang F, Chen Y, Li W, Pastor-Pareja JC, Xue L. 2015. Toll pathway modulates TNF-induced JNK-dependent cell death in Drosophila. Open Biol 5(7):140171 DOI: 10.1098/rsob.140171

Xu Q, Lu A, Xiao G, Yang B, Zhang J, Li X, Guan J, Shao Q, Beerntsen BT, Zhang P, Wang C, Ling E. 2012. Transcriptional profiling of midgut immunity response and degeneration in the wandering silkworm, *Bombyx mori*. PLoS One 7(8):e43769 DOI 10.1371/journal.pone.0043769

Xue, Q.G., Schey, K.L., Volety, A.K., Chu, F.L., La Peyre, J.F. 2004. Purification and characterization of lysozyme from plasma of the eastern oyster (*Crassostrea virginica*). Comp Biochem Physiol B Biochem Mol Biol 139(1):11-25 DOI: 10.1016/j.cbpc.2004.05.011

Yang PJ, Zhan MY, Ye C, Yu XQ, Rao XJ. 2017. Molecular cloning and characterization of a short peptidoglycan recognition protein from silkworm Bombyx mori. Insect Mol Biol. 26(6):665-676 DOI 10.1111/imb.12330

Zaidman-Rémy A1, Hervé M, Poidevin M, Pili-Floury S, Kim MS, Blanot D, Oh BH, Ueda R, Mengin-Lecreulx D, Lemaitre B. 2006. The Drosophila amidase PGRP-LB modulates the

immune response to bacterial infection. Immunity 24(4):463-73 DOI 10.1016/j.immuni.2006.02.012

Yang J, Wang X, Tang S, Shen Z, Wu J. 2015. Peptidoglycan recognition protein S2 from silkworm integument: characterization, microbe-induced expression, and involvement in the immune-deficiency pathway. J Insect Sci 15. pii: 20 DOI 10.1093/jisesa/iev007

Zhao GY, Wang J, Han YP, Teng WL, Sun GL, Li WB. 2008. Identification of QTL underlying the resistance of soybean to pod borer, *Leguminivora glycinivorella* (Mats.) obraztsov, and correlations with plant, pod and seed traits. Euphytica 164: 275-282 DOI 1016/S2095-3119

Zhou J, Zhao S, Fang WH, Zhou JF, Zhang JX, Ma H, Lan JF, Li XC. 2017. Newly identified invertebrate-type lysozyme (Splys-i) in mud crab (*Scylla paramamosain*) exhibiting muramidase-deficient antimicrobial activity. Dev Comp Immunol 74:154-166 DOI: 10.1016/j.dci.2017.04.017

Figure legends

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 percentages (1,000 replicates) are provided next to the branches. The first two letters of each PGRP name indicate the species (Dm, *D. melanogaster*; Lg, *L. glycinivorella*).

Figure 2 The analysis of Toll receptors of the *L. glycinivorella. A*) Phylogenetic relationships among Toll receptors from *Leguminivora glycinivorella*, *Bombyx mori* and *Drosophila melanogaster*. The phylogenetic tree was constructed using MEGA5.0 with a neighbour-joining approach. *Lg*, *L. glycinivorella*; *Bm*, *Bombyx mori*; *Dm*, *D. melanogaster*; TLR, Toll like receptor. (B) Predicted domains of the *L.glycinivorella* Toll receptors. The domain organizations were predicted using the SMART program (http://smart.embl.de/). The extracellular leucine-rich repeats (LRRs), rectangles; LRR C-terminal domain, small ellipses; intracytoplasmic TIR domains, big ellipses; signal peptides, red rectangles; transmembrane domain, blue bar.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

Figure 3 Mortality of larvae fed an artificial diet supplemented with dsRNA (10 μg/g) for 11 candidate RNA interference target genes. Columns represent mean±SE. Different letters above the column indicate significant difference ($p < 0.0009$).

Figure 4 Body weight of larvae fed an artificial diet supplemented with dsRNA (10 μg/g) for 11 candidate RNA interference target genes. Columns represent mean±SE. Different letters above the column indicate significant difference ($p < 0.0009$).

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. (A) The larvae fed an artificial diet containing dsRNA for *LgPGRP-LB*. (B) The larvae fed an artificial diet containing dsRNA for *LgPGRP-LB2a*. (C) The larvae fed an artificial diet containing dsRNA for *LgPGRP-LB2b*. (D) The larvae fed an artificial diet containing dsRNA for *LgToll-5-1a*, only one survived after 15 d. (E) The larvae fed an artificial diet containing dsRNA for Lg*Toll-5-1b*. (F) The larvae fed an artificial diet containing dsRNA for *LgTLR-6-1b* .(G) The larvae fed an artificial diet containing dsRNA for *LgTLR-7*.(H) The larvae fed an artificial diet containing dsRNA for *LgTLR-3*. (I) The larvae fed an artificial diet containing dsRNA for *LgChaoptin*. (J) The larvae fed an artificial diet

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

containing dsRNA for *LgSerpin-2*. (K) The larvae fed an artificial diet containing dsRNA for *LgItype-1*. (L) The larvae fed an artificial diet containing dsRNA for *GFP*.

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. Columns represent mean±SE. Different letters above the column indicate significant difference ($p < 0.002$)

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

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 percentages (1,000 replicates) are provided next to the branches. The first two letters of each PGRP name indicate the species (Dm, *D. melanogaster*; Lg, *L. glycinivorella*).

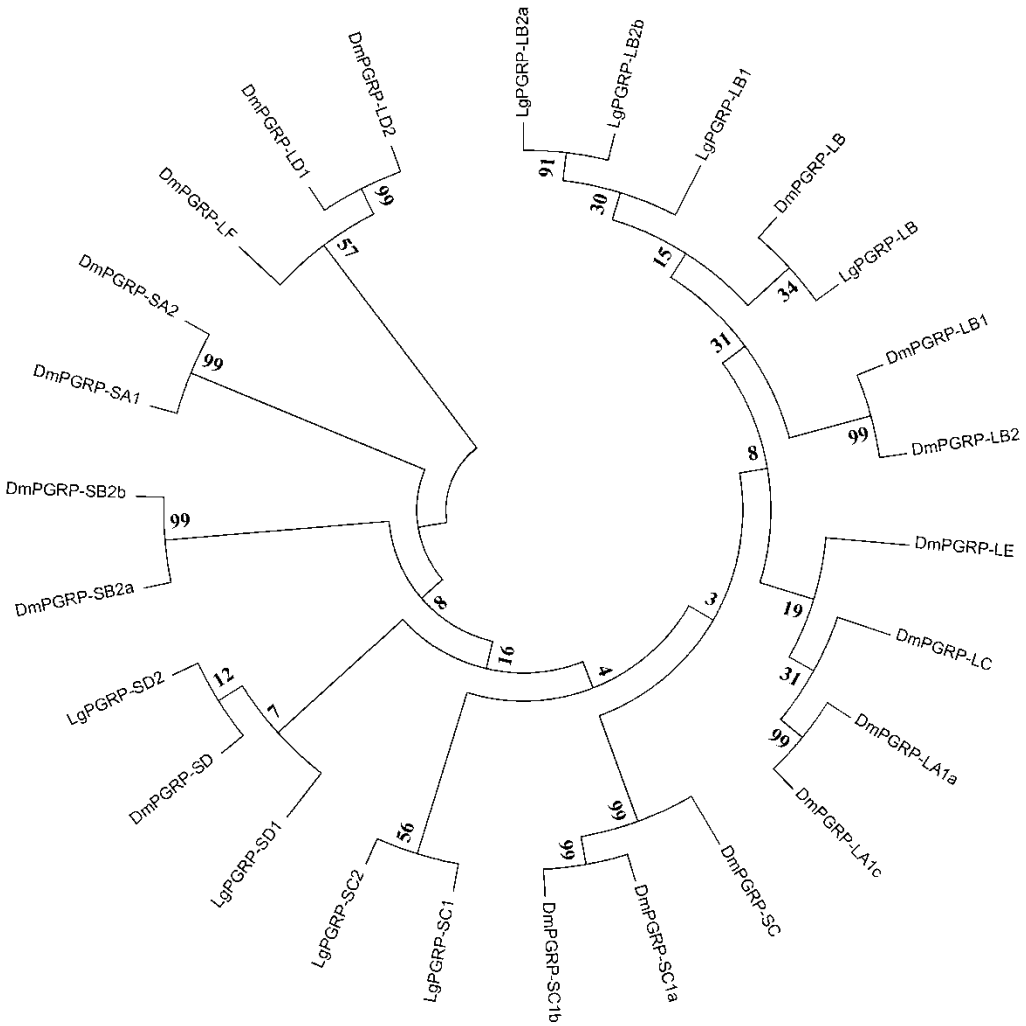


Figure 2 The analysis of Toll receptors of the *L. glycinivorella*. *A*) Phylogenetic relationships among Toll receptors from *Leguminivora glycinivorella*, *Bombyx mori* and *Drosophila melanogaster*. The phylogenetic tree was constructed using MEGA5.0 with a neighbour-joining approach. *Lg*, *L. glycinivorella*; *Bm*, *Bombyx mori*; *Dm*, *D. melanogaster*; TLR, Toll like receptor. (B) Predicted domains of the *L.glycinivorella* Toll receptors. The domain organizations were predicted using the SMART program (http://smart.embl.de/). The extracellular leucine-rich repeats (LRRs), rectangles; LRR C-terminal domain, small ellipses; intracytoplasmic TIR domains, big ellipses; signal peptides, red rectangles; transmembrane domain, blue bar.

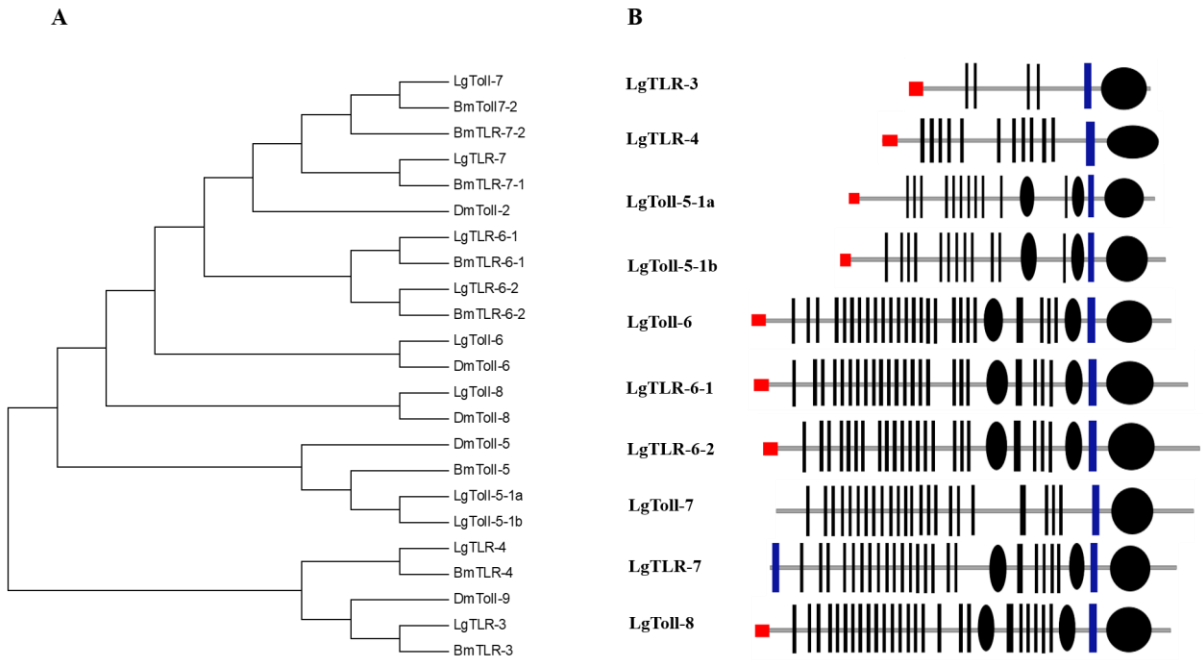


Figure 3 Mortality of larvae fed an artificial diet supplemented with dsRNA (10 μg/g) for 11 candidate RNA interference target genes. Columns represent mean±SE. Different letters above the column indicate significant difference ($p < 0.0009$).

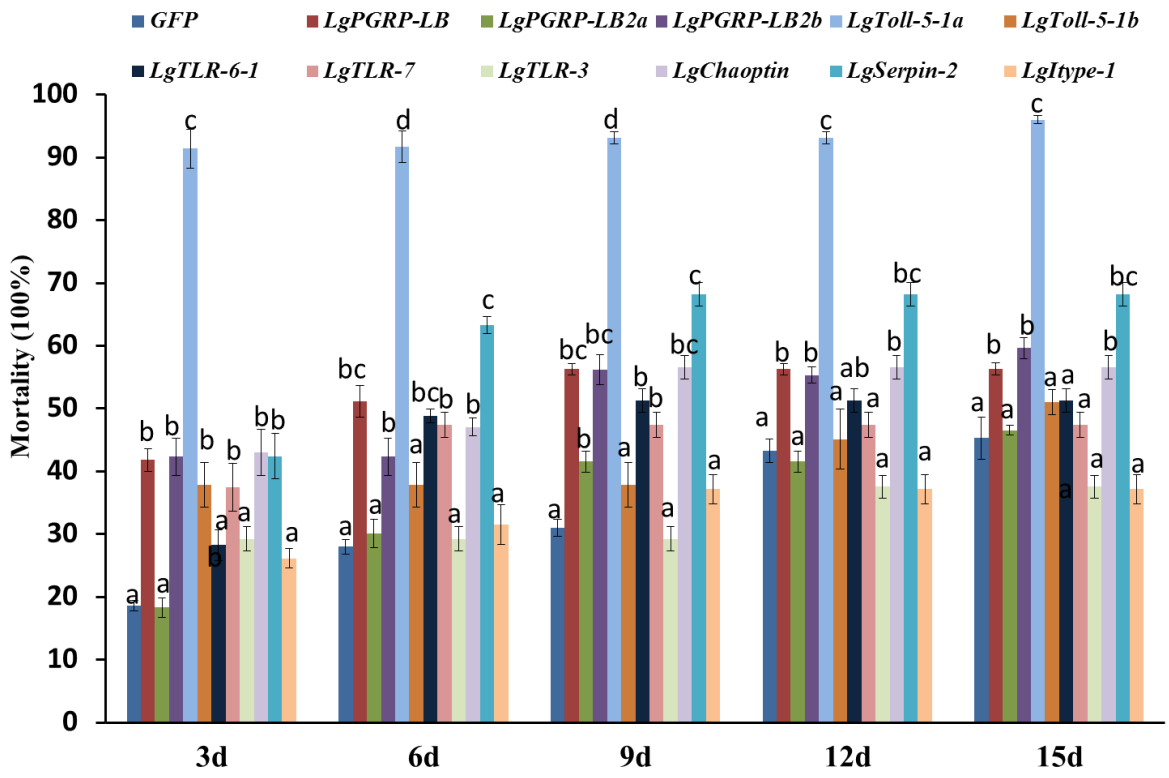


Figure 4 Body weight of larvae fed an artificial diet supplemented with dsRNA (10 μg/g) for 11 candidate RNA interference target genes. Columns represent mean±SE. Different letters above the column indicate significant difference ($p < 0.0009$).

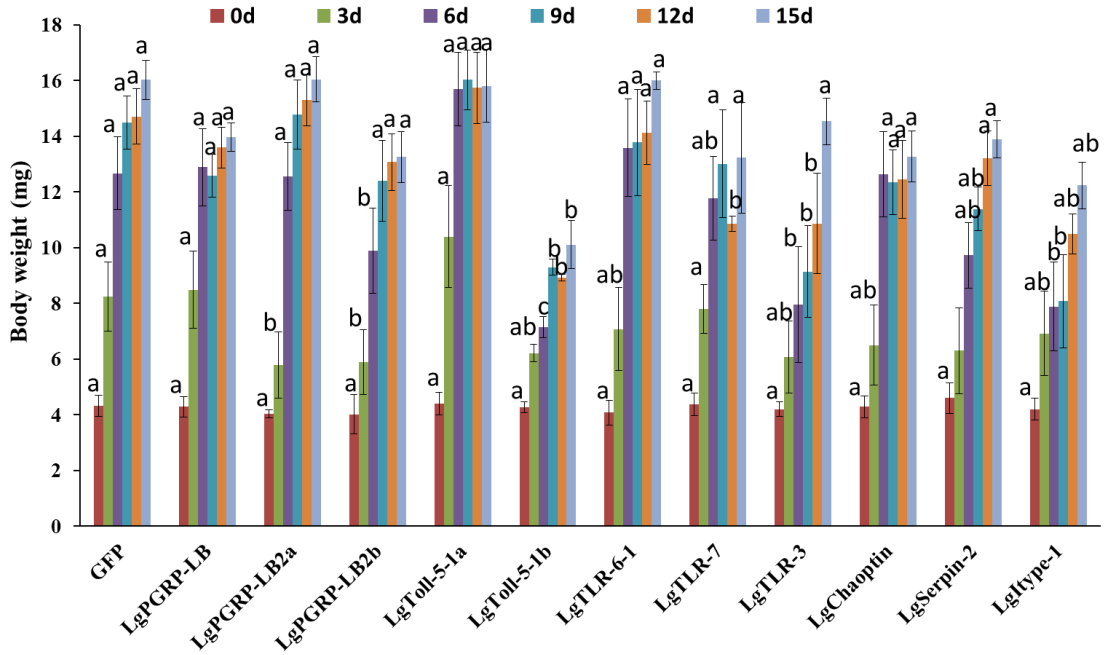


Figure 5 Images of larvae fed an artificial diet supplemented with dsRNA (10 μg/g) for 11 candidate RNA interference target genes for 15 days. (A) The larvae fed an artificial diet containing dsRNA for *LgPGRP-LB*. (B) The larvae fed an artificial diet containing dsRNA for *LgPGRP-LB2a*. (C) The larvae fed an artificial diet containing dsRNA for *LgPGRP-LB2b*. (D) The larvae fed an artificial diet containing dsRNA for *LgToll-5-1a*, only one survived after 15 d. (E) The larvae fed an artificial diet containing dsRNA for Lg*Toll-5-1b*. (F) The larvae fed an artificial diet containing dsRNA for *LgTLR-6-1b* .(G) The larvae fed an artificial diet containing dsRNA for *LgTLR-7*.(H) The larvae fed an artificial diet containing dsRNA for *LgTLR-3*. (I) The larvae fed an artificial diet containing dsRNA for *LgChaoptin*. (J) The larvae fed an artificial diet containing dsRNA for *LgSerpin-2*. (K) The larvae fed an artificial diet containing dsRNA for *LgItype-1*. (L) The larvae fed an artificial diet containing dsRNA for *GFP*.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018

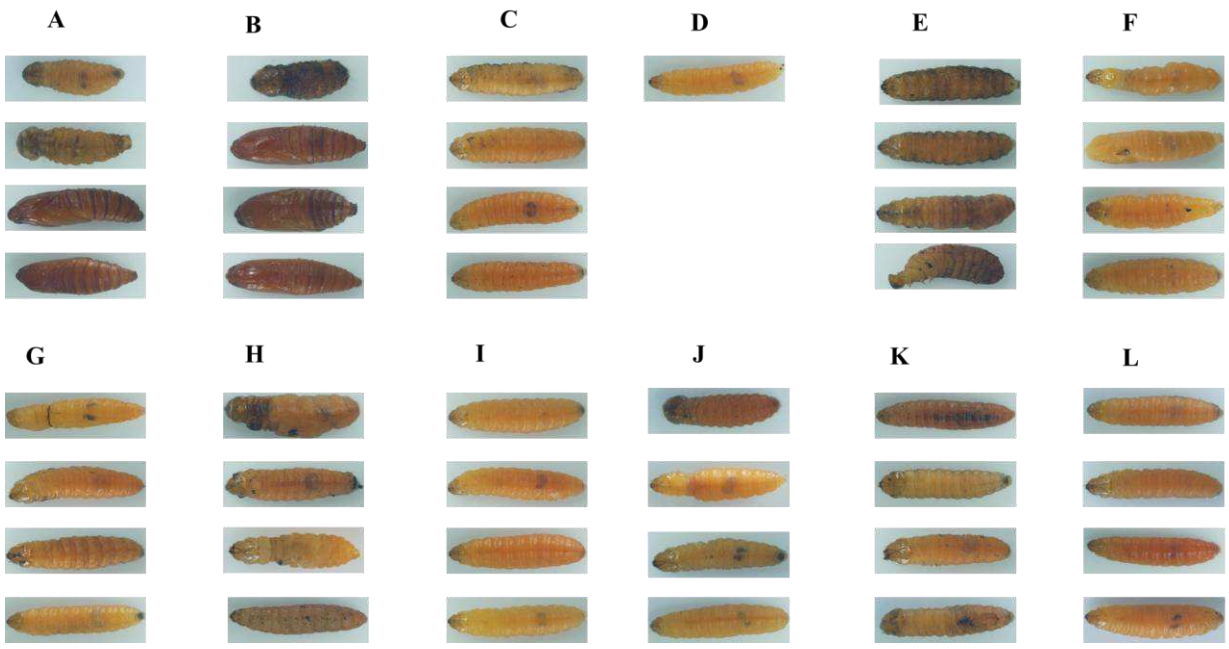
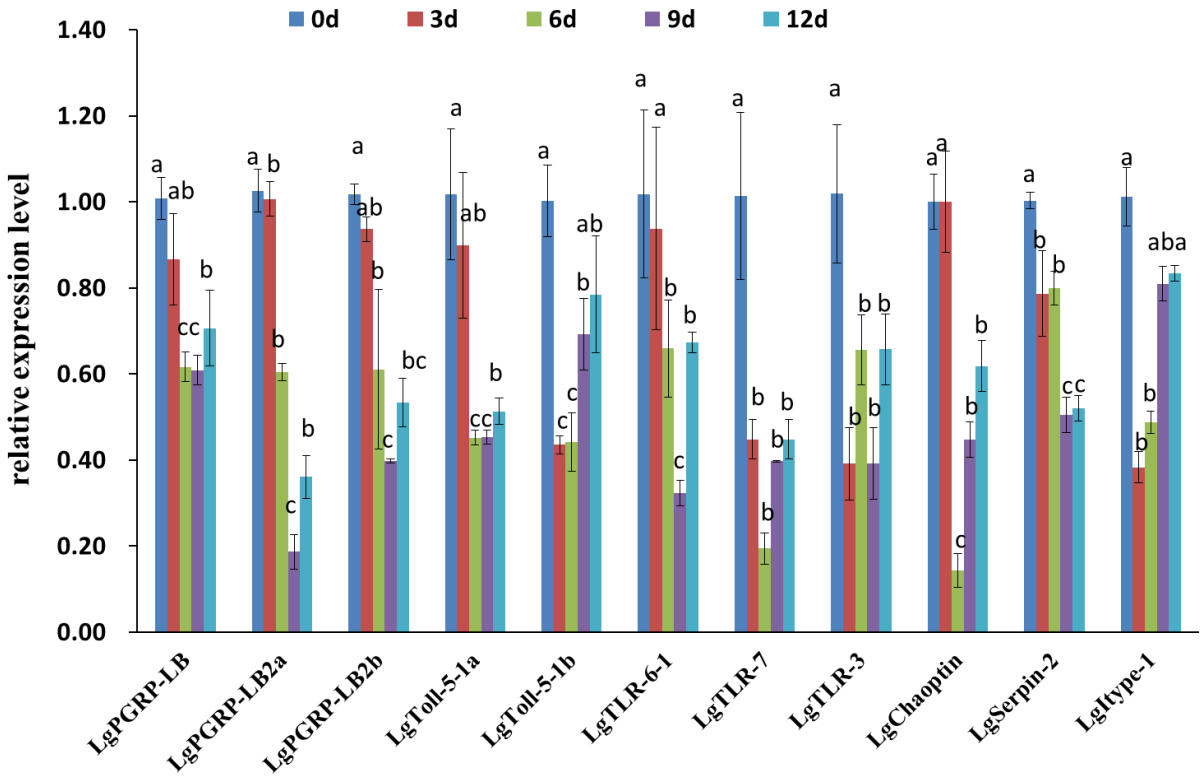


Figure 6 Relative expression levels of 11 candidate RNA interference target genes at different time points after larvae were fed an artificial diet containing dsRNA (10 μg/g). Quantitative real-time polymerase chain reactions were completed using total RNA extracted from surviving larvae. Columns represent mean±SE. Different letters above the column indicate significant difference ($p < 0.002$)



PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26923v2 | CC BY 4.0 Open Access | rec: 11 May 2018, publ: 11 May 2018