Ras-like family small GTPases genes in *Nilaparvata lugens*: Identification, phylogenetic analysis, gene expression and function in nymphal development

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Abstract

Twenty-nine cDNAs encoding Ras-like family small GTPases (RSGs) were cloned and sequenced from *Nilaparvata lugens*. Twenty-eight proteins are described here: 3 from Rho, 2 from Ras, 9 from Arf and 14 from Rabs. These RSGs from *N. lugens* have five conserved G-loop motifs and displayed a higher degree of sequence conservation with orthologues from insects. RT-qPCR analysis revealed *NlRSGs* expressed at all life stages and the highest expression was observed in hemolymph, gut or wing for most of *NlRSGs*. RNAi demonstrated that eighteen *NlRSGs* play a crucial role in nymphal development. Nymphs with silenced *NlRSGs* failed to molt, eclosion or development arrest. The qRT-PCR analysis verified the correlation between mortality and the down-regulation of the target genes. The expression level of nuclear receptors, Kr-h1, Hr3, FTZ-F1 and E93 involved in 20E and JH signal pathway was impacted in nymphs with silenced twelve *NlRSGs* individually. The expression of two halloween genes, *Cyp314a1* and *Cyp315a1* involved in ecdysone synthesis, decreased in nymphs with silenced *NISar1* or *NIArf1*. *Cyp307a1* increased in nymphs with silenced *NIArf6*. In *N. lugens* with silenced *NISR6*, *NISar1* and *NIRab2* at 9th day individually, 0.0% eclosion rate and almost 100.0% mortality was demonstrated. Further analysis showed *NISR6* could be served as a candidate target for dsRNA-based pesticides for *N. lugens* control.

Introduction

The rice brown planthopper (*Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae)) is one of the most destructive monophagous insect pest of rice, *Oryza sativa*, it draw nutrients from the phloem of rice plants and cause huge yield losses every year in rice grown throughout tropical, subtropical and temperate areas in Asia[1,2]. RNAi (targeted silencing of gene expression by injection of corresponding dsRNA) has become a major tool in functional analysis of genes in insects [3, 4]. A decade of research on RNAi in insects has demonstrated that dsRNA could be
developed as tailor-made pesticides for its sequence specificity and ability to suppress genes critical for insect survival [5]. *N. lugens* has a robust RNAi response to dsRNAs [6]. This offers great opportunities to analyze gene function by RNAi and search target for dsRNA-based pesticides in *N. lugens*.

Ras-like family small GTPases (RSGs) are components of signaling pathways that link extracellular signals via trans-membrane receptors to cytoplasm and touch on virtually every aspect of cell biology, including growth, differentiation, morphogenesis, cell division and motility, cytokinesis, and trafficking through the golgi apparatus, nucleus, and endosomes [7–9]. RSGs share a conserved structure and biochemical properties, acting as binary molecular switches turned on by binding GTP and off by hydrolyzing GTP to GDP. They are grouped into five major subfamilies Ras, Rab, Ran and Arf based on their sequence and functional similarities [10, 11]. Members from different subfamilies share a maximum of 40% identity. The functional diversity of these proteins is based on minor modifications in sequence, structure, and/or regulatory posttranslational modifications [12]. Moreover, members of the same family differ from each other in their “variable” membrane targeting domains, which dictate subcellular localization and dynamic spatiotemporal regulation. Conversely, distinct members of the RSGs transact and interconnect to each other through complex signaling networks [13]. Ras genes were first identified and characterized as transduced oncogenes in human tumor cells, Their dysfunction plays a crucial role in the pathogenesis of serious human diseases, including cancer and developmental syndromes[14, 15]. Since then, RSGs genes are described or predicted in diverse insect species, mostly based on sequence similarity, and demonstrated to participate in sorting and amplification of transmembrane signals, translocation of proteins, vesicular traffic, cell polarity, autophagy, cellular immune response etc [16–19]. In *Aedes aegypti*, Rheb GTPase was required for amino acid mediated activation of the TOR pathway that controls egg development. When Rheb was down-regulated, the egg development was severely hindered [20]. In Drosophila, Rheb is required for both cell growth (increase in mass) and cell cycle progression and the effects of Rheb are mediated by TOR [21, 22]. Arf1 is required for normal wing development and formation of secretory granules [23]. Rac2 is required for normal feeding and mating behaviour [24]. Rho GTPase signaling in *Apis mellifera* mushroom body mediated foraging-dependent growth [25]. Rab4b takes part in metamorphosis by regulating gene transcription and glycogen level in the insulin and 20E signal pathways in *Helicoverpa armigera* [26]. Knock down of Ran by RNAi resulted in the suppression of 20E regulated genes including EcR-B1, USP1, E75B, BR-CZ2, and HR3 [27]. These results suggest that RSGs involved in fundamental developmental process and showed functional diversity in insects. However, almost all the researches of RSGs focused on homometabolous insect development, their functions in hemimetabolous are still unknown.

In this study, twenty-nine RSGs genes were cloned and characterized in hemimetabolous insect *N. lugens*. The patterns of gene expression in different tissues and at different developmental stages were examined. Furthermore, the importance of each RSGs gene in *N. lugens* development was studied by RNAi. To our knowledge, this is the first report to describe and functionally analyze RSGs in *N. lugens*. Our results could provide basic information for understanding RSGs function in *N. lugens*.

**Materials and methods**

**Insects rearing and samplings**

*N. lugens* used in this study were originally collected from rice field in China National Rice Research Institute, Hangzhou, China, in 2008. The insects were reared on rice variety Tai-chung Native1 (TN1, a *N. lugens* -susceptible rice cultivar) in nylon mesh cages under
conditions (28°C, 85% relative humidity and 16 h light/8 h darkness). For temporal expression analysis, the first- through fifth-instar nymphs and female, male adults were sampled respectively. Each sample contained 10 individuals and was repeated in biological triplicate. The tissues including salivary gland, gut, fat body, ovary, leg, wing, integument and hemolymph were dissected from 100 adult females, 2 days after eclosion, under a Leica S8AP0 stereomicroscope. Each sample was repeated in biological triplicate. The samples were frozen in liquid nitrogen and stored at -80°C until use. The third instar (one-day old) nymphs were used for dsRNA treatment and subsequent analysis.

RNA extraction and qRT-PCR analysis

Total RNAs were isolated from whole bodies of the first- through fifth-instar nymphs, adults and from tissues or the nymph survivors of the dsRNA bioassay with RNeasy mini kit (Qiagen, Germany) according to manufacturer’s instruction. The potential genomic DNA contamination was removed by a treatment with DNase I kit (Qiagen Germany) after the RNA extraction. RNA concentration and quality was determined using a Nanodrop spectrophotometer (Thermo Scientific, USA). The first-strand cDNA were synthesized by reverse transcriptase using ReverTra AceqPCR RT Kit (ToYoBo, Osaka, Japan). The 10-fold diluted first-strand cDNA (2.0 μL) was used as template for qRT-PCR.

mRNA abundance of each gene was tested in three technical replicates for each of three biological replicates. qRT-PCR were performed using a qPCR master mix SYBR® Premix (Toyobo, Osaka, Japan) on ABI 7500 System (Applied Biosystems). The accompanying software was used for qPCR data normalization and quantification. Relative value for the expression level of target gene in development stages and tissues was calculated by the equation

\[ Y = 10^{(C_{\text{internal}} - C_{\text{target}})/3} \times 100\% \]

[28], using the geometric mean of 18S rRNA (JN662398) and β-actin (EU179846) as internal [29]. Duncan’s Multiple Comparison was used to determine differences among tissues and stages. Values of p < 0.01 were considered significant.

Identification, cloning and sequence analysis of RSGs genes

To identify the genes related to RSGs, we conducted sequence search in EST data (http://bphest.dna.affrc.go.jp/) based on molecular function and transcriptome database of N. lugens based on the functional annotation. Twenty-nine cDNA sequences in N. lugens similar to RSGs were obtained and assembled. The assembled sequences were used as queries to find homologs in the N. lugens genome (GenBank assembly GCA_000757685.1) by BLAST search. The correctness of sequences with complete open reading frames (ORFs) was substantiated by common PCR techniques using the gene-specific primers. The gene-specific primers were designed using the Primer Premier 5.0 program based on the assembled sequences as shown in S1 Table. All the primers were synthesized by Invitrogen. Each PCR products specific to the target genes was individually cloned into the pCRII-TOPO vector (Invitrogen). The plasmids were extracted from several independent subclones with high pure plasmid isolation kit (Roche, Germany) and sequenced at Shanghai Boshang Ltd. Translations of cDNAs and predictions of the deduced proteins were conducted using DNASTar software (DNASTAR Inc., Madison, USA). Sequences were aligned using ClustaW and then a phylogenetic tree was generated using the neighbor joining method with MEGA 5.10 software (http://www.megasoftware.net/). Robustness of the branches was assessed by performing a bootstrap analysis of 1000 replications. Prediction of transmembrane helices and motif discovery in proteins were conducted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and SMART (http://smart.embl-heidelberg.de/). Exon/intron organization of each Ras family
gene was checked by aligning ORF with genomic DNA using the spidey program [http://www.ncbi.nlm.nih.gov/spidey/spideyweb.cgi] and GSDS online [http://gsds.pku.edu.cn].

RNA interference and bioassay
dsRNAs of target genes and GFP were synthesized in vitro with PCR-generated DNA templates using the MEGAscript T7 Transcription Kit (Ambion, Austin, TX, USA). The sequence including full ORF regions were chosen for dsRNA synthesis for the twenty-eight genes. The specific primers used to generate these DNA templates are shown in S1 Table and a 23 base T7 promoter sequence (TAATACGACTCACTATAGGG) was added to the 5' end of each gene specific dsRNA synthesis primer. dsRNAs for each of twenty-eight genes were adjusted into the concentration of 700ng/μL and injected into the third instar (1-day old) nymph with 0.1 μL according to previously described techniques [29, 30]. In parallel, N. lugens injected with GFP dsRNA or double distilled water were used as the negative control. After a 12-hour recovery period, the survived nymphs (at least 30 individuals) were selected and reared on 30- to 35-day-old plants of rice variety TN1 in one cage. Four days after injection, RNA was isolated from five survivors to test the efficiency of RNAi knockdown using qRT-PCR as described above. The mRNA levels of target gene, eight nuclear receptor genes (NlFTZ-F1, NlKr-h1, NIECR, NIMet, NlBr-C, NIE75, NlHr3 and NIE93) and five Halloween genes (NlCyp302a1, NlCyp306a1, NlCyp307a1, NlCyp315a1 and NlCyp314a1) were normalized to the endogenous reference gene 18S rRNA and actin. The relative amounts of gene transcripts were expressed as a ratio between treated group and control group by 2^{-ΔΔCt} method [31]. Three replicate injections were established in each of the dsRNA treatments and control. Duncan’s tests were used to determine difference between treatment and control. Values of p < 0.05 were considered significant.

Mortality and development defects of dsRNA injected insects were recorded at 48 h time intervals until adult eclosion. Three independent biological replicates were carried out. To test for an effect of treatment, ANOVAs were performed using the percentage of survival nymphs or the cumulative percentage of eclosion as the dependent variable and treatment dsGFP injection, Duncan’s tests were used to determine differences among groups when treatment effects were detected.

Results
Identification of RSGs genes in N. lugens and sequence analysis
Based on the transcriptome and EST data, twenty-nine cDNA sequences encoding putative RSGs were assembled and amplified with PCR. BLAST analysis revealed that the putative amino acid sequence shows high homology to corresponding RSGs in other insects. We named these genes according to the names of their orthologues in Zootermopsis nevadensis or Acyrthosiphon pisum. The putative start codon was preceded by an in-frame stop codon in all this twenty-nine sequences. Among the twenty-nine genes, one Ran gene has been described previously [32]. The cDNA of NlRSGs contains an entire ORF range from 540 to 845bp in length. The predicted molecular weights of these proteins range from 20.2 to 30.3 kDa, with PI from 4.78 to 9.51. Sequences cloned in the present study were submitted to Genebank and their accession numbers, length of ORF, number of exon, gene loci, the PI and molecular weight (MW) of deduced amino were listed in Table 1. Based on sequence homology and domain organization, NlRSGs were divided into five subfamilies: Rab subfamily (including 14 genes), Rho subfamily (including 1 Rho, 1 Rac and 1Cdc42), Ras subfamily (including 1 NlRheb and 1 K-Ras), Ran subfamily and Arf subfamily (including 1 Sar1, 1 SRβ, 3 Arf and 4Arl).
Analysis of the genomic position and structure showed that the twenty-six NlRSGs ORF sequences were aligned to N.lugens genome, with NlRab1 and NlRab6 remained on as yet unmapped scaffolds. Interestingly the deduced amino acid of the two genes has the lowest PI with 4.78 and 4.95 respectively. The sequence from 1 to 255 of NlArl5B was matched on KN154202.1 (full length 139047bp) of N.lugens genome between 109344 to 100169, while from 254–540 was matched on KN153648.1 (full length 205899bp) between 29534 to 31028. So we proposed that KN154202.1 and KN153648.1 of N.lugens genome are in same scaffold. The ORF from 1–35 of NlRho showed no similarities in N.lugens genome. Because of the gap of genome in N.lugens, the number of copy or exon in NlRab1, NlRab6, NlArl5B, NlRho and

Table 1. Ras-famil y genes in N.lugens and their sequence characteristics.

| Gene name   | Accession Number | cDNA | ORF (bp) | exon NO. | Length of Gene | Gene loci (ORF coordinates) | MW (D) | PI  |
|-------------|------------------|------|----------|----------|----------------|----------------------------|--------|-----|
| NlRab30     | KT984790         | 612  | 5        | 12365    | KN152431.1(1–612) | 23.3 | 5.63 |
| NlRab35     | KT984791         | 594  | 5        | 13318    | KN152285.1(1–594) | 22.5 | 8.54 |
| NlRab32     | KT984792         | 723  | 5        | 12678    | KN153722.1(1–740) | 27.5 | 8.57 |
| NlRab1     | KT984793         | 585  | 5        | 12895    | KN152447.1(1–585) | 21.8 | 6.5  |
| NlRab8     | KT984794         | 624  | 5        | 8067     | KN151947.1(1–624) | 23.5 | 5.51 |
| NlRab2     | KT984795         | 627  | 5        | 20300    | KN154377.1(1–627) | 23.9 | 9.05 |
| NlRab7     | KT984796         | 639  | 5        | 11732    | KN152218.1(1–639) | 23.7 | 6.35 |
| NlRab7L    | KT984797         | 621  | 3        | 9906     | KN153079.1(1–621) | 23.3 | 5.3  |
| NlRab11    | KT984798         | 574  | 5        | 11871    | KN152751.1(1–574) | 24.4 | 5.8  |
| NlRho      | KT984800         | 582  | /        | /        | KN152640.1(36–572) | 21.8 | 8.03 |
| NlRab23    | KT984801         | 705  | /        | /        | • KN152203.1(33–705) | 6.6  |
| NlRab1     | KT984802         | 615  | /        | /        | / | KN152640.1(36–572) | 22.8 | 4.78 |
| NlRab14    | KT984803         | 648  | 6        | 6184     | KN152075.1(1–648) | 24.2 | 6.74 |
| NlR1f      | KT984804         | 549  | 3        | 6884     | KN152117.1(1–549) | 20.7 | 6.4  |
| NlRab7L    | KT984805         | 845  | 5        | 7002     | KN152606.1(1–845) | 30.3 | 5.82 |
| NlCdc42    | KT984806         | 576  | 5        | 9632     | KN152251.1(1–576) | 21.2 | 6.38 |
| NlRab6     | KT984807         | 603  | 4        | 11432    | KN152265.1(1–603) | 22.8 | 9.51 |
| NlRab1     | KT984808         | 546  | /        | /        | • KN152926.1(1–443) | 20.4 | 8.8  |
| NlRab2     | KT984809         | 540  | 1        | 540      | KN152837.1(1–540) | 20.4 | 8.39 |
| NlRab1     | KT984810         | 549  | 5        | 21602    | KN152467.1(1–549) | 20.5 | 5.66 |
| NlArf5     | KT984811         | 540  | /        | /        | • KN154202.1(1–255) | 20.4 | 6.01 |
| NlRab6     | KT984812         | 627  | /        | /        | / | KN152640.1(36–572) | 23.5 | 4.95 |
| NlRab2     | KT984813         | 555  | 5        | 5377     | KN154257.1(1–555) | 20.9 | 8.57 |
| NlRab39    | KT984814         | 660  | 4        | 11601    | KN152962.1(1–660) | 24.9 | 5.63 |
| NlRab1     | KT984815         | 543  | 3        | 5275     | KN153275.1(1–543) | 20.2 | 5.96 |
| NlRac      | KT984816         | 579  | 6        | 15368    | KN153087.1(1–579) | 21.5 | 7.92 |
| NlSRβ      | KU566374         | 762  | 5        | 15195    | KN151966.1(1–762) | 28.3 | 8.97 |
| NlRan      | KT313028         | 642  | 4        | 4147     | KN153157.1(1–642) | 24.5 | 7.31 |

bp, base pair; MW, Molecular weight; D, Dalton. Gene loci are obtained from blasting on database N. lugens genome (GenBank assembly GCA_000757685). The high identity region of ORF with genome was given in bracket.

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NlRab23, NlArl3 was uncertain. The gene length and loci corresponding on *N. lugens* genome were listed in Table 1.

Phylogenetic tree was constructed using the NJ method to evaluate the molecular evolution relationships of the twenty-nine RSGs proteins of *N. lugens* and other RSGs from representative insect species. In the phylogenetic tree, twenty-nine NIRSGs were clustered into two classes. Rab, Ran, Rho and Ras were clustered together, Rab32 and Rab7L fall slightly outside the main Rab cluster. Sar1, SRβ and Arf formed another separate class. SRβ is distantly related to Arf and Sar1. Rab proteins represent the largest branch of the RSGs gene (Fig 1). Except for the genes *NlRab1, NlRab6, NlArl5B, NlRho, NlRab23* and *NlArf3* that are absent or do not have enough genomic information, the structure of the other twenty-three genes was characterized and shown to contain between 0 and 6 introns (S1 Fig). Among twenty-three RSGs genes, only *NlArf2* possesses no intron, which is similar with that from *A. pisum*, *Tribolium castaneum* and *A. mellifera*. Other NIRSGs genes were divided into many segments by introns. Introns are located either between codons (phase 0) or within codons (phase 1 and 2) [33].

Deduced amino acid sequence showed more than 76% identity with their respective amino acid of other insects except NISRβ and NlRab7L. The best matched sequence for NISRβ is that SRβ from *Megachile rotundata*, with 56% identity and 90% coverage at amino acid level, 68% identity and 56% coverage at nucleotide level. The best matched sequence for NlRab7L is that Rab7L from *Halyomorpha halys*, with 67% identity and 82% coverage at protein level, 75% identity and 62% coverage at nucleotide level. The multiple alignments of twenty-nine *N. lugens* proteins were shown in Fig 2. All these newly cloned proteins have four conserved GTP-binding or GTPase regions of the small G protein superfamily (G1, G3, G4, and G5 loop), as well as an effector site (G2 loop) and a universal conformational switch (switch I and II) [11]. RSGs consist of six-stranded β sheet (β1–β6), five parallel strands (α1–α5) according to the three-dimensional structures [34, 35]. Transmembrane helix was predicted only in protein NISRβ at residues 21–43 as reported by Miller [36]. Rho family members (Rho, Rac and Cdc42) are defined by the presence of a Rho-specific insert about 13 amino acids located between the G4 and the G5 loop and involved in the binding to effectors and regulators [37]. Another important biochemical feature of a majority of RSGs is their post-translational modification by lipids. The modification dictates their specific subcellular locations and interactions with distinct membrane compartments. The C-termini motif of Rab, Ras and Rho and N-termini glycines of Arf family are modified by lipids. The C-termini motif of RSGs from *N. lugens* differ in length from 27 to 43 and can be divided into five types Cxxx, CC, CxC, CCx or CCxxx, where x is any other amino acid (Fig 2).

The expression of NIRSGs genes in *N. lugens*
The expression of all NIRSGs did not show obvious variation at various developmental stages. The highest expression level was found in newly emerged adults and majority of the NIRSGs genes showed higher expression level in females than in males (data not shown).

The results of qRT-PCR in tissue showed that the NIRSGs expressed in all tested tissues, including gut, haemolymph, fat body, salivary gland, ovary, integument, leg and wing, except *NlArl2* was not expressed in integument and wing. In addition, three NIRSGs (*NlRab32, NIK-Ras* and *NlArf2*) expressed with the highest level in gut. Five NIRSGs expressed with the highest level only in haemolymph. Eleven NIRSGs expressed with the highest level in gut. A particularly high expression level of *NlArl2* was found in haemolymph and leg. *NlArf3* showed higher expression level in fat body and haemolymph than that in other tissues. *NlRab23* expressed highly in ovaries (Fig 3). The different expression pattern in tissues may provide clues of the biological functions of NIRSGs in *N. lugens*. 

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**Table 1**

| Gene      | Length | Loci Corresponding |
|-----------|--------|--------------------|
| NlRab23   |        |                    |
| NlArl3    |        |                    |
| NlRab1    |        |                    |
| NlRab6    |        |                    |
| NlArl5B   |        |                    |
| NlRho     |        |                    |
| NlRab23   |        |                    |
| NlArf3    |        |                    |

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Fig 1. Unrooted phylogenetic tree of RSGs from *N. lugens* and representative insect species. An unrooted phylogenetic tree was constructed by the neighbour-joining method, together with 1000 bootstrap replicates based on the protein sequence alignments. *N. lugens* proteins in Class I were marked black circle. *N. lugens* in Class II was marked with black triangle. Gross Ras subfamily groupings were shown on the right. (See S2 Table for details of GeneDB accession numbers).

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Expression of target genes is suppressed by the injection of its specific dsRNA

About 454-898bp dsRNA fragments were injected into 3\textsuperscript{rd} instar nymph respectively to ascer-tain the biological function in insect development. There was no difference at the gene expres-sion level between nymphs injected with double distilled water and dsGFP, so we use the nymphs injected dsGFP as control. After dsRNA injection, mRNA abundance of target gene in the survival nymphs was examined by qRT-PCR. The mRNA level of \textit{NlCdc42} and \textit{NlSRβ} was investigated over seven days after the injection. As expected, \textit{NlCdc42} expression levels in nymphs decreased by 68.7%, 75.9%, 65.9%, 91.6%, 84.0%, 83.5% and 82.5% respectively (S2 Fig). \textit{NlSRβ} expression levels in nymphs decreased by 4.3%, 72.8%, 68.6%, 83.8%, 77.2%, 80.7% and 80.1% respectively, when compared with that in dsGFP- injected nymphs (S2 Fig). The relative expression of \textit{NlSRβ} expression was not reduced in one day after dsRNA injection. The maximum reduction for target gene was occurred at the 4\textsuperscript{th} day after injection. Because of high mortality, there were no enough samples for analysis the RNAi efficiency from the seventh day onward. The RNAi efficiency was demonstrated for other \textit{NlRSGs} genes at the 4\textsuperscript{th} day post injection. At the same concentration (70ng/nymph) of injected dsRNA, qRT-PCR showed that the transcript level of target genes reduced by 39.2%-99.5%, comparing with the dsGFP con-trol. Among the eighteen \textit{NlRSGs} which have lethal effect in \textit{N. lugens} when silenced, the expression level decreased more than 60.0% for fifteen \textit{NlRSGs} genes (Fig 4C1–4C3). Less than 50% decrease for gene \textit{NlRho} (Fig 4C1), \textit{NlArf6} (Fig 4C2) and \textit{NlArf2} (Fig 4C3).

RNAi screen to identify \textit{NlRSGs} genes with crucial functions in the development of \textit{N. lugens}

To examine the role of individual \textit{NlRSGs} in the nympha development of \textit{N. lugens}, we synthe-sized dsRNA for twenty-eight \textit{NlRSGs} genes and lowered its expression by RNAi individually. The phenotypic defects in morphological and lethal characteristics were almost not detectable in dsGFP-injected or water-injected nymphs throughout the test period. The nymphs in the water or dsGFP controls were successfully molted to adult at 5\textsuperscript{th} day and 90.0% individuals...
Fig 3. Tissue expression patterns of *N. lugens* genes encoding RSGs. Total RNA was extracted from the salivary gland (SG), gut (GT), fat body (FB), ovary (OV), hemolymph (HM), integument (IT), leg (LG) and wing (WG). Three biological replications (mean ± SE) were carried out for each gene based on independent samples and relative value for the expression level of target gene was calculated by the equation \( Y = 10^{\left(\frac{Ct_{\text{internal}} - Ct_{\text{target}}}{3}\right)} \times 100\% \), using the geometric mean of 18S rRNA and \( \beta \)-actin as internal control. Different letters indicate significant differences among different tissues by Duncan’s multiple range test at \( P < 0.01 \).

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were survival to adult at 11th day after injection. In contrast, a large-scale RSGs RNAi screen identified the suppression of eighteen NlRSGs transcription levels resulted in lethal effect in N. lugens. The survival rate of nymphs began to decrease 3 days after injecting dsRNA of NlRab2, NlSar1, NlArf1 or NlRho. The surviving rate was 52.0–78.0% at 3rd day, which dramatically dropped to less than 20.0% at 5th day, Nine days after injecting dsRNA of NlRab2 or NlSar1, the survival rate of nymphs decreased to 0.0%, yet the dsGFP control remained high (90.0%) (Fig 4A1). The survival rate of nymphs began to decrease 3 days after injecting dsRNA of NlSRβ, NlCdc42, NlRab7, NlArf6 or NlArf3. The surviving rate was 30.0–45.0% at 5th day, which dramatically dropped to less than 15% at 7th day after injection. Nine days after injecting dsRNA of NlSRβ, the survival rate of nymphs decreased to 0.0% (Fig 4A2). More than 80.0% of dsNlArf2 and dsNlRab1-treated nymphs survived at 5th day after injection. However, the surviving rates decreased at 7th day and dramatically declined to less than 10% and 20% respectively at 11th day after injection (Fig 4A3). No nymphs with injected dsNlSRβ, dsNlRab2,
dsNlSar1 or dsNlRab1 eclosed to adult. Less than 17.6% of dsNlRab7, dsNlRab6, dsNlRab39, dsNlRheb, dsNlRho, dsNlCdc42, dsNlArf1, dsNlArf6 or dsNlArl-treated nymphs were survived to eclose (Fig 4B1 and 4B2). Many of the deaths occurred during molting stage in nymphs with silenced NlSar1, NlSar1, NlArf6 or NlRab2 (A, B) which did not molt into next stage and old nymphal exoskeletons remained on the tips of legs and abdomens and died later. The typical phenotype of nymphs with silenced NlRheb, NlRab1, NlRab39 or NlRac (C, D) which started to eclose but failed to shed the exuvia completely and showed deficiencies in the extension of the wings.

Fig 5. Effect of dsRNA injection on the nymphal molting. The typical phenotype of nymphs with silenced NlSar1, NlSar1, NlArf6 or NlRab2 (A, B) which did not molt into next stage and old nymphal exoskeletons remained on the tips of legs and abdomens and died later. The typical phenotype of nymphs with silenced NlRheb, NlRab1, NlRab39 or NlRac (C, D) which started to eclose but failed to shed the exuvia completely and showed deficiencies in the extension of the wings.

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dsNlSar1 or dsNlRab1 eclosed to adult. Less than 17.6% of dsNlRab7, dsNlRab6, dsNlRab39, dsNlRheb, dsNlRho, dsNlCdc42, dsNlArf1, dsNlArf6 or dsNlArl-treated nymphs were survived to eclose (Fig 4B1 and 4B2). Many of the deaths occurred during molting stage in nymphs with silenced NlRab2, NlSar1, NlArf6 or NlSar1. A typical phenotype is that the nymphal cuticles remained on the tips of legs and were not completely shed (Fig 5A and 5B). Many of the deaths occurred during nymph-adult ecdysis in nymphs with silenced NlRheb, NlRab39, NlRab1 or NlRac. The typical phenotype was that the wings wrinkled, puckered or bent and...
the nymphal cuticles cannot be completely drained away (Fig 5C and 5D). About 59.3% nymphs with silenced NiRab23 survived to adult emergence, the mortality at 11th day was high to 96.3% (Fig 4A3). This suggested that NiRab23 plays an important role in nymph and adult development. About 20% nymphs with silenced NiRab1 were survival at 11th day, while no nymphs can adult eclosion (Fig 4A3 and 4B3). So we proposed NiRab1 plays an important role in nymph-adult transition. By contrast, the suppression of other ten genes did not lead to abnormal effects in Nilaparvata lugens, although more than 90% decrease in the transcript levels following RNAi application were observed.

**Function of NiRSGs in the 20E and JH signal pathway**

JH and 20E play critical signaling roles during insect development and adulthood and are essential for normal metamorphosis and reproduction [38, 39]. To examine the possible function of RSGs in the 20E or JH signal pathway, we conducted qRT-PCR experiment to examine expression levels of eight nuclear receptor genes involved in the 20E and JH signal pathway and five Halloween genes involved in 20E biosynthesis in nymphs with silenced NiRSGs. Of those eighteen genes which have lethal effect on nymph development after dsRNA injection, twelve genes were proposed to be related with 20E or JH signal pathway. The expression of NiFTZ-F1, NiHr3, NiKr-h1 or NIE93 and three Halloween genes were significantly decreased or increased when compared to their mRNA levels in the nymphs with dsGFP (Fig 6A–6G). The expression level of NiKr-h1 was decreased by 89.6–74.4% in nymphs with silenced eight genes respectively (including NiSar1, NiRab2, NiArf1, NiCdc42, NiRab6, NiRab39, NiArf11 or NiSRβ). The expression level of NIE93 was decreased by 85.6–74.6% in nymphs with silenced six genes respectively (including NiSar1, NiRab2, NiArf1, NiArf6, NiRab39 or NiArf11) as compared to nymphs injected with dsGFP. From our result, we conclude that the expression of at least two nuclear receptors was decreased in nymphs with silenced NiRab2, NiArf11 or NiArf2. At least the expression of three genes including nuclear receptors and Halloween genes was decreased in nymphs with silenced NiSar1 and NiRab39. The nymphs with silenced NiArf1 demonstrated 4–5 folds reduction in five genes expression including two Halloween genes NiCyp315a1 and NiCyp314a1 and three nuclear receptors NiKr-h1, NiFTZ-F1 and NIE93. The nymphs with silenced NiSRβ demonstrated an approximate 3.3 folds reduction in NiKr-h1 gene expression. In contrast, the gene expression of NiFTZ-F1 was increased about 5 folds when compared with the controls. The nymphs with silenced NiArf6 demonstrated an approximate 3–4 folds reduction in NiKr-h1 and NIE93, while the transcript level of NiCyp307a1 was increased about 6.5 folds. The transcript level of other nuclear receptors including NIECR, NiMet, NiBr-C and NIE75 and Halloween genes NiCyp306a1, NiCyp302a1 was only slightly or without influenced in RNAi-treated nymphs (data not shown). Of those eighteen genes, down-regulation of NiRho, NiArl3, NiRab1, NiArf2, NiRab23 and NiRac has no significant effect on transcript level of tested nuclear receptors and Halloween genes.

**Identification of efficient RNAi target genes for Nilaparvata lugens control**

Three potential effective RNAi target genes NiRab2, NiSar1 and NiSRβ genes, which were marked by 0.0% eclosion rate and almost 100.0% mortality on day nine post injection, were extensively studied by alignment protein sequences from seven insects including A. pisum, T. castaneum, A. aegypti, Z. nevadensis D. melanogaster, B. mori and A. mellifera. The results indicated that NiRab2 and NiSar1 are both highly conserved among different insects (S3 and S4 Figs). However, NiSRβ is not highly conserved among different insects (S5 Fig). The analysis of sequence pair distances with Megalign software suggested that NiSRβ share relatively low sequence similarity with other insects, while sequence identity at both the nucleotide and the
amino acid level is very high for NlSar1 and NlRab2 (Table 2). And then we searched the nucleotide sequences against the well annotated NCBI transcriptome databases for >19 bp long stretches of identity. The result showed no 19nt overlap (data not shown), and thereby prevented RNAi-induced silencing in the non-target species.

Table 2. Sequence pair distances of three genes.

| Gene name  | Dm     | Am     | Bm    | Aa     | Zn     | Tc     | Ap     |
|------------|--------|--------|-------|--------|--------|--------|--------|
| NlRab2     | 89.2/75.6 | 88.7/64.0 | 88.2/72.8 | 90.1/73.1 | 95.8/77.9 | 91.0/72.0 | 89.6/70.6 |
| NlSar1     | 63.2/62.1 | 69.6/65.3 | 73.5/70.3 | 73.7/70.1 | 68.8/66.0 | 71.3/65.8 | 70.1/63.0 |
| NlSRβ      | 29.4/37.3 | 53.2/47.2 | 38.8/39.8 | 37.2/34.6 | 51.3/44.2 | 45.1/43.1 | 47.9/41.3 |

Sequence pair distances were calculated by the ClustalV method of Megalign software between insects. Degree of identity at protein level in percentage is given in left of slash and the degree of identity at DNA level in percentage is given in right of slash. The data sources for all Ras family GTPase are listed in S3 Table. Ap, Acrhoposiphon pisum; Bm, Bombyx mori; Zn, Zootermopsis nevadensis; Aa, Aedes aegypti; Dm, Drosophila melanogaster; Am, Apis mellifera; Tc, Tribolium castaneum.

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Fig 6. qRT-PCR analysis of genes expressions in the nymphs injected with dsGFP and dsRSGs. Error bars represent the standard deviation in three independent RNAi experiments. Different letters indicate a significant difference at P <0.05.

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To determine the sensitivity of \( N.lugens \) to dsNlSR\( \beta \), different concentration gradient experiments were performed. In the third-instar nymphs, injection of dsNlSR\( \beta \) (0.1\( \mu \)L) at the concentrations of 0.7, 7.0, 70.0 and 700.0ng/\( \mu \)L suppressed NlSR\( \beta \) expression level by 42.9%, 55.0%, 73.7% and 83.7%, respectively, comparing with the levels in dsGFP injected nymphs (Fig 7A). The survival rates decreased from 3\textsuperscript{rd} day and dramatically dropped to 0.93% and 1.3% at the 5\textsuperscript{th} day for nymphs injected with concentrations of 70.0 and 700.0ng/\( \mu \)L dsNlSR\( \beta \), respectively. No nymphs were survived at the 9\textsuperscript{th} day. As the dose of dsNlSR\( \beta \) used decreased to 7.0 and 0.7 ng/\( \mu \)L, the survival was increased and reached to 30.7% and 59.4% at the 9\textsuperscript{th} day respectively (Fig 7B). The result suggested that no nymphs were survived in the dsNlSR\( \beta \)-treated group when the dose of dsNlSR\( \beta \) given was higher than 7ng/per insect.

**Discussion**

The RSGs take part in numerous and diverse cellular processes and act as switches to regulate protein activity or localization. Protein localization to their correct subcellular compartments is critical for eukaryotic cells to maintain the high degree of order required for life.

**Identification of RSGs genes in \textit{N. lugens}**

In yeast, \textit{Trypanosoma brucei}, \textit{C.elegans}, \textit{D.melanogaster} and humans, the number of genes for RSGs is 30, 40, 56, 90, and 174, respectively [40, 41]. In this study, we cloned the cDNA of twenty-nine RSGs from \textit{N. lugens}. These proteins were clustered into two class and five sub-families. The largest class is the Rab family with 14 members followed by Arf, with 11 members including Sar1 and SR\( \beta \). Homology analysis revealed that NIRSGs were conserved among different insect species. This suggests that the biological functions may be similar to the corresponding RSGs in other model organisms. Sequence alignments showed that all NIRSGs shared a common G domain, two switch regions (I and II) and significant sequence similarity. The five types cysteine motif boxes (Cxxc, CC, CxC, CCx or CCxxx) and C-termini polybasic region of Rab, Ras and Rho play important roles in subcellular localization and small GTPase
function[42,43]. The Arf family proteins are characterised by being anchored to the bilayer in their GTP-bound state by an N-terminal myristoylation at Gly2 found at the N-terminal amphipathic helix (α1 helix) rather than the C-terminal lipid anchor used by most other members of the Ras superfamily of small G proteins [44–46]. These acylations localize the RSGs to cellular membranes, where their signaling activities take place [43]. Sar1 lacks N-myristoylation but has a highly conserved bulky hydrophobic patch (NIW in NlSar1) within N-terminal amphipathic helix that mediates recruitment of Sar1 to ER membranes and can also induce their curvature in vitro [47, 18]. In contrast to Arf proteins, SRβ is a membrane-bound protein via its N-terminal transmembrane helix and has a distinct sequence-conservation pattern in the switch II region. The proline residues in switch II region is phylogenetically well conserved in all SRβ orthologues, which undergoes cis/trans isomerization as part of this G protein switch [48]. Transmembrane helix was predicted in protein NlSRβ at residues 21–43. The proline residues of 102 position in NlSRβ is strictly conserved as glycine in the Arf-type proteins. This subtle difference to the Arf-type switch suffices to create a quite different switch mode that regulates the protein targeting process. Rans are closest to Arfs. They also lack cysteines at the C-ends and are not posttranslationally modified at all. Ran has an elongated COOH-terminal element (EDDEDL (209–214) in NlRan) crucial for its function in nuclear transport [35].

Tissue specificity analysis showed the various expression patterns of RSGs genes in N. lugens. Three NlRSGs expressed at the highest level in gut. Five NlRSGs expressed at the highest level only in haemolymph. Since gut is an important organ related to the absorption and transport of nutrients. Intracellular transport is more active here than in other tissues. Haemolymph plays a central role in insect immune response and defend against pathogen invasion. RSGs are postulated to be implicated in the hemocyte cellular processes to perform phagocytosis, nodulation, and encapsulation behaviors [19, 49]. This might indicate that the possible intracellular transport or immune-related functions of NlRSGs which highly expressed in the gut and haemolymph. The wing we dissected included the direct flight muscles at the base of the wing. Our result showed that eleven NlRSGs (eight belong to Rab subfamily) expressed with the highest level in wing. Insect wing muscle is a strictly aerobic tissue and contains a large number of endoplasmic reticulum or sarcoplasmic reticulum [50]. Rab GTPases associate with distinct endomembrane compartments and are essential for the transport of proteins and membrane through the endomembrane system to their destination [51]. The NlRSGs highly expressed in wing may be associated with ER and involved in functional regulation of muscles and wings.

**RNAi induces target gene repression and lethal effect in N. lugens nymphs**

When nymphs were injected with dsRNA, levels of transcripts of the targeted genes were reduced. RNAi revealed the functional importance of eighteen NlRSGs in nymphal development, which showed lethal effect. Most nymphal death was occurred during nymph molting or nymph-adult ecdysis. While the other ten genes were not the key factor in nymphal development of N.lugens. RNAi studies showed that some Rab subfamily genes such as NlRab2, NlRab7, NlRab6, NlRab23, NlRab1 and NlRab39 are essential, whereas others are dispensable. They may have only partially overlapping functions in membrane traffic. Although only 39.2–49.5% decrease of gene expression was observed for RNAi of NlArf6, NlArf1 and NlRho, the adverse effect on N.lugens implies the importance of the three genes in N.lugens development. All the Arf subfamily genes except Arf15B and Rho subfamily genes are essential for N.lugens development. We demonstrated that NlRab2, NlSar1 or NlSRβ is an indispensable factor during the molting or eclosion process. The silencing of NlRab2, NlSar1 or NlSRβ expression...
which highly expressed in tissue haemolymph, leg, wing or integument leads to nymphal cuticles shed incompletely and 100% mortality at 9th day.

In insects, the life cycle is characterized by a series of moltings, including larval/nymph molting and metamorphic molting. The molting process is regulated by steroid hormones including 20E and JH acting via nuclear receptors [39]. Five halloween genes coding for the P450 enzymes that control the 20E biosynthesis pathway. Nuclear receptors EcR, Br-C, E75, Hr3, FTZ-F1 and E93, Kr-h1 participated in 20E and JH regulatory cascade respectively [52]. And also the appropriate timing of their expression is almost certainly essential for their correct function [52]. Knock-down of some NIRSGs inhibited nymphs molting or nymphal–adult transition. The phenotypic defects similar to insect whose ecdysteroid-mediated signaling had been inhibited [53–56] or whose ecdysteroid synthesis had been disturbed [57, 58]. Therefore the transcription level of nuclear receptors and halloween genes were tested in RNAi-treated nymphs. RNAi of twelve NIRSGs individually resulted in the transcriptional down or up-regulation of nuclear receptors (Kr-H1, Hr3, FTZ-F1 and E93) or ecdysteroid synthesis genes (Cyp307a1, Cyp314a1 or Cyp315a1) in N. lugens. When the gene NlArf1 was suppressed, not only the expression of NlArf1 decreased, but also the expressions of other genes involved in the 20E biosynthesis and signal transduction pathway were affected, such as Cyp315A1, Cyp314a1, E93, Kr-h1 and FTZ-F1. When the gene NlSar1 was suppressed, the expression of Cyp314a1, E93 was affected. While the gene NlArf6 was suppressed, the expression of Cyp307a1, E93 and Kr-h1 was affected. These results suggested that NlArf1, NlArf6 and NlSar1 are involved in regulation of transcription of 20E biosynthesis genes and nuclear receptors. While most NIRSGs are necessary for nuclear receptors involved in the transcription of 20E or JH signal pathway. Different members of the RSGs can activate multiple signaling pathways leading to the activation of transcription factors, such as the activation of nuclear factor by members of Rho family [59, 60]. We proposed that the problems to complete the molting process in dsNIRSGs insect were partially due to disruption of the steroid hormones signal pathway via a) failure regulatory function on ecdysteroid synthesis or nuclear receptors transcription; b) destruction the consistence and appropriate timing expression patterns; c) improper localization proteins participated in ecdysteroid synthesis or signal pathway.

**NISRβ a better candidate as a target for pest control purpose**

To discover potential RSGs as pesticide targets, a large-scale RNAi screen was performed by injecting dsRNA into developing nymph in this study. Eighteen RSGs were found involved in nymphal growth, molting and metamorphosis. The identified RSGs may serve as potential insecticide targets for controlling N. lugens and other related pest species. Among these eighteen RSGs genes, severe RNAi-induced phenotypes were observed for NISRβ, NISar1 or NlRab2. Specifically, knock-down resulted in 100% mortality and 0% eclosion rate within 9 days. In order to protect non-target organisms it would be desirable to use dsRNA fragments that are specific to the pest species and do not contain sequences targeting genes in non-target organisms (off targets). dsRNA are processed by the enzyme Dicer into 21-23nt long short interfering RNAs, they serve as template to recognize the complementary mRNA and target it for destruction [61]. This short interfering RNAs with an exact sequence identity of ≥19nt can already induce off target effect [62]. Therefore, the sequence conservation of three potential RNAi target genes were analyzed. The analysis suggested NISRβ could be served as a target for dsRNA-based pesticides for N. lugens control. Firstly, NISRβ, as an integral membrane protein, is constitutively expressed in almost all tissues. The SRβ is required for the cotranslational targeting of both secretory and membrane proteins to the ER membrane [36]. Protein translocation across and insertion into membrane is essential to all life forms. It is not surprising,
therefore, that any changes in SRβ expression and function have profound effects on many cellular functions. Secondly, injection only 7.0 ng/nymph dsRNA of NlSRβ during nymphal development was sufficient to induce 100% mortality and hindered eclosion completely. The expression of Kr-H1 and FTZ-F1 was interfered by RNAi of NlSRβ. Thirdly, NlSRβ shared relatively low sequence similarity (34.6–44.2%) over the entire gene’s length and showed no >19 bp overlap with other insects, thereby it was relatively easy to design dsRNA sequences that were unique to N.lugens and prevented RNAi-induced silencing in the non-target species. Therefore, NlSRβ seems a better candidate as a target for pest control purpose.

In summary, we identified twenty-nine NIRSGs genes and demonstrated the importance of eighteen NIRSGs in nymphal development of rice pest N.lugens. NlSRβ was proposed to be a potential effective target for RNAi-based pest control. Attempts to analysis the function of NIRSGs in reproduction and cellular immune are in progress in our laboratory.

Supporting information

S1 Fig. Phylogenetic tree (A) and intron–exon structures (B) of RSGs genes in N.lugens. The number of branches on the phylogenetic tree indicated the bootstrap values. The phylogenetic tree was constructed by maximum likelihood based on the full-length cDNAs. cDNA and genomic sequences were compared putative exon–intron map. Exons and introns are indicated with the black boxes and black lines, respectively. The number indicated intron phase. Lengths are roughly at scale. (TIF)

S2 Fig. Silencing efficiency of injection dsNlCdc42 (A) and dsNlSRβds (B). The relative transcript level for each sample was measured daily from 3 independent pools of 5 nymphs. P < 0.01 was considered statistically significant (***) different from dsGFP treatment. (TIF)

S3 Fig. Amino acid sequence alignments of Rab2 from eight species. Blank residues indicate identical amino acids. Gray residues represent conserved substitutions. Sequences were aligned using ClustalW. Nl,N.lugens;Ap, Acyrthosiphon pisum;Bm, Bombyx mori;Zn, Zootermopsis nevadensis;Aa, Aedes aegypti;Dm, Drosophila melanogaster;Am, Apis mellifera;Tc, Tribolium castaneum. The data sources for all Ras family GTPase are listed in S3 Table. (TIF)

S4 Fig. Amino acid sequence alignments of Sar1 from eight species. Blank residues indicate identical amino acids. Gray residues represent conserved substitutions. Sequences were aligned using ClustalW. Nl,N.lugens;Ap, Acyrthosiphon pisum;Bm, Bombyx mori;Zn, Zootermopsis nevadensis;Aa, Aedes aegypti;Dm, Drosophila melanogaster;Am, Apis mellifera;Tc, Tribolium castaneum. The data sources for all Ras family GTPase are listed in S3 Table. (TIF)

S5 Fig. Amino acid sequence alignments of SRβ from eight species. Blank residues indicate identical amino acids. Gray residues represent conserved substitutions. Sequences were aligned using ClustalW. Nl,N.lugens;Ap, Acyrthosiphon pisum;Bm, Bombyx mori;Zn, Zootermopsis nevadensis;Aa, Aedes aegypti;Dm, Drosophila melanogaster;Am, Apis mellifera;Tc, Tribolium castaneum. The data sources for all Ras family GTPase are listed in S3 Table. (TIF)

S1 Table. Primers used in this study. F,forward primer,R,reverse primer. (DOC)
S2 Table. Protein sequences sources used in Fig 1.

(DOCX)

S3 Table. Sequences sources used in Table 2 and S1 Fig.

(DOC)

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Investigation: FXL.
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