Running head: A salivary effector in rice brown planthopper

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A salivary endo-β-1,4-glucanase acts as an effector that enables the brown planthopper to feed on rice

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Summary

A salivary endo-β-1,4-glucanase in the rice brown plant hopper Nilaparvata lugens facilitates access to the phloem by degrading cellulosics in plant cell walls.

Footnotes:

Author contributions
Y.L. conceived the original research plans and supervised the experiments; Y.L., R.J. and W.Y. designed the experiments and analyzed the data; R.J., W.Y., H.C., J.Z., H.L., H.Y. and J.L. performed the experiments; Y.L. and R.J. wrote the manuscript; all authors reviewed the manuscript.

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Abstract

The rice brown planthopper (BPH) *Nilaparvata lugens* (Stål) is one of the most destructive insect pests on rice in Asia. After landing on plants, BPH rapidly accesses plant phloem and sucks the phloem sap through unknown mechanisms. We discovered a salivary endo-β-1,4-glucanase (NlEG1) which has endoglucanase activity with a maximal activity at pH 6 at 37°C and is secreted into rice plants by BPH. NlEG1 is highly expressed in the salivary glands and midgut. Silencing NlEG1 decreases the capacity of BPH to reach the phloem and reduces its food intake, mass, survival, and fecundity on rice plants. By contrast, NlEG1 silencing had only a small effect on the survival rate of BPH raised on artificial diet. Moreover, NlEG1 secreted by BPH did not elicit the production of defense-related signal molecules -- salicylic acid, jasmonic acid (JA), and jasmonoyl-isoleucine (JA-Ile) -- in rice, although wounding plus the application of the recombination protein NlEG1 did slightly enhance the levels of JA and JA-Ile in plants compared to in the corresponding controls. The data suggest that NlEG1 enables the BPH’s stylet to reach the phloem by degrading celluloses in plant cell walls (PCWs), thereby functioning as an effector that overcomes the PCW defense in rice.
INTRODUCTION
To protect themselves from attack by herbivores, plants have developed a set of resistance mechanisms, including constitutive and induced defenses (Felton and Tumlinson, 2008; Erb et al., 2012; Stam et al., 2014; Schuman and Baldwin, 2016). Constitutive defenses are physical and chemical defensive traits that plants express regardless of the presence of herbivores. By contrast, induced defenses are activated only when plants are infested by herbivores (Wu and Baldwin, 2010). Defense induction starts with the recognition of specific herbivore-associated molecular patterns and is followed by the activation of a complex signaling network, such as mitogen-activated protein kinase cascades, and jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile), salicylic acid (SA), and ethylene signaling pathways; the expression of defense-related genes; and the production of defensive chemicals (Erb et al., 2012; Stam et al., 2014; Schuman and Baldwin, 2016).

In response, herbivores have evolved the capacity to suppress and circumvent these plant defenses through the release of effectors (Elzinga and Jander, 2013). Plant cell walls (PCWs), for instance, are thick, rigid structures that mainly consist of a pectin-embedded network of cellulose and hemicellulose (Calderón-Cortés et al., 2012); these structures not only act as physical defenses against herbivores by enhancing the mechanical hardness of plant tissues, but they also reduce the digestibility of food for herbivores (Santiago et al., 2013), thereby functioning as the first layer of defense against herbivores. Herbivores can secrete salivary PCW-degrading enzymes such as cellulases (consisting of endo-β-1,4-glucanases and β-glucosidases) and pectinases to degrade PCWs (Backus et al., 2012; Calderón-Cortés et al., 2012). Herbivores can also secrete other effectors to overcome plant defenses. C002, for instance, is a salivary protein identified from the salivary glands of the pea aphid *Acyrthosiphon pisum*; this protein is essential for sustained phloem feeding (Mutti et al., 2008). Expressing C002 from the green peach aphid *Myzus persicae* in *Nicotiana benthamiana* increased aphid reproduction on these plants, whereas reducing C002 expression in aphids by plant-mediated RNA interference reduced aphid fecundity (Bos et al., 2010; Pitino et al., 2011). Other salivary proteins, such as glucose oxidase from corn earworm *Helicoverpa*...
zea (Musser et al., 2002), calcium-binding proteins from the vetch aphid *Megoura viciae* (Will et al., 2007), Mp10 and Mp55 from the green peach aphid (Bos et al., 2010; Elzinga et al., 2014), structural sheath proteins from the grain aphid *Sitobion avenae* (Abdellatef et al., 2015), Me10 and Me23 from the potato aphid *Macrosiphum euphorbiae* (Atamian et al., 2013), and Armet from the pea aphid (Wang et al., 2015) have also been found to increase herbivore performance. Thus, herbivore effectors play a central role in overcoming plant defenses and helping the herbivore establish a population on host plants. However, the mechanisms underlying the effector-mediated promotion of herbivore abilities to overcome plant defenses remain mostly unknown (Elzinga and Jander, 2013).

The brown planthopper (BPH, *Nilaparvata lugens* (Stål)), one of the most destructive insect pests of the rice plant (*Oryza sativa* L.) in Asia, causes substantial losses in rice yield every year (Heong et al., 2014). As a piercing-sucking insect, BPH secretes two primary kinds of saliva during feeding: coagulable and watery. Coagulable saliva forms salivary sheaths around the insect’s stylets which help to stabilize and protect the stylets and may suppress plant defense responses to components of the watery saliva (Miles, 1999; Abdellatef et al., 2015). Watery saliva, which contains a mixture of amino acids, proteins, and digestive enzymes, assists in the digestion of plant material and helps suppress plant defense responses (Miles, 1968; Miles, 1999; Harmel et al., 2008; Carolan et al., 2009; Hogenhout and Bos, 2011; Nicholson et al., 2012; Elzinga and Jander, 2013). Thus, BPH saliva plays an important role in BPH feeding. The morphology, transcriptomes and secreted proteins of BPH salivary glands have been analyzed and identified (Sogawa, 1968; Konishi et al., 2009; Ji et al., 2013). Moreover, several BPH salivary proteins, such as a catalase-like protein Kat-1, NlShp, salivap-3, and annexin-like5, have been reported to be secreted into rice and play an important role in salivary sheath formation and/or BPH feeding (Petrova and Smith, 2014; Huang et al., 2015; Huang et al., 2016). However, whether other salivary proteins are also involved in BPH feeding and how these proteins regulate BPH feeding remain unclear.

By screening 352 reported genes encoding putative secreted proteins of salivary gland of BPH (Ji et al., 2013), we identified a BPH gene *NIEGI* which encodes a
putative endo-β-1,4-glucanase. Given the role of salivary cellulases in degrading PCWs stated above, we thought that NIEG1 may play a role in BPH feeding by influencing the formation of the salivary sheaths and/or the defense in rice. Therefore, we chose NIEG1 and explored its role in rice-BPH interactions. Through a combination of molecular biology and behavioral experiments, we show that NIEG1 is an effector that enables BPH to feed on rice plants and simultaneously circumvents plant defenses.

RESULTS
Isolation and characterization of NIEG1
Based on the data from BPH salivary gland transcriptomes (Ji et al., 2013), the full length of cDNA of the gene NIEG1 (1454 bp), including an open reading frame (ORF) of 1386 bp, was obtained by reverse transcription–polymerase chain reaction (RT-PCR) (Fig. 1, GenBank: KM459012). Its deduced amino acid sequence revealed that NIEG1 encodes a protein of 461 amino acids with a calculated relative molecular mass of 52.2 kDa. The protein possesses an extracellular signal peptide and has no transmembrane domains, suggesting a putative secreted protein. Moreover, two potential O-glycosylation sites and two N-glycosylation sites were identified (Fig. 1). Protein alignment followed by phylogenetic tree analysis revealed that NIEG1 is homologous to insect endo-β-1,4-glucanases and belongs to the glycosyl hydrolase family 9 (GHF 9); this family is characterized by catalytic domains, including catalytic nucleophile (77 Asp), a probable secondary nucleophile (80 Asp) and proton acceptor (535 Glu), and two signature motifs (Nakashima et al., 2002; Kim et al., 2008; Willis et al., 2011) (Fig. 1; Supplemental Figs. S1 and S2). NIEG1 shares the highest homology (72%) with the endo-β-1,4-glucanase from Isoptera (Zootermopsis nevadensis KDR16731.1) and Phthiraptera (Pediculus humanus corporis XP_002426465.1), followed by that from Hemiptera (A. pisum XP_001944774.2) (71%) and Hymenoptera (Apis florea XP_003690676.1) (69%).

To confirm that NIEG1 has endoglucanase activity, the recombination protein NIEG1 was produced in Pichia pastoris (Fig. 2A). The mass of the recombination
protein NlEG1 was about 60 kDa (Fig. 2A). Enzyme activity assays demonstrated that
NlEG1 acted hydrolytically on carboxymethyl cellulose (CMC) and showed the highest
activity at pH 6 at 37°C (Fig. 2, B and C). Moreover, NlEG1 also acted hydrolytically
on filter paper and cellulose from rice plants (0.55 and 0.79 U/mg at pH 6 at 37°C,
respectively) but had no activity on crystalline cellulose (Avicel), curdlan, laminarin
and xylan. The catalytic activity of NlEG1 against CMC showed a Michaelis constant
(Km) of 6.72 mg/ml with a maximal reaction rate (Vmax) of 9.91 U/mg. QRT-PCR
analysis revealed that NlEG1 was expressed in most life stages of BPH (Fig. 2D) and
highly expressed in the salivary gland, midgut, fat body, and ovary (Fig. 2E).

**NlEG1 is secreted into rice during BPH feeding**
To explore whether BPH excretes NlEG1 through salivation during feeding, rice stems
were individually infested with 200 fourth- and fifth-instar nymphs for 24 h, after which
the outer three leaf sheaths were harvested and the proteins extracted. Western blot
analysis was performed using polyclonal anti-NlEG1 rabbit anti-bodies. As shown in
Figure 3A (lane 2), a band of about 50 kDa was detected in plants attacked by BPH. The
same band was also detected in extracts from BPH salivary glands (Fig. 3A, lane 1). On
the other hand, the band of NlEG1 was not detected in control plants (non-infested
plants) (Fig. 3A, lane 3). The results indicate that NlEG1 is transferred from BPH
salivary glands to the plant during feeding. A band of about 38 kDa was also detected in
extracts of the salivary glands (Fig. 3A, lane 1) but could not be detected in the plant
extracts either before or after BPH feeding (Fig. 3A, lanes 2 and 3). It appears that the
rabbit used for raising antibodies against the protein NlEG1 polypeptide also contained
antibodies that matched another salivary gland protein of 38kDa. These results show
that NlEG1 accumulates in rice plants after BPH feeding and is therefore likely excreted
into the plant by the herbivore.

**Silencing NlEG1 impairs BPH feeding and fecundity**
To explore the function of NlEG1, we used RNA interference (RNAi) as described in
Liu et al. (2010). Injection with dsRNA of NlEG1 decreased the transcript levels of
NlEG1 in the whole body, salivary gland, midgut, fat body, and ovary of BPH by
81%-95% over a period of 6 days (2-8 d post injection; Fig. 3, B and C). Silencing also
reduced the abundance of the protein in the salivary glands and the enzyme activity of NlEG1 (Fig. 3D). Silencing NlEG1 did not influence the body length and pronotum width of BPH nymphs (Fig. 4A; Supplemental Fig. S3). However, the mass of NlEG1-silenced nymphs and the number of eggs in the ovaries of NlEG1-silenced female adults were reduced by about 21% and 54-57%, respectively, although eggs were normal (Fig. 4, B-D).

To study the effect of NlEG1 on BPH feeding, we used the electrical penetration graph (EPG) technique, a powerful method used to profile the feeding behavior of piercing-sucking insects (Seo et al., 2009; Cao et al., 2013). Five main phases of feeding can be distinguished by EPG: non-penetration, the pathway phase (including penetration initiation, salivation and stylet movement, and extracellular activity near the phloem), intracellular activity in the phloem, phloem sap ingestion, and the xylem phase. Representative EPG traces from BPH, including the different phases, are shown in Fig. 5A (upper panel). NlEG1 silencing significantly increased the non-penetration (Fig. 5A, NP) and the pathway phase (Fig. 5A, PP) time. On the other hand, the phloem intracellular activity and ingestion phases were significantly reduced (Fig. 5A, N4-a, and N4-b). Eleven of the 15 tested NlEG1 silenced individuals did not reach the phloem during the 6 h trial period, whereas all control individuals did. The few silenced individuals that reached the phloem still spent significantly less time ingesting phloem sap than did controls (Fig. 5A, N4-b’). Silencing NlEG1 also significantly and consistently reduced the amount of secreted honeydew, which is an indicator of the amount of food intake (Fig. 5B). Moreover, in accord with the result on the number of eggs in the ovaries, the number of eggs laid by NlEG1-silenced female adults was decreased by 50% (Fig. 5D). Collectively, these results show that NlEG1 is required for cell wall penetration and feeding as well as fecundity.

**NlEG1 is indispensable for BPH survival on rice**

To further test whether the presence of NlEG1 influences BPH survival rates on rice and whether this influence is related to the effect of NlEG1 on the ability of BPH to reach the phloem, we compared the performance of BPH nymphs on different food matrices.
NlEG1 silencing generally reduced the survival rate (Fig. 6), and the effect was most pronounced on rice plants: Three days after dsRNA injection, the survival rate of BPH on rice dropped significantly and was 23% 7 days post-injection (Fig. 6A). In contrast, the survival rate of BPH nymphs with silenced NlEG1 was much higher in insects raised on artificial diet than in those raised on rice; moreover, the survival rate of BPH nymphs with knocked-down NlEG1 raised on artificial diet was only slightly reduced compared to that of control BPH nymphs 7 days after the start of the experiment (Fig. 6B). Compared with that of BPH nymphs with silenced NlEG1 raised on artificial diet, the corrected survival rate of BPH nymphs with silenced NlEG1 raised on rice was significantly lower 4-7 days after injection (Fig. 6C). These results demonstrate that NlEG1 contributes to the survival rate of BPH nymphs raised on rice.

**NlEG1 secreted by BPH does not induce defense responses in rice**

Plant hormones – namely, JA, JA-Ile and SA -- play major roles in rice defense against herbivores (Zhou et al., 2009; Lu et al., 2011; Lu et al., 2015). To determine if the salivary protein NlEG1 influences the production of these phytohormones and thus modulates defense responses in rice, we investigated the levels of SA, JA and JA-Ile in rice after the plant was either infested by fifth-instar BPH nymphs whose ability to produce NlEG1 had been silenced or treated with the recombination protein NlEG1. The results showed that BPH nymph feeding did not induce, or very weakly induced, the production of SA, JA and JA-Ile from 8 to 48h after feeding (Fig. 7A-C). Silencing NlEG1 did not alter the effect of BPH nymph feeding on SA, JA, and JA-Ile levels in plants: levels of these signals in all of the three treatments -- feeding by nymphs, by nymphs with injected dsRNA of GFP or by nymphs with knocked-down NlEG1 -- and in controls were similar (Fig. 7A-C). However, compared to plants that were treated with wounding plus the purified elution products from the empty vector, plants treated with wounding plus the application of the recombination protein NlEG1 showed slightly higher levels of JA (8h and 24h after treatment) and JA-Ile (24h after treatment) but not SA (Fig. 7D-F). These findings indicate that the NlEG1 secreted by BPH nymphs during feeding did not elicit JA- and JA-Ile-mediated defense responses in rice, although the recombination protein NlEG1 did slightly induce the production of these
signals in plants that had been mechanically wounded.

**DISCUSSION**

Our experiments demonstrate that NlEG1 acts as an herbivore effector that enables BPH to overcome the defense of PCWs of rice plants. NlEG1 has endo-β-1,4-glucanase activity but no exoglucanase activity (Fig. 2, B and C) and is injected into rice during BPH feeding (Fig. 3A). Knocking down NlEG1, which significantly reduced the transcript and protein levels as well as enzyme activity of NlEG1 (Fig. 3B-D), caused BPH to spend more time in non-penetration and the pathway phase, and less time feeding on phloem (Fig. 5A); this in turn decreased the amount of food intake, nymph mass, survival rate and fecundity of BPH fed on rice (Figs. 4-6). By contrast, NlEG1 silencing did not affect the early ability of BPH to feed on artificial diet without cellulose and rigid PCWs, that is, for the first 6 days. These results suggest that NlEG1 aids phloem access through cell wall penetration by degrading PCW celluloses.

Endo-β-1,4-glucanases play an important role in degrading PCWs by randomly cleaving amorphous sites of cellulose chains. Thus far, endogenous endo-β-1,4-glucanases have been reported in 16 insect orders; most belong to the GHF 9 and are mainly expressed in the salivary glands and midguts (Calderón-Cortés et al., 2012). NlEG1 is also classified as a GHF 9 protein, and is most highly expressed in the midgut and salivary glands of BPH (Fig. 2E and Supplemental Fig. S1), an expression pattern that fits its biological functions well. Interestingly, NlEG1 was also found to be highly expressed in the fat bodies and ovaries of BPH (Fig. 2E), with highest expression levels in female adults (Fig. 2D). This suggests that NlEG1 may have other biological functions. NlEG1 produced in the ovaries may, for instance, be secreted into the plants by the ovipositor of the female adult to soften plant tissues and assist egg deposition; such was found to be the case in *Deraeocoris nebulosus*, which uses salivary pectinases to soften plant materials before oviposition (Boyd et al., 2002). Our silencing experiments also illustrate that the gene is directly required for egg production in the ovaries (Fig. 4, B and C). NlEG1 expression in the fat body might be related to the detoxification of plant defense chemicals, as has been reported for some PCW-degrading enzymes in insects (Calderón-Cortés et al., 2012). Further research will
be necessary to elucidate these roles in \textit{NIEG1}.

Using the \textit{P. pastoris} expression system, we obtained the purified recombinant NIEG1 with an estimated relative molecular mass of about 60 kDa by SDS-PAGE (Fig. 2A); this observed mass was about 10 kDa bigger than the predicted mass of mature NIEG1, 49.6 kDa. This inconsistency can be explained by the 36 additional amino acids from the expression vector (which caused the mass of the recombinant protein to increase to 53.9 kDa) and the mobility retardation due to the His-tag consisting of six histidine residues in the recombinant NIEG1. That the molecular mass of a His-tag fusion protein determined by SDS-PAGE is greater than expected has been reported by many researchers (Niu and Guiltinan, 1994; Qiu et al., 2010; Yin et al., 2013; Wang et al., 2016); and the His-tag is known to decrease the mobility of a His-tag fusion protein (Tang et al., 2000). In a specific enzyme activity assay, we found that NIEG1 acted hydrolytically on CMC, filter paper and rice cellulose, but not on crystalline cellulose, glucose polymers with β-1,3 or β-1,6 linkages and xylose polymers, suggesting a similar substrate specificity of NIEG1 to insect GHF9 endo-β-1,4-glucanases, such as rCfEG5 and rCfEG3a from \textit{Coptotermes formosanus} (Zhang et al., 2009; Zhang et al., 2011).

Using CMC as a substrate, the $K_{m}$ value of NIEG1 (6.72 mg/ml) was similar to the values of other purified GHF9 enzymes from termites – for example, rCfEG5 (2 mg/ml), rCfEG3a (4.67 mg/ml) and RsEG (15 mg/ml) from \textit{Reticulitermes speratus}; however, the $V_{\text{max}}$ of NIEG1 (9.91 U/mg) was much lower than the $V_{\text{max}}$ of the three enzymes stated as above (548, 590 and 89 U/mg, respectively) (Ni et al., 2010; Zhang et al., 2011). This difference may reflect the various efficiency levels among insect species in degrading celluloses (Oppert et al., 2010; Zhang et al., 2011). As a piercing-sucking herbivore, BPH might not have to possess cellulase activity at levels comparable to those of wood-feeding herbivores, such as termites. We also investigated the optimal temperature and pH condition of NIEG1. The result showed that the pH optimum of NIEG1 was 6, which was consistent with the pH optimum of cellulases reported in most insects that had an optimal pH between 4 and 6 (Tokuda et al., 1997; Willis et al., 2011). The optimal temperature of NIEG1 was 37°C, near the lower limit value of the range of the optimal temperature (37-65°C) reported in insect endo-β-1,4-glucanases (Tokuda et
This difference may reflect the adaptation of NlEG1 to the environmental conditions in which BPH lives.

It has been reported that cellulases and/or their degraded cell wall fragments, such as oligosaccharides, can induce plant defense responses (Martinez et al., 2001). Similarly, here we found that wounding plus the application of the recombination protein NlEG1 elicited slightly higher levels of JA and JA-Ile in rice than did wounding plus the addition of the purified elution products from the empty vector (Fig. 7D-F). However, levels of JA, JA-Ile and SA in plants infested by BPH nymphs whose NlEG1 had been knocked down were similar to levels in plants infested by control BPH nymphs and in control plants (Fig. 7A-C), suggesting that NlEG1 secreted by BPH did not induce these signal-mediated defense responses in rice. This discrepancy is probably related to the feeding behavior of BPH, namely, its use of a stylet to penetrate intercellular spaces, allowing it to suck phloem sap (Seo et al., 2009); this behavior causes little tissue damage and circumvents plant defenses. Moreover, the salivary sheath and some components of the watery saliva secreted and/or formed during the piercing-sucking insect feeding can also reduce the chance that plants will produce defensive responses (Miles, 1968; Miles, 1999). These reasons may also explain why BPH nymph feeding had little induction on the production of JA, JA-Ile and SA in rice (Fig. 7A-C). Whether NlEG1 elicits the biosynthesis of other defense-related signals, such as ethylene, and thus also acts as a plant defense elicitor needs to be elucidated. However, our present experiments show that the benefit of NlEG1 as an effector outweighs its potential costs as an elicitor.

**CONCLUSIONS**

In summary, our study shows that NlEG1, a salivary endo-β-1,4-glucanase of BPH, is an effector that enables BPH to feed on rice by degrading PCW celluloses and, simultaneously, to circumvent JA- and JA-Ile-mediated defense responses in rice. The finding reveals the molecular basis of how piercing-sucking insects overcome PCW-based resistance traits in plants and provides a plausible mechanism that helps to explain the extraordinary success and impact of BPH as a rice pest since the green revolution started in the 1960s; that revolution resulted in extensive plantations of
semi-dwarf rice varieties whose GA pathways had been impaired, varieties that have lower levels of lignin and cellulose than those with normal GA pathways (Okuno et al., 2014; Huang et al., 2015).

MATERIALS AND METHODS

Plant growth and insect rearing

Mudgo, a rice variety containing the resistance gene Bph1, was used for experiments. Plants were grown as described by Lu et al. (2011), and 35- to 40-day-old plants individually planted in 500-ml hydroponic plastic pots were used. Colonies of BPH were originally provided by the Chinese National Rice Research Institute (Hangzhou, China), and maintained on Mudgo at 27 ± 1°C and 70 ± 10% relative humidity under a 14/10 h light/dark photoperiod.

Cloning, sequence alignment and phylogenetic analysis of NIEG1

The full-length cDNA of NIEG1 was obtained by RT-PCR from total RNA isolated from salivary glands of adult BPH females. The primers (Supplemental Table S1) were designed based on the consensus sequence of endo-β-1,4-glucanase genes in insects and transcriptome data of BPH salivary glands and whole bodies (Xue et al., 2010; Ji et al., 2013). PCR-amplified fragments were cloned into the pMD19-T vector (TaKaRa, Otsu, Japan) and sequenced. The NIEG1 sequence was translated and analyzed with the compute pl/MW tool to predict the isoelectric point (pl) and molecular weight of the predicted protein (http://expasy.org/tools). NetOGlyc 4.0 (http://www.cbs.dtu.dk/services/NetOGlyc/) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc) were used to predict O- and N-glycosylation sites, respectively. Predictions of signal peptide and transmembrane domain were made using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), respectively. Amino acid sequences of insect endo-β-1,4-glucanase sequences downloaded from NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) were aligned using ClustalX2. Phylogenetic relationships were determined using MEGA 5.0 with the neighbor-joining method.

RNA preparation and quantitative real-time PCR (qRT-PCR)
Total RNA was extracted from the following materials: 1) whole bodies of BPH at different developmental stages (from first- to fifth-instar nymphs, newly emerged brachypterous male and female adults); 2) specific tissue of BPH: salivary glands, thorax muscles, integuments, midguts, ovaries, legs, and fat bodies that had been dissected from newly emerged brachypterous female adults. Total RNA was isolated using the SV Total RNA Isolation System (Promega, Fitchburg, MA, USA), according to the manufacturer’s instructions. One microgram of each total RNA sample was reverse-transcribed using the PrimeScript RT–PCR Kit (TaKaRa). The qRT-PCR reactions were performed on a CFX96™ Real-Time System (Bio-Rad, Hercules, USA) using SsoFast™ probes supermix (Bio-Rad). A linear standard curve, threshold cycle number versus log (designated transcript level), was constructed using a series dilutions of a specific cDNA standard and the relative levels of the transcript of the target gene in all unknown samples were determined according to the standard curve. A BPH actin gene (GenBank: EU179848) was used as an internal standard to normalize cDNA concentrations. The primers and probes used for qRT-PCR for all tested genes are provided in Supplemental Table S1. Three to four independent biological replicates were analyzed in each experiment.

**Expression of NIEG1 in Pichia pastoris**

The full-length ORF of NIEG1 was PCR-amplified using a pair of primers (Supplemental Table S1) and cloned into the Pichia expression vector pPICZα A (Invitrogen, Carlsbad, CA, USA). The recombinant vector NIEG1:pPICZα A and empty vector pPICZα A (as a control) were transformed into Pichia pastoris strain KM71 (Invitrogen). Expression was induced by adding 100% methanol to a final concentration of 1%. The expressed products from the empty vector and recombinant vector were purified by using Ni-NTA columns and following the instructions of Ni-NTA Superflow Cartridge Handbook (Qiagen, Valencia, USA), and the purified products were concentrated with a YM-10 Microcon centrifugal filter device (Millipore, Billerica, MA, USA) to remove imidazole. The final purified concentrated products from P. pastoris cells with the empty vector and recombinant vector were mixed with 2×SDS loading buffer, respectively, separated by SDS/PAGE on a 12% gradient gel, and stained with
0.025% Coomassie blue R-250 in water. The predicted mass of the mature recombinant protein NlEG1, including 6 C-terminal His-tags, is 53.9 kDa.

**Polyclonal antibody preparation and western blot analysis**

Based on the Optimum Antigen™ design tool, a polypeptide (WRGDSSLNDRGLKGC) of NlEG1 was selected as the antigen to produce the rabbit polyclonal antibodies, and the polyclonal antibodies were purified by GenScript™ (GenScript, Nanjing, China). The following protein samples used for western blot analysis were prepared: 1) Proteins extracted from salivary glands of BPH. The salivary glands of one hundred newly emerged adult females were collected and homogenized in 1 ml PBS. The extract was centrifuged at 12,000 × g for 5 min at 4 °C, and the supernatant was collected as samples. 2) Proteins from rice leaf sheaths, infested by BPH or not. Rice stems were individually confined within glass cylinders (diameter 4.00 cm, height 8.00 cm, with 48 small holes, diameter 0.800 mm) in which 200 fourth- or fifth-instar nymphs were released, and 24 h later, the herbivore was removed. Plants in empty glass cylinders were used as controls. For each rice stem, the outer three leaf sheaths were harvested, and the entire leaf sheaths (0.900 g) from 3 rice stems were merged and homogenized in 4 ml PBS in liquid nitrogen. The extract was centrifuged at 12,000 × g for 5 min at 4°C, and the supernatant was collected and concentrated to 200 µL by using a YM-3 Microcon centrifugal filter device (Millipore, Billerica, MA, USA). SDS/PAGE loading buffer (2 × ) was added to samples, and these samples were then subjected to SDS/PAGE on a 12% gradient gel and transferred onto a PVDF membrane. Nonspecific binding sites were blocked with 5% instant nonfat dry milk, and membranes were incubated with the purified polyclonal antibody. The antigen–antibody complexes were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (Multisciences, Hangzhou, China) at a dilution of 1:5,000 at 37°C for 1h followed by extensive washing for 30 minutes with frequent changes of TBST and detected by FluorChem FC2 (Alpha Innotech, San Leandro, CA, USA).

**Endo-β-1,4-glucanase activity assay**

The endo-β-1,4-glucanase activity of the protein samples was determined, using CMC as a substrate, by a reducing sugar releasing assay as described previously with some
modifications (Li et al., 1998). Briefly, 400 μL of 1.00% CMC-Na solution in 50.0 mM sodium acetate buffer (pH 6.0) mixed with 15 μL of the sample was incubated at certain temperatures (see below) for 30 min. We first used this method to investigate the optimal pH and temperature condition of NIEG1 produced from *P. pastoris*. For the optimum temperature, the enzyme activity was measured at 28°C, 37°C and 50°C at pH 6.0, and for the optimum pH, the activity was determined at 37°C at pH 5.0, 6.0 and 7.0 (adjusted by 50.0 mM sodium acetate buffer solution). Based on these experimental data, we found that NIEG1 has its highest activity at 37°C at pH 6.0. Thus, we investigated the enzyme activity of the proteins extracted from newly emerged adult females 3 days after injection at 37°C at pH 6.0. For the extraction of BPH proteins, newly emerged BPH female adults were homogenized in 50.0 mM sodium acetate buffer (pH 6.0) on ice, and then the solution was centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant (proteins) was collected as samples. Endo-β-1,4-glucanase activity was defined as units per mg of protein, where one unit of enzyme activity was defined as the amount of enzyme that produced 1µmol of reducing sugars (glucose equivalent) per min. The concentration of the proteins was measured in triplicate by using Bio-Rad Bradford Protein Assay (Bio-Rad).

**Specific enzymatic activity and kinetics of NIEG1**

Using the reducing sugar assay, the specific enzyme activity of NIEG1 was measured. Six substrates were used in the assay, including Whatman No. 1 filter paper (Whatman Ltd, Maidstone, UK), cellulose extracted from rice plants using the method as described by (Rosa et al., 2012), Avicel (PH101-type crystalline cellulose; Sigma-Aldrich, St Louis, MO, USA), curdlan (β-1,3-glucan; Sigma-Aldrich), laminarin (β-1,3;β-1,6-glucan; Sigma-Aldrich) and xylan (poly β-1,4-xylopyranose; Sigma-Aldrich). An aliquot of 400 μL of 1% substrate solution in 50 mM sodium acetate buffer (pH 6.0) was mixed with 10 μL NIEG1 (500 ng), and then the mixture was incubated for 1h at 37°C.

The enzyme kinetics of NIEG1 was measured using serial concentrations of CMC (from 1 to 10 mg/ml) in 50.0 mM sodium acetate buffer (pH 6.0). An aliquot of 20 μL of NIEG1 (1000 ng) was added into 400 μL of CMC solution, and then the mixture was
incubated for 5 min at 37°C. Lineweaver–Burk plots were drawn using Microsoft Excel 2010, and $K_m$ and $V_{max}$ were determined.

**RNAi experiment**

A 215 bp fragment of *NlEG1* and a 657 bp fragment of control gene *GFP* were amplified by RT-PCR with primers including a T7 promoter sequence (Supplemental Table S1). The PCR products were used to synthesize dsRNA *in vitro* by using the MEGAscript RNAi kit (Ambion, Austin, TX, USA). Third- or fifth-instar nymphs (see details in different experiments) were injected as described previously (Liu et al., 2010). Each nymph was injected with about 0.100 μg dsRNA of *NlEG1* or *GFP* (control), or not injected (control). To determine the efficiency of gene silencing after dsRNA injection, the levels of *NIEG1* transcripts in the whole body, salivary gland, midgut, ovary, and fat body of the insect that had been injected with *NIEG1* or *GFP* dsRNA, or not injected, were investigated at 2, 4, 6, and 8 days after injection.

**BPH bioassays**

To measure survival rates of BPH, third-instar nymphs injected with *NlEG1* or *GFP* dsRNA, or kept non-injected, were allowed to feed on rice plants or artificial diet. The treated insects were first kept on rice seedlings at 27 ± 1°C with 70 ± 10% RH and a 14:10 h (light/dark) photoperiod to recover for 1 day, and then the healthy ones were used for the following bioassay: Stems of rice plants (one plant per pot) were individually confined within glass cylinders as stated above into which 15 third-instar BPH nymphs were released. In the artificial diet experiment, 15 third-instar BPH nymphs were introduced into individual feeding chambers (9 cm long and 2 cm in diameter) as described previously (Fu et al., 2001). The number of surviving BPH nymphs in each cylinder or feeding chamber was recorded every day. The survival rates of each BPH treatment and the corrected survival rates of BPH nymphs with injected *NIEG1* dsRNA, using BPH nymphs with injected *GFP* dsRNA as controls, on rice or artificial diet were calculated. The experiment was repeated four times.

To investigate the influence of *NIEG1*-knockdown on the growth phenotype of BPH nymphs, third-instar nymphs injected with *NlEG1* or *GFP* dsRNA were allowed to feed on rice plants. Six days later, the body length and pronotum width of BPH nymphs
(using MshotDigital Imaging System) as well as nymph mass (to an accuracy of 0.1 mg) were measured. Photographs of nymphs were also taken under a light stereomicroscope (Olympus SZX7, Tokyo, Japan). The measurement for body length and pronotum width was repeated 11 times. The mass measurement was replicated five times; each time the total mass of 20 nymphs was measured and the average individual mass was calculated.

To assess the effect of the knock-down of \textit{NlEG1} on BPH feeding, a brachypterous female adult at 3 (newly emerged) and 5 days after the injection of \textit{NlEG1} or \textit{GFP} dsRNA, or no injection (fifth-instar nymphs were injected), was placed into a small parafilm bag (6.00×5.00 cm), which was then fixed on the stem of a rice plant. The amount of honeydew excreted by a female adult was weighed (to an accuracy of 0.100 mg) at 24 h after the start of the experiment. The experiment was replicated 15 times.

The effect of \textit{NlEG1}-knockdown on the fecundity of BPH female adults was also investigated. Stems of rice plants (one plant per pot) were individually confined within the glass cylinders into which were released one newly emerged BPH female adult, 2 days after the injection of \textit{NlEG1} or \textit{GFP} dsRNA or no injection (fifth-instar nymphs were injected), and one newly emerged BPH male adult without treatment. Eleven days later, the insect was removed and the number of eggs laid by female adults in each rice plant was counted under a microscope. The number of eggs in the ovary of each female adult from the three treatment groups, at 3, 6, and 9 days after eclosion, was also counted under a microscope. The experiment was repeated 11-15 times.

\textbf{EPG recording of BPH feeding behavior}

The feeding behavior of BPH was recorded on a direct current-EPG system (Wageningen Agricultural University, Wageningen, The Netherlands). The method was the same as described by Cao et al. (2013). All experiments were carried out at 26±1℃ and 70±10% RH under continuous light conditions. The feeding behavior of individual newly emerged adult females, 4 days after the injection of \textit{NlEG1} or \textit{GFP} dsRNA, or not injected (fifth-instar nymphs were injected), on rice was monitored for 6 h. For each treatment (group), 15-19 replications were recorded. The signals recorded were analyzed using the PROBE V. 3.4 software (Wageningen Agricultural University). The output signals from EPG recordings were classified into five typical waveforms.
associated with the stylet penetration behavior of BPH (Seo et al., 2009; Cao et al., 2013), including NP for non-penetration, PP (N1+ N2 + N3) for the pathway phase (including penetration initiation, salivation and stylet movement, and extracellular activity near the phloem), N4-a for an intracellular activity in the phloem, N4-b for phloem sap ingestion, and N5 for the xylem phase (Fig. 5A). The durations of each sequential waveform event for each insect were measured, and the average waveform duration per insect (in minutes) for each waveform was calculated for each treatment (Cao et al., 2013). Another variable used in this experiment was the duration of N4-a’, N4-b’, and N5’, meaning the duration excluding the numerical value of zero in N4-a, N4-b, and N5, respectively.

JA, JA-Ile, and SA Analysis

Potted plants (one per pot) were randomly assigned to the following treatments: 1) infestation by different BPH nymph groups. Plant stems were individually confined in glass cylinders into which 25 fifth-instar nymphs that had been injected with NiEG1 or GFP dsRNA, or kept non-injected for 3 days, were released. 2) NiEG1 treatment. Plants were individually pierced 200 times on the lower part of the stems (about 2 cm) with a #00 insect pin and treated with 20µL of either the recombinant protein NiEG1 (32.7 ng/µL), the purified products of the empty vector (Vector), or water (W), or they were kept non-manipulated (control). The outer three leaf sheaths of stems were harvested at different time points (see details in Fig. 7) after the start of the treatment. Samples were ground in liquid nitrogen, and SA, JA, and JA-Ile were extracted with ethyl acetate spiked with labeled internal standards (2D4-SA, 2D6-JA, and 2D6-JA-Ile), then analyzed with HPLC/mass spectrometry/mass spectrometry system following the method as described in Lu et al. (2015).

Data analysis

Differences in BPH performance, expression levels of the gene and JA, JA-Ile and SA levels between treatments were determined by analysis of variance (ANOVA) (Student’s t-tests for comparing two treatments). All tests were carried out with Statistica (Statistica, SAS, Institute Inc., Cary, NC, USA, http://www.sas.com/).

Supplemental Materials
The following supplemental materials are available.

**Supplemental Figure S1.** Protein alignment of GHF 9 enzymes from insects.

**Supplemental Figure S2.** Phylogenetic tree for amino acid sequences of NlEG1 and reported insect endogenous endo-β-1,4-glucanases.

**Supplemental Figure S3.** The growth phenotype of BPH nymphs 6 days after they had been injected with either GFP (left) or NlEG1 (right) dsRNA. Representative photographs are shown.

**Supplemental Table S1.** Primers and probes used for qRT-PCR and PCR.

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**Figure legends**

**Figure 1.** Nucleotide sequence of NlEG1 and its deduced amino acid sequence.

The arrow indicates the signal peptide cleavage site. Predicted N-glycosylation and O-glycosylation sites are underlined in black and highlighted in gray, respectively. Square region denotes proton–donor catalytic region. The catalytic nucleophile, probable secondary nucleophile, and proton acceptor (Glu) of the conserved catalytic domain for GHF 9 members are represented as black diamond, gray diamond, and black circle regions, respectively. Two GHF 9 signature motifs are underlined in bold gray.

**Figure 2.** Molecular characterization of NlEG1.

A, Expression and purification of NlEG1. Samples for western blot (lanes 1 and 2) and SDS-PAGE analysis (lanes 3-6) were as follows: concentrated supernatant from Pichia pastoris with the empty vector pPICZα A (lanes 1 and 4; control); concentrated supernatant from P. pastoris with the recombinant vector NlEG1:pPICZα A (lanes 2 and 3); purified recombinant protein NlEG1 (lane 5); protein maker (lane 6). The black
Figure 3. NlEG1 in rice and its silencing efficiency by RNAi.
A, Detection of protein NlEG1 in rice infested by BPH nymphs. Protein samples for western blot analysis were as follows: the extract from the salivary glands (lane 1); the extracts from rice plants that were infested by nymphs (lane 2) or kept non-infested (lane 3). The black arrow represents the target band.

B and C, Mean transcript levels (+SE, n = 3) of NlEG1 in whole bodies on different days (B) and in different tissues of newly emerged brachypterous female adults 3 days after they (fifth-instar nymphs) had been injected with dsRNA of NlEG1 (dsNlEG1) or GFP (dsGFP), or kept non-injected (control). Sg, salivary gland; Mg, midgut; Ov, ovary; Fb, fat body.

D, Mean endo-β-1,4-glucanase activities (+SE, n = 4) in the whole body 3 days after the insects received the same treatments as above. Insert: western blot analysis for NlEG1 in the salivary glands of BPH received the same treatments as above. Letters indicate significant differences among different treatments (p<0.05, Duncan’s multiple range test).

Figure 4. Growth phenotypes of BPH nymphs that were injected with dsRNA of NlEG1 or GFP, or kept non-injected.
A and B, Mean body length and pronotum width (+SE, n=11; A) as well as individual mass (+SE, n=5; B) of BPH nymphs 6 days after they had been injected with dsRNA of NlEG1 (dsNlEG1) or GFP (dsGFP). Asterisks indicate significant differences between treatments (*, p < 0.05; Student’s t-test).
C, Mean number of eggs (+SE, n=11-15) in the ovary of a female adult that had been injected with dsRNA of \textit{NlEG1} (\textit{dsNlEG1}) or \textit{GFP} (\textit{dsGFP}), or kept non-injected (control) at the fifth-instar nymph stage. Letters indicate significant differences among different treatments (p<0.05, Duncan’s multiple range test).

D, Photographs of ovaries of female adults at 3 and 6 days after eclosion that received the same treatments as in C, showing that knocking down \textit{NlEG1} reduces the number of eggs in the ovaries of female adults but does not result in deformities. The bar represents 500µm.

\textbf{Figure 5}. Knocking down \textit{NlEG1} reduces the feeding and fecundity of female BPH adults.

A, Overall typical view of EPG waveforms generated by the feeding behavior of BPH on rice (upper panel) and mean duration (+SE, n=15-19) spent at different feeding phases of female adults (under panel) that had been injected with dsRNA of \textit{NlEG1} (\textit{dsNlEG1}) or \textit{GFP} (\textit{dsGFP}), or kept non-injected (control) at the fifth-instar nymph stage. NP: non-penetration; PP: the pathway phase (N1+ N2 + N3), including penetration initiation (N1), salivation and stylet movement (N2), and extracellular activity near the phloem (N3); N4-a: intracellular activity in the phloem region; N4-b: phloem sap ingestion; N5: the xylem phase. N4-a’, N4-b’, and N5’ indicate mean duration (+SE, n=4-19), excluding the numerical value of zero in N4-a, N4-b, and N5 respectively. EPGs were recorded for 6h per insect.

B, Mean amount of honeydew per day (+SE, n=15) secreted by a female BPH adult that received the same treatments as above. FA, female adult.

C, A gravid female adult of BPH on rice, showing its feeding, excreted honeydew, and laying eggs.

D, Mean number of eggs (+SE, n=11-15) laid by a female adult on plants that received the same treatments as above. Letters indicate significant differences among different treatments (p<0.05, Duncan’s multiple range test).

\textbf{Figure 6}. Knocking down \textit{NlEG1} decreases survival rates among BPH nymphs.

A and B, Mean survival rates (+SE, n=4) of BPH nymphs which had been injected with
dsRNA of *NIEG1* (*dsNIEG1*) or *GFP* (*dsGFP*), or kept non-injected (control) at third-instar nymph stage, feeding on rice (A) or AD (artificial diet, B). Letters indicate significant differences among different treatments (p<0.05, Duncan’s multiple range test).

C, Mean corrected survival rates (+SE, *n*=4) of BPH nymphs with injected *NIEG1* dsRNA, using BPH nymphs with injected *GFP* dsRNA as controls, feeding on rice or AD. Asterisks indicate significant differences between treatments (**, p < 0.01; Student’s *t*-test).

**Figure 7.** NIEG1 secreted by BPH doesn’t affect levels of SA, JA, and JA-Ile in rice.

A, B, and C, Mean levels (+SE, *n*= 5) of SA (A), JA (B), and JA-Ile (C) in rice plants at 8 and 24h after they were kept non-manipulated (control) or were individually infested by 25 fifth-instar nymphs, which had been injected 3 days earlier with dsRNA of *NIEG1* (*dsNIEG1*) or *GFP* (*dsGFP*), or kept non-injected (BPH). Insert: mean levels (+SE, *n*= 5) of SA, JA, and JA-Ile in rice plants 48h after they received same treatments as stated above. This experiment was not done at the same time as did the above experiment.

D, E, and F, Mean levels (+SE, *n*= 5) of SA (D), JA (E) and JA-Ile (F) in rice plants at 3, 8 and 24h after they were kept non-manipulated (control) or were individually treated with wounding plus 20μl of either the purified recombinant protein NIEG1 (NIEG1), the purified products of the empty vector (Vector), or water (W).

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**LITERATURE CITED**


Backus EA, Andrews KB, Shugart HJ, Greve LC, Labavitch JM, Alhaddad H (2012) Salivary enzymes are injected into xylem by the glassy-winged sharpshooter, a vector of *Xylella fastidiosa*. J Insect Physiol 58: 949-959


characterization of a glycosyl hydrolase family 9 cellulase distributed throughout the digestive tract of the cricket *Teleogryllus emma*. Comp Biochem Physiol B 150: 368-376


antifreeze protein His-MpAFP149 from the desert beetle (Microdera punctipennis dzungarica) in
Escherichia coli. Mol Biol Rep 37: 1725-1732
Rosa SML, Rehman N, Miranda MIGD, Nachtigall SMB, Bica CID (2012) Chlorine-free extraction
of cellulose from rice husk and whisker isolation. Carbohydr Polym 87: 1131-1138
Pests and Diseases. Int J Mol Sci 14: 6960-6980
61: 373-394
the actual positions of the stylet tips of Nilaparvata lugens in rice tissue. J Asia-Pacific Entomol
12: 89-95
Entomol Zool 3: 13-25
multiple insect herbivores: from community to genes. Plant Biol 65: 689
weight of His-tag fusion proteins by SDS-PAGE. Acta Photophysiological Sinica 26: 64-68
termite, Nasutitermes takasagoensis (Shiraki): distribution of cellulases and properties of
endo-β-1, 4-glucanase. Zool Sci 14: 83-93
JM (2015) Armet is an effector protein mediating aphid-plant interactions. FASEB J 29:
2032-2045
qdvp001: genome sequence and endolysin with a modular structure. Arch Virol: 1-8
saliva. Proc Natl Acad Sci USA 104: 10536-10541
Willis JD, Oppert B, Oppert C, Klingeman WE, Jurat-Fuentes JL (2011) Identification, cloning, and
expression of a GHF9 cellulase from Tribolium castaneum (Coleoptera: Tenebrionidae). J Insect
Physiol 57: 300-306
Rev Genet 44: 1-24
Transcriptome analysis of the brown planthopper Nilaparvata lugens. PLoS One 5: e14233
preparation, and characterization of a functional iron-related transcription factor IRO2 from
Malus xiaojinensis. Plant Physiol Biochem 67C: 63-70
Biol 41: 211-218
C-terminal tagged-form cellulase derived from Coptotermes formosanus and expressed in E. coli.
Insect Biochem Mol Biol 39: 516-522
susceptible to chewing herbivores, but enhances resistance to a phloem feeder. Plant J 60: 638-648
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Rosa SML, Rehman N, Miranda MIGD, Nachtigall SMB, Bica CID (2012) Chlorine-free extraction of cellulose from rice husk and whisker isolation. Carbohydr Polym 87: 1131-1138

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