Secretome Analysis and In Planta Expression of Salivary Proteins Identify Candidate Effectors from the Brown Planthopper *Nilaparvata lugens*

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The brown planthopper (BPH), *Nilaparvata lugens* (Stål), is a phloem sap-feeding insect. During feeding on rice plants, BPH secretes salivary proteins with potential effector functions, which may play a critical role in the plant–insect interactions. However, a limited number of BPH effector proteins have been identified to date. Here, we sequenced the salivary gland transcriptomes of five BPH populations and subsequently established a *N. lugens* secretome consisting of 1,140 protein-encoding genes. Secretome analysis revealed the presence of both conserved and rapidly evolving salivary proteins. A screen for potential effectors that elicit responses in the plant was performed via the transient expression analysis of 64 BPH salivary proteins in *Nicotiana benthamiana* leaves and rice protoplasts. The salivary proteins NI12, NI16, NI28, and NI43 induced cell death, whereas NI40 induced chlorosis and NI32 induced a dwarf phenotype in *N. benthamiana*, indicating effector properties of these proteins. Ectopic expression of the six salivary proteins in *N. benthamiana* upregulated expression of defense-related genes and callose deposition. Tissue expression analysis showed a higher expression level of the six candidate effectors in salivary glands than in other tissues. Subcellular localization and analysis of the domain required for cell death showed a diverse structure of the six effectors. NI28, NI40, and NI43 are *N. lugens* specific; in contrast, NI12, NI16, and NI32 are conserved among insects. The NI40 family has numerous isoforms produced by alternative splicing, exemplifying rapid evolution and expansion of effector proteins in the BPH. Our results suggest a potential large effector repertoire in BPH and a higher level of effector conservation exist in BPH compared with that in plant pathogens.

Nilaparvata lugens (Stål), commonly known as the brown planthopper (BPH), is a major rice pest that has coevolved with

rice for approximately 0.25 million years (Sezer and Butlin 1998). Serious damage of BPH to rice can result in “hopper burn”. During feeding on rice, BPH use their stylets to puncture the plant epidermis, followed by probing and penetration of the plant cell wall to access the phloem sap (Wang et al. 2008). During this process, BPH secretes both gelling and watery saliva from their salivary glands into plant cells. Gelling saliva quickly solidifies following secretion and forms a continuous salivary sheath in the plant that provides mechanical stability, lubrication, and protection against plant defense chemicals for the insect, whereas watery saliva contains digestive, hydrolyzing, and cell-wall-degrading enzymes (Miles 1999). The saliva of herbivorous insects plays a crucial role in their interaction with plants, and determines the compatibility between feeding insects and host plants (Miles 1999). Broadly, salivary proteins and other molecules secreted into plant cells by insects that can alter host structures and functions are defined as effectors (Hogenhout et al. 2009). Many sap-feeding insects cause extensive losses in modern-day agriculture. Only recently has the characterization of effectors from sap-feeding insects been a research focus, and studies have primarily focused on aphids and the Hessian fly. Several insect effectors have been identified in the past few years (Jaouannet et al. 2014; Kaloshian and Walling 2016; Mutti et al. 2008; Naessens et al. 2015; Rodriguez et al. 2014; Wang et al. 2015a; Zhang et al. 2015), providing insights into the mechanisms underlying plant–insect interactions.

To overcome plant defense responses, plant parasites such as bacteria, fungi, oomycetes, nematodes, and insects secrete a repertoire of effectors into plant cells. In total, 758 secreted proteins were predicted in the genome of the fungal pathogen *Ascochyta rabiei*. Secretome analysis identified several secreted proteins with numerous tandem repeats, which provides insights into the necrotrophic nature of fungal phytopathogens (Verma et al. 2016). A bioinformatics approach identified 486 secreted proteins in the fungal pathogen *Sclerotinia sclerotiorum*, and further analysis of the secretome revealed 78 secreted proteins associated with positive selection, recent gene duplication, *S. sclerotiorum*-specific effectors, and effector candidates associated with broad-host-range necrotrophy (Guyon et al. 2014). In insects, a dual transcriptomic and proteomic approach identified 324 secreted proteins in the aphid *Acyrtosiphon pisum*. Furthermore, it was demonstrated that multiple-copy genes in *A. pisum* genome are under positive selection and many secreted proteins appear to evolve at a faster rate compared with that of proteins homologous to those in other insects (Carolan et al. 2011).

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Many pathogen effectors may evolve rapidly, thus exhibiting a high level of sequence polymorphism and species specificity (Guyon et al. 2014; Raffaele et al. 2010). However, insects, as multicellular eukaryotes at an evolutionary level much higher than that of bacteria, fungi, and oomycetes, have a lower evolutionary rate than the latter. Several effector genes identified in insects display high-level sequence conservation among different species, despite their different function in their respective host plants. The first salivary protein characterized to suppress host defenses was glucose oxidase (GOX) (Musser et al. 2002), which is conserved among many caterpillar species and sap-feeding insects such as aphids (Eichenseer et al. 2010; Harmel et al. 2008). A comparative analysis of the salivary proteins in blood- and plant-feeding insects revealed a number of common types such as apyrases, calreticulins, and peroxiredoxins that interfere with early host defense signaling (Guiguet et al. 2016). Recent studies have identified several conserved effectors in aphids. Many vertebrate parasites, including protozoa, nematodes, and ticks, secrete macrophage migration inhibitory factor (MIF) to modulate host defense responses (Augustijn et al. 2007; Bowen et al. 2010; Vermeire et al. 2008). MIF secreted by a plant-parasitic aphid inhibits plant defense responses and is crucial for the survival and feeding of the aphid on the host plant (Naessens et al. 2015). Armet, a conserved protein in many animal species, has been detected in aphid watery saliva and mediates aphid-plant interactions. Armet facilitates pea aphid feeding on bean plants (Wang et al. 2015a). Angiotensin-converting enzymes (ACEs), which are conserved in animals, are highly expressed in the *A. pisum* salivary glands. Simultaneous knockdown of *ACE1* and *ACE2* confers a higher mortality rate than the control group in aphids feeding on plants, indicating that *ACE1* and *ACE2* function together to modulate aphid-plant interactions (Wang et al. 2015b). Mp10, a chemosensory protein (CSP) in the aphid *Myzus persicae*, induces chlorosis and triggers defense responses in *Nicotiana benthamiana* (Bos et al. 2010; Rodriguez et al. 2014). Furthermore, the CSP family displays a notable level of conservation in insects.

Past studies have reported the identification of either 202 or 107 BPH salivary proteins via mass spectrometric analysis of saliva collected in vitro (Huang et al. 2016; Liu et al. 2016). However, only a few BPH effectors have been identified (Huang et al. 2017; Ji et al. 2017; Shangguan et al. 2018; Ye et al. 2017). Pathogen recognition in plants is often accompanied by the hypersensitive response, a form of programmed cell death. Transient expression of secreted proteins in *N. benthamiana* combined with cell death assays has been developed as a convenient tool to identify candidate pathogen effectors (Chen et al. 2018; Kettles et al. 2017; Wang et al. 2011). In this study, we characterized the BPH secretome containing 1,140 potential secreted proteins. In total, 64 salivary proteins were analyzed using *N. benthamiana* cell death assays. Six were identified as candidate effector proteins and subsequently applied to function analysis. Particular attention was paid to the differences between insect effectors and pathogen effectors, including subcellular localization, sequence, and structure features. Our results indicate that the effector repertoire in BPH is large and that many BPH effectors are conserved proteins.

RESULTS

Sequencing of the BPH salivary gland transcriptome and prediction of secreted proteins.

The cDNA libraries produced using the salivary glands of five BPH populations, which were reared on either plants of the rice varieties TN1 (TN1 population), Mudgo (M population),

ASD7 (A population), and YHY15 (Y population) or *Leersia* (L population) plants, were sequenced using an Illumina platform, which generated a total of 40,001,367, 10,0591,728, 100,249,608, 40,000,103, and 98,534,746 reads, respectively. Sequences were assembled using SOAPdenovo software (Li et al. 2009), resulting in 31,645, 34,245, 25,535, 41,676, and 29,119 unigenes, respectively. Finally, sequence data from the five libraries were combined and assembled into 41,883 unigenes. All the raw transcriptome sequencing data were deposited in the SRA database (National Center for Biotechnology Information [NCBI]) under accession numbers SRX532392 (TN1 population), SRX2882231 (M population), SRX2882276 (A population), SRX2882277 (Y population), and SRX2882278 (L population).

Secreted proteins are likely deposited in the saliva through the eukaryotic endoplasmic reticulum-Golgi pathway (Cherqui and Tjallingii 2000). Therefore, all amino acid sequences were assigned to predict secretory signal peptides using SignalP 4.1 (Petersen et al. 2011), and 1,315 unigenes were predicted to encode proteins containing a secretory signal peptide. Finally, following the removal of 175 unigenes containing a transmembrane domain in addition to the signal peptide, 1,140 potential secreted proteins were isolated, forming a *Nilaparvata lugens* secretome (Supplementary Table S1). The size of the *N. lugens* secretome reported here is considerably larger than that previously predicted (Ji et al. 2013), and resembles the numbers of secreted proteins reported for the spider mites *Tetranychus urticae* (1,493) and *T. evansi* (1,121) (Villarroel et al. 2016).

Past studies have reported 83 *N. lugens* salivary proteins that contain a signal peptide by mass spectrometry (Huang et al. 2016; Liu et al. 2016). Following the removal of redundant proteins, we obtained a dataset of 65 salivary proteins with signal peptide. We used these 65 salivary proteins to BLAST against the *N. lugens* secretome (1,140) with a cut-off E-value of 10^{-5} , which resulted in 61 sequence matches, indicating that this secretome is of high quality.

Characterization of the BPH secretome.

Rapid effector evolution and extensive expansion of specific effector families has been demonstrated in the genome of the oomycete *Phytophthora infestans* (Haas et al. 2009). Therefore, we clustered proteins identified in the BPH secretome into protein families using orthoMCL (Li et al. 2003). In total, 746 proteins were clustered into 174 protein families with more than 2 proteins (Supplementary Table S2). The largest protein family consisted of 25 proteins without annotation. Annexin proteins, previously identified in the saliva of BPH (Huang et al. 2016), constituted the second-largest protein family. Furthermore, particular attention was paid to large protein families which have highly expressed members. With the application of this condition, groups 3, 6, 19, 31, 70, and 97 of the 174 protein families were selected for further investigation.

Insects share a number of salivary proteins that interfere with early host defense signaling (Guiguet et al. 2016). To identify insect-shared salivary proteins in BPH, we used the *N. lugens* secretome (1,140 proteins) to search the now-published secretome of two insect species, *T. urticae* secretome (1,493 proteins) (Villarroel et al. 2016) and *A. pisum* secretome (324 proteins) (Bos et al. 2010; Carolan et al. 2011), using the BLAST tool with an E-value of 10^{-5} , which obtained 245 and 229 homologous proteins, respectively (Supplementary Table S3). In order to identify *N. lugens*-specific salivary proteins, we used the *N. lugens* secretome to search the Nr, Swiss-Prot database and the transcriptomes of six insect species (*A. pisum*, *Tribolium castaneum*, *Drosophila melanogaster*, *Nasonia vitripennis*, *Manduca sexta*, and *Locusta migratoria*) representing

six different insect orders. As a result, 461 proteins (40.44%) could not match proteins of other species in these databases and were considered to be *Nilaparvata lugens*-specific proteins, and the rest 679 (59.56%) were regarded as conserved proteins.

BPH secretes both watery and gelling saliva, and the latter solidifies to form a feeding sheath. Recently, 10 (NCBI accession numbers BAP87097.1 to BAP87106.1) and 13 (Huang et al. 2016) gelling saliva proteins with signal peptides from BPH were identified. After removing repetitive proteins, 20 gelling saliva proteins were finally obtained (Supplementary Table S4). Among these, 19 were identified in our *N. lugens* secretome. Comparative analysis showed that 30 of the top 50 highly expressed genes in our secretome are gelling saliva proteins, indicating that many salivary sheath proteins are highly expressed in salivary glands. Furthermore, 13 of the 20 feeding sheath proteins shared no similarity with proteins from other organisms contained in the NCBI database.

Amino acid tandem repeats exist in many eukaryotic proteins (Huntley and Golding 2000). Although the function of amino acid tandem repeats remains unclear, natural selection drives the accumulation of amino acid tandem repeats by modulating repeat size and number (Mularoni et al. 2010). Amino acid tandem repeats were identified in the *N. lugens* secretome, with 254 proteins (22.24%) containing at least two amino acid tandem repeats of two residues or more (Supplementary Table S5). The presence of amino acid tandem repeats was much higher in the highly expressed proteins than in the entire secretome. Among the 20 highly expressed salivary sheath proteins, 9 contain amino acid tandem repeats, typically are mucin-like protein (accession number BAP87097.1) and vitellogenin (accession number BAP87098.1), in which tandem repeat sequences are abundant. Furthermore, when the secreted proteins were ranked according to gene expression level (T_{FPKM}), 42 genes in the top 100 highly expressed genes were found to contain amino acid tandem repeats. This result suggests the rapid evolution of the highly expressed salivary proteins in *N. lugens*.

Most fungal effector proteins identified to date are small secreted cysteine-rich proteins (SSCPs), which are small in size (<200 amino acids [aa]) and have a high cysteine content (>2%) (Saunders et al. 2012; Stergiopoulos and de Wit 2009). The cysteines in SSCP are often involved in the formation of disulfide bonds, which stabilize protein structures (Saunders et al. 2012). SSCP are recognized as a common source of fungal effectors that trigger defense responses in host plants. Thus, we characterized the cysteine content of all *N. lugens* secretome proteins and identified 432 (37.89%) cysteine-rich proteins for which 2% or more of amino acid residues are cysteine.

Identification of BPH candidate effectors.

Previous studies have shown that many pathogen effector proteins can induce cell death in nonhost plants (Wroblewski et al. 2009). To identify candidate BPH effectors, we used transient expression of BPH salivary proteins in *Nicotiana benthamiana* via agroinfiltration to screen for those salivary proteins that induce cell death.

Candidate effectors were identified in the *Nilaparvata lugens* secretome using selection criteria: high expression level in salivary glands, member of a large protein family (i.e., 13 genes from groups 3, 6, 19, 31, 70, and 97 were selected), homolog of known effectors in other organisms, and *N. lugens*-specific genes. The cDNAs of 64 salivary proteins were successfully cloned from BPH biotype I (Supplementary Table S6). Because salivary proteins may be secreted in both the plant cell and apoplast, for each of these genes, two plasmids were constructed: one contained an open reading frame with signal

peptide (ORF+SP) and the other contained a truncated open reading frame without signal peptide (ORF-SP).

Transient expression assays showed that 6 of the 64 salivary proteins (namely, NI12, NI16, NI28, NI32, NI40, and NI43) induced cell death, chlorosis, or a dwarf phenotype in *Nicotiana benthamiana* (Fig. 1A), indicating that these proteins may function as effectors that are recognized by the plant immune system (Wang et al. 2011). NI12, NI16, and NI28 induced cell death in *N. benthamiana* only when expressed without a signal peptide (Fig. 1A). In contrast, NI40 induced chlorosis and NI43 induced cell death in *N. benthamiana* when expressed with a signal peptide (Fig. 1A). Interestingly, NI32 expressed both with and without a signal peptide induced a dwarf phenotype in *N. benthamiana* (Fig. 1A). Immunoblot analysis confirmed the expression of these proteins in *N. benthamiana* (Supplementary Fig. S1).

To further confirm candidate BPH effector functionality, the six proteins described above were coexpressed with the luciferase reporter protein in rice protoplasts to determine whether they induced cell death in rice (Chen et al. 2006). Compared with that in the green fluorescent protein (GFP) control, expression of NI12, NI28, and NI43 caused a significant reduction in luciferase activity whereas NI16 caused a slight reduction

Fig. 1. Brown planthopper (BPH) candidate effectors induce cell death in *Nicotiana benthamiana* and rice protoplasts. **A**, Analysis of BPH effector functionality in *N. benthamiana*. Six candidate BPH effectors were transiently expressed in *N. benthamiana* leaves by agroinfiltration with PVX vectors alongside green fluorescent protein (GFP) that was included as a control. Pathology symptoms were documented with photos taken 7 days postinfiltration (DPI) for NI12, NI16, NI28, NI40, and NI43 and 15 DPI for NI32. **B**, Analysis of BPH effector functionality in rice protoplasts. Six candidate BPH effectors and GFP were coexpressed with the luciferase reporter protein in rice protoplasts. Values are mean ($n = 3$) relative luciferase activity in tissue expressing a candidate effector normalized against tissue expressing GFP, the latter of which was set to one. Cell death induced by candidate effectors was reflected by a reduction in relative luciferase activity. Error bars show $\pm$ standard deviation and asterisks (**) significant difference, $P < 0.01$ (Student's t test). Luciferase activity was measured using a luciferase assay system (Promega Corp.) 40 h after transformation.

(Fig. 1B), confirming that these proteins induce cell death in rice protoplasts. Transient expression of NI32 and NI40 did not affect luciferase activity (Fig. 1B).

Tissue expression profiles of BPH candidate effectors.

Some salivary proteins of BPH may be exclusively expressed in the salivary glands. To investigate the expression patterns of the six candidate effectors in BPH, different tissues, including salivary glands, midguts, fat bodies, and the remaining parts, were dissected from BPH adults and were used for real-time quantitative polymerase chain reaction (RT-qPCR) analysis. NI12 and NI16 were mainly expressed in the salivary glands but low expression levels were also detected in other tissues (Fig. 2A and B). NI28 was detected at high expression levels in both the salivary glands and midguts (Fig. 2C). As for NI32, transcripts were extensively detected in all test tissues but were highest in the salivary glands (Fig. 2D). NI40 and NI43 were exclusively expressed in the salivary glands and almost undetectable in other tissues (Fig. 2E and F).

BPH candidate effectors activate plant defense responses.

To analyze whether the six cell death-inducing proteins NI12, NI16, NI28, NI32, NI40, and NI43 affected the plant immune system, we transiently expressed these proteins in *N. benthamiana* leaves and then analyzed defense-related gene expression and callose deposition.

The relative expression of the salicylic acid (SA)-related marker genes *pathogenesis related 1* (*PR1*) and *PR2* and the jasmonic acid (JA)-related marker genes *PR3* and *PR4* were determined using RT-PCR analysis performed 2 days post-infiltration (DPI) (Keller et al. 1996; Maimbo et al. 2010).

Fig. 2. Expression analysis of brown planthopper (BPH) candidate effectors. Relative expression levels of BPH candidate effectors in different tissues were normalized against β -actin gene expression. The remaining parts dissected from BPH was used as reference. Error bars denote $\pm$ standard deviation of three repeats. **A**, NI12; **B**, NI16; **C**, NI28; **D**, NI32; **E**, NI40; and **F**, NI43. Different letters above the bars indicate significant differences, $P < 0.05$ (Tukey's honestly significant difference test). SG = salivary glands, Mg = midguts, FB = fat bodies, and Others = the remaining parts.

Surprisingly, we found that expression of NI12 induced approximately 9- and 59-fold expression upregulation of the JA-related marker genes *PR3* and *PR4*, respectively, and NI16 expression induced approximately 16-fold expression upregulation of SA-related marker genes *PR1* and *PR2* (Fig. 3A). Moreover each SA- and JA-related marker gene displayed significant expression upregulation following NI32 expression whereas NI28 caused significant and simultaneous expression upregulation of *PR1*, *PR2*, and *PR4* (Fig. 3A). In contrast, the remaining BPH candidate effectors NI40 and NI43 caused a slight reduction of both SA- and JA-related marker genes expression (Fig. 3A).

Callose deposition is a hallmark of plant defense responses and is typically triggered by pathogen-associated molecular patterns (PAMPs) (Luna et al. 2011). Aside from PAMPs, pathogen effectors and injury caused by insects can trigger callose depositions (Kuśnierczyk et al. 2008; Naessens et al. 2015; Voigt 2014). Callose deposition assays were performed in *N. benthamiana* leaves, as described previously (Dietrich et al. 1994; Naessens et al. 2015). We observed that NI16, NI28, NI32, and NI40 expression caused enhanced callose deposition at 2 DPI (Fig. 3B). Furthermore, rapid and strong callose deposition was detected at 2 DPI for NI12 and NI43 (Fig. 3B).

Subcellular localization of BPH candidate effectors in plant cells.

During feeding on rice, BPH penetrates the plant tissue with the stylets and delivers salivary proteins into plant cells (Wang et al. 2008). Identifying the subcellular localization of effector proteins inside the plant cell can provide information about their mode of action (Downen et al. 2009; Schornack et al. 2010).

To determine the subcellular localization of the six BPH candidate effectors in the plant cell, the signal peptide in each of the six proteins was removed; then, C-terminal fusion proteins with the yellow fluorescent protein (YFP) were created and expressed in rice protoplasts. A strong fluorescence signal for NI12-YFP, NI16-YFP, and NI28-YFP was observed in both the cytoplasm and the nucleus (Fig. 4). NI32-YFP was primarily localized in the nucleus, with slightly fluorescence signal observed also in the cytoplasm (Fig. 4). The observed localization pattern of NI32 differed from that of the homolog effector Mp10 in *Myzus persicae*, which is localized in the cytoplasm when expressed in *N. benthamiana* (Rodriguez et al. 2014). Fluorescence observed for NI40-YFP and NI43-YFP indicated that they were both localized in the nucleus (Fig. 4).

We also investigated the subcellular localization of the six BPH candidate effector-YFP fusion proteins in *N. benthamiana*, with results similar to those observed in rice protoplasts, except that NI40-YFP was both nuclear and chloroplast localized in *N. benthamiana* and NI32 was only localized in the nucleus (Supplementary Fig. S2). From the above results, we concluded that the six BPH candidate effectors (NI12, NI16, NI28, NI32, NI40, and NI43) localized in the nucleus or nucleus and cytoplasm, similar to the subcellular localization pattern of oomycete RXLR (a conserved motif in oomycete effectors) effectors (Caillaud et al. 2012). However, in silico analysis showed that none of the six proteins carry a canonical nuclear localization signal. Western blot using GFP antibodies showed the expression of the effector-YFP fusion proteins (Supplementary Fig. S3).

Structural analysis of BPH candidate effectors required for cell death.

The structure of protein is closely related to its function. To identify the functional protein domains in the BPH candidate effectors required to induce plant responses, a series of effector deletion mutants were constructed and analyzed via cell death assays.

NI12 is a protein disulfide isomerase (PDI) and consists of the four thioredoxin-like domains a, a', b, and b'; an N-terminal signal peptide; and a C-terminal endoplasmic reticulum (ER) retention signal (Fig. 5A) (Ben Achour et al. 2002; Meng et al. 2015). Cell death assay results showed that NI12 induced cell death only when the signal peptide was truncated, indicating a secretion function of this protein in plant cells when it had a signal peptide and an ER retention signal simultaneously, whereas the ER retention signal KDEL was not required for NI12-induced plant symptoms (Fig. 5A). A minimum of three PDI domains were required for NI12 function, and the engineered versions of NI12 containing domain combinations a-b-b' or b-b'-a' were able to induce cell death in *N. benthamiana* (Fig. 5A). Thus, it appears that the domains a and a' share a similar role in NI12 function.

NI16 is an apolipoprotein-III composed of a signal peptide and an ApoLp-III-like domain that spans the majority of the protein (30 to 179 aa, 79%). The ApoLp-III-like domain alone was sufficient for NI16 function in *N. benthamiana* (Fig. 5B). Furthermore, the minimal functional sequence within the ApoLp-III-like domain was identified as 61 to 140 aa, which induced more extensive cell death compared with that resulting from full-length NI16 without its signal peptide (NI16-SP) (Fig. 5B).

NI28 is an SSCP. NI28 induced weak symptoms in *N. benthamiana* (Fig. 1A). However, cell death was significantly enhanced following the expression of NI28 with a C-terminal hemagglutinin (HA) tag fusion. Therefore, we used the NI28-HA in further experiments. The NI28 protein sequence contains no predicted domains, and therefore we created successive protein truncations reducing the amino acid sequence by 50 aa, 30 aa, and 10 aa. This narrowed the NI28 functional region to 101 to 167 aa located in the C-terminus (Fig. 5C).

NI32 is a CSP that contains an OS-D domain (insect pheromone-binding family) located in the middle of the amino acid sequence, which spans a large proportion of the protein (28 to 118 aa, approximately 70%). Compared with the a dwarf phenotype induced by NI32 (Fig. 1A), the OS-D domain C-

terminal truncated form (28 to 111 aa) had much weaker effects, and the OS-D domain N-terminal truncated form (38 to 118 aa) did not induce a plant response, indicating that the complete OS-D domain is required for NI32 function in *N. benthamiana* (Fig. 5D).

NI43 induced extensive cell death in *N. benthamiana* when expressed with its endogenous signal peptide (Fig. 5E). The NI43 protein sequence contains no predicted domains and, therefore, we divided the protein into an N-terminal and a C-terminal segment (1 to 140 and 141 to 244 aa, respectively), each with a signal peptide. Functional assessment of sequential 20-aa protein truncations showed that the minimal NI43 region required for the induction of cell death was the C-terminal 141 to 244 aa (Fig. 5E). We did not carry out the "deletion mutant" experiments for NI40, because chlorosis induced by NI40 is too weak and cannot induce cell death in rice protoplast.

Sequence analysis and homology search of the BPH candidate effectors.

Sequence analysis and homologs of the BPH candidate effectors in other organisms may provide some insight into the function of these proteins.

NI12. NI12 is a member of the PDI family, which is conserved in eukaryotic organisms. Through reciprocal BLAST analysis, 13 NIPDI family members were identified in the BPH transcriptome and 5 PDI family members were identified in the *Nilaparvata lugens* secretome (Supplementary Table S7). NI12 shares 37, 56, and 57% similarity with gene homologs in *P. parasitica* (PpPDI), *Oryza sativa*, and *Nicotiana benthamiana*, respectively. Sequence alignment showed that PDIs in insects, oomycetes, fungi, and plants were highly conserved. (Supplementary Fig. S4).

NI16. NI16 is an apolipoprotein-III protein. Apolipoprotein-III is a canonical apolipoprotein with an ApoLp-III-like domain, which is conserved in many insect species (Supplementary Fig. S5). Homology searches in our transcriptome revealed no homologous gene for NI16.

Fig. 3. Brown planthopper (BPH) candidate effectors activate plant defense responses. **A**, BPH candidate effectors affect the expression of defense-related genes in *Nicotiana benthamiana*. Following the transient expression of six candidate BPH effectors in *N. benthamiana*, relative expression of the salicylic acid- and jasmonic acid-related marker genes pathogenesis-related (PR) genes *PR1/PR2* and *PR3/PR4*, respectively, were measured by real-time polymerase chain reaction at 2 days postinfiltration. Values are mean ($n = 3$) relative expression in tissue expressing a candidate effector normalized against tissue expressing green fluorescent protein (GFP), the latter of which was set to one. Error bars denote $\pm$ standard deviation and ** indicates significant difference, $P < 0.01$ (Student's t test). **B**, BPH candidate effectors induce callose deposition in *N. benthamiana* leaves. Callose deposition was detected in detached leaves stained with aniline blue. GFP (left panel) was infiltrated as a control. Numbers indicate $\pm$ standard deviation of mean values ($n = 3$). For each experiment, callose deposition was quantified in a minimum of 10 randomly selected microscopy fields using ImageJ software. Scale bars = 100 μ m.

NI28. NI28 is an SSCP for which no homologous gene was identified in the NCBI database and BPH transcriptome. NI28 (NI28-1) has a particularly high cysteine content of 34%. Therefore, we selected an additional four SSCPs with a cysteine content higher than 15% (Supplementary Fig. S6) and observed cell-death symptoms following their transient expression in *N. benthamiana*, which revealed that three of these SSCPs induced a weak cell death.

NI32. CSPs, a family of small water-soluble proteins with an OS-D domain, are conserved among different insects (Pelosi et al. 2005). Eleven CSPs (NICSP1 to -11) have been previously identified in BPH (Zhou et al. 2015). We identified five CSPs (namely, NICSP1 [NI32], NICSP5, NICSP6, NICSP7, and NICSP8) in the *Nilaparvata lugens* secretome. Of these, NICSP1 (NI32) and NICSP5 were highly expressed in salivary glands.

NI40. NI40 is a *N. lugens*-specific salivary protein which has no homolog in other organisms. NI40 shares 80% similarity with a salivary protein (accession number KU365932) detected in both the watery and gelling saliva of BPH (Huang et al. 2016). NI40 was categorized into group 6 alongside 14 unigenes in our *N. lugens* secretome. To investigate the level of NI40 polymorphism in BPH, 100 NI40 cDNA sequences were obtained from 10 BPH individuals. Four ORF deletions were

detected and various combinations of these formed seven isoforms (Supplementary Data S1). We performed a BLAST search against the BPH genome (Xue et al. 2014) with the longest NI40 ORF version and identified that the NI40 genomic sequence (NLH13 scaffold689, accession number KN152627.1) is composed of 12 exons and 11 introns (Fig. 6A). The fidelity of the NI40 genomic sequence was verified by Sanger sequencing. Furthermore, we determined that the seven NI40 isoforms arise from alternative gene splicing (Fig. 6B), which is a widespread phenomenon in eukaryotes and promotes transcriptome diversification that can potentially lead to speciation (Ast 2004). In addition to NI40 (NI40-1), the homolog NI40-2 (Fig. 6C) located adjacent to NI40 in the BPH genome has 10 isoforms (Fig. 6D) that also arise from alternative gene splicing, bringing into being a new gene family.

NI43. NI43 is an uncharacterized protein that exhibits partial similarity to only one other protein (uncharacterized protein LOC105699590, *Orussus abietinus*, accession number XP_012280147.1) in the Nr database. In addition, NI43 shares 28% similarity with an alternative unigene (CL2572.Contig1_All) that also possesses a signal peptide and is unannotated in the transcriptome.

BPH candidate effectors of conserved families induced cell death with specificity.

Among the six candidate effectors, NI16 and NI32 are conserved in insects, NI12 is conserved in eukaryotes, and two (NI12 and NI32) belong to conserved protein families.

NI12 is a member of the *N. lugens* PDI (NIPDI) family. In total, 13 PDIs were identified in BPH and 5 of these presented in the *N. lugens* secretome. Thus, we examined the ability of the five NIPDI family members to induce cell death. The results showed that, whereas NI12-1 induced intense cell death in *Nicotiana benthamiana* at 2 DPI, the remaining four NIPDI family members did not induce any symptoms (Fig. 7A), indicating that the NI12-induced response is specific. Because NI12-2 was not detected in *N. benthamiana* (Supplementary Fig. S7), we could not rule out the possibility that the absence of cell death is due to the instability of this protein.

The widespread CSP families in insects have been characterized to varying extents. In a previous study, 11 CSPs were identified in BPH (NICSP1-11) (Zhou et al. 2015). To test the ability of these 11 CSPs to induce altered growth phenotype in *N. benthamiana*, we first examined the expression of these proteins in *N. benthamiana* and showed that all of them could be detected in *N. benthamiana*. Our results revealed that six CSPs (NICSP1, NICSP2, NICSP4, NICSP5, NICSP8, and NICSP11) induced effects in *N. benthamiana* similar to that caused by Mp10 (Fig. 7B). The remaining five CSPs did not induce a dwarf phenotype (Fig. 7B). A sequence alignment between 11 NICSPs and Mp10 was performed using clusterx, which revealed high-level similarity among the 12 proteins (Supplementary Fig. S8).

DISCUSSION

At present, transgene or gene knockout cannot be performed in BPH; therefore, the identification and functional analysis of effectors in BPH is not as straightforward as in plant pathogens. Nevertheless, the procedure described in this experiment proved to be effective in describing the secretome and screening for candidate effectors based on their ability to cause cell death in a nonhost plant (Fig. 8). In total, the *Nilaparvata lugens* salivary gland transcriptomes predicted 1,140 secreted salivary proteins that formed the BPH secretome. Out of the 64 tested salivary proteins, 6 were found to induce cell death, chlorosis, or dwarf phenotype. Four of them induced cell death

Fig. 4. Subcellular localization of brown planthopper (BPH) candidate effectors in rice protoplasts. C-terminal yellow fluorescent protein (YFP) fusion proteins were made for each of the six candidate BPH effectors lacking their endogenous signal peptides, which were then cotransformed with a cyan fluorescent protein (CFP)-tagged nuclear marker into rice protoplasts. YFP and CFP fluorescence signals were detected by confocal microscopy at 24 h after transformation. Scale bars = 5 μ m.

in rice protoplasts. In other similar studies, 11 of 169 *P. sojae*-secreted proteins, 7 of 95 nematode-secreted proteins, and 14 of 63 wheat pathogen-secreted proteins induced cell death or chlorosis in *Nicotiana benthamiana* (Chen et al. 2018; Kettles

et al. 2017; Wang et al. 2011). In addition, 5 of 42 *Magnaporthe oryzae*-secreted proteins were shown to induce cell death in rice protoplasts (Chen et al. 2013). The number of cell-death-inducing candidate effectors in BPH is high, similar to that of

Fig. 5. Structural analysis of brown planthopper (BPH) candidate effectors that elicit plant cell death. Deletion mutants of six candidate BPH effectors were constructed and transiently expressed in *Nicotiana benthamiana* leaves. Amino acids numbers for residues contained in each protein version are indicated on the left side of each panel, next to their corresponding sequence structure. The corresponding plant phenotypes induced by expression of each protein version are shown on the right of each panel. The predicted domains or motifs of candidate BPH effectors are represented by different shaded boxes underneath the panels. Plant phenotypic effects were documented with a representative photo taken at 7 days postinfiltration (DPI) for NI12, NI16, NI28, and NI43, and at 15 DPI for NI32. PDI = protein disulfide isomerase and ER = endoplasmic reticulum. Symbols +, +-, and - indicate obvious cell death or dwarf phenotype, weak cell death or dwarf phenotype, and no cell death or dwarf phenotype, respectively.

other plant pathogens. Based on secretome analysis and the high proportion of cell-death-inducing candidate effectors in BPH, we propose that there is a large effector repertoire in BPH. A large effector repertoire may simultaneously facilitate the adaptation of BPH to rice and production of resistance genes in rice, thus contributing to rice–BPH interactions and coevolution.

There are many conserved salivary proteins in the *Nilaparvata lugens* secretome. Of the 1,140 secreted proteins, 679 have homologs in other species. Comparative analysis revealed that 245 and 229 proteins in the *N. lugens* secretome showed homology with proteins in *A. pisum* and *Tetranychus urticae* secretome, respectively. Moreover, a number of effector proteins in other insects also display high-level conservation, such as Mp10, MIF, Armet, and ACE identified in aphids and GOX in caterpillar species. Here, among the six characterized BPH candidate effectors, NI12, NI16, and NI32 showed conservation among insects. The degree of effector conservation in BPH is higher than that observed for other plant pathogens. However, a number of salivary proteins may evolve quickly and are specific to *N. lugens*. Of the 1,140 secreted proteins, 461 were identified as *N. lugens*-specific proteins. Among the 100 highest expressed genes in the *N. lugens* secretome, many are salivary sheath proteins and possess numerous amino acid tandem repeats, indicating their high evolution rate and positive selection.

These highly expressed salivary proteins may play a crucial role in the rapid adaptation of BPH to rice. NI28, NI40, and NI43 are *N. lugens* specific and belong to these kind of genes.

We characterized the six identified BPH candidate effectors and compared their subcellular localization, sequence, and structure features with known pathogen effectors. Of the six candidate effectors, NI12, NI40, and NI43 were identified in BPH saliva by mass spectrometric analysis (Huang et al. 2016; Liu et al. 2016). All six candidate effectors showed a higher expression level in salivary glands than in other tissues. Two of the three *N. lugens*-specific genes, NI40 and NI43, were exclusively expressed in the salivary glands. In contrast, the three conserved genes NI12, NI16, and NI32 were more extensively expressed in different tissues. The six candidate effectors localized in the nucleus or nucleus and cytoplasm of plant cell. It should be noted that small proteins may diffuse into the nucleus and the actual localization of these candidate effectors when they are delivered into the plant cell by insects might be different from when they are artificially expressed in the plant cell. Sequence analysis showed that the six candidate BPH effectors have highly diverse structures. NI12, NI16, NI28, and NI32 induced cell death without signal peptide and located in the nucleus and cytoplasm of plant cells, whereas NI40 and NI43 induced chlorosis or cell death with the presence of a signal peptide. Among the six BPH candidate effectors, NI12, NI16,

Fig. 6. Splicing patterns of genes in the NI40 family. **A**, Genomic model of NI40-1 containing 12 exons and 11 introns. Different shaded boxes represent the alternatively spliced exons 3, 5, 6, and 9. **B**, NI40-1 isoforms 1 to 7, which represent the structure of the seven transcripts produced by alternative splicing of the four exons. White boxes indicate the positions of exons which are absent in alternatively spliced isoforms. Each exon is presented in a size in proportion to its sequence length. **C**, Genomic model of NI40-2 containing 14 exons and 13 introns. Different shaded boxes represent the seven alternatively spliced exons 3 to 9. **D**, NI40-2 isoforms 1 to 10, which represent the structure of the 10 transcripts produced by alternative splicing of the seven exons. White boxes indicate the positions of exons which are absent in alternatively spliced isoforms. Each exon is presented in a size in proportion to its sequence length.

and NI32 display protein conservation and two (NI12 and NI32) belong to large gene families. We did not identify an NI28 homolog in the NCBI database, and found NI28 to resemble SSCPs which are commonly fungal effectors. NI43 exhibits partial similarity to only one other protein in the Nr database. We did not identify homologs of the *NI40* family in other organisms; however, there are two *NI40* family members in the BPH genome and a variety of isoforms in the transcriptome arising from alternative splicing. As a multicellular eukaryote, alternative splicing is more prevalent in BPH compared with bacteria, fungi, and oomycetes (Ast 2004), thus increasing the diversity within the effector repertoire and contributing to the complex interaction between BPH and rice.

NI12 is a member of the PDI. In recent years, PDIs have been identified in the saliva of a number of insects such as ticks and BPH (Huang et al. 2016; Liao et al. 2007; Tirloni et al. 2015). Pathogen PDIs are important virulence factors that function during infection (Stolf et al. 2011). PpPDI1, the *P. parasitica* NI12 effector homolog, induces substantial cell death in *Nicotiana benthamiana*. In addition, overexpression of PpPDI1 enhances *P. parasitica* virulence during infection (Meng et al. 2015). NI16 is an apolipophorin-III protein. Apolipophorin-III

belongs to a large family of exchangeable apolipoproteins that play vital roles in lipid transport and lipoprotein metabolism (Wang et al. 2002). Recently, apolipophorin was identified in the saliva of rice leafhopper and aphids and in the salivary glands of planthoppers (Konishi et al. 2009; Nicholson et al. 2012; Vandermoten et al. 2014). Lipids and free fatty acids, which function as plant defense signaling compounds, may be potential targets for apolipophorin (Madey et al. 2002). As for NI32, recent findings have revealed novel functions of CSPs. In two *Helicoverpa* spp., CSP4 is uniquely and abundantly expressed in the proboscis and, once secreted, acts as a surfactant to reduce the surface tension of aqueous solutions, thus reducing the pressure of feeding (Liu et al. 2014). In *M. persicae*, the chemosensory protein Mp10 was shown to be a salivary effector protein that was delivered into the plant mesophyll cell cytosol during feeding, where it triggered plant defense responses, thus reducing aphid fecundity (Bos et al. 2010; Mugford et al. 2016; Rodriguez et al. 2014). Our findings suggest that NI12 and NI32 may share similar functions with their homologous effectors in *N. benthamiana* and that distinct sap-feeding insects may share a number of salivary proteins and common mechanisms to disarm plant defense systems.

Fig. 7. Members of two brown planthopper (BPH) candidate effector families induce cell death in a specific manner in *Nicotiana benthamiana*. **A**, Five members of the NIPDI family were transiently expressed in *N. benthamiana* leaves via agroinfiltration. A single member (NI12-1) of the NIPDI family induced cell death. **B**, In all, 11 members of NICSP family were transiently expressed in *N. benthamiana* leaves. Six members (NICSP1, NICSP2, NICSP4, NICSP5, NICSP8, and NICSP11) of the NICSP family induced a dwarf phenotype. Green fluorescent protein (GFP) was infiltrated as a control. The effect of each BPH candidate effector on plant phenotype was documented with a representative photo taken at 7 days postinfiltration (DPI) for the NIPDI family and at 15 DPI for the NICSP family.

We observed that BPH salivary proteins N112, N116, N128, and N143 induced cell death in rice protoplasts and *N. benthamiana* leaves, and immunity responses in *N. benthamiana*, reflecting some sort of conserved recognition by plant immune receptors. However, the exact function of these BPH candidate effectors and recognition of the mechanism in plants remain to be elucidated. Although these BPH candidate effectors may induce plant defense responses, these responses may be suppressed by other effectors, thus preventing any adverse impact on the BPH survival. Moreover, it should be noted that nonhost plants may react to effectors more frequently and intensely than host plants (Wroblewski et al. 2009) and, at present, there is no evidence that cell death occurs in the process of rice defense against BPH. The *Nilaparvata lugens* secretome described here provides a valuable platform to identify effectors that suppress rice defense systems and those that interact with rice resistance genes.

MATERIALS AND METHODS

Sequence analysis.

PDIs from the following species were obtained from GenBank: *P. parasitica* (XP_008914616.1), *M. oryzae* (XP_003710672.1), *Oryza sativa* (XP_015627045.1), *Nicotiana benthamiana* (XP_019255435.1), *A. pisum* (XP_008183165.1), and *Nilaparvata lugens* (ANJ04677.1). Apolipoprotein-III sequences from the following seven insect species were downloaded from the Swiss-Prot database: *Pararge aegeria* (S4PD79), *Epiphyas postvittana* (Q9GPN5), *Danaus plexippus*

(G6CIC8), *Apis cerana* (V9ILX0), *Dufourea novaeangliae* (A0A154PL18), and *Triatoma infestans* (A0A170YUN3). The transcriptomes of six insect species (*Acyrtosiphon pisum*, *Tribolium castaneum*, *Drosophila melanogaster*, *Nasonia vitripennis*, *Manduca sexta*, and *L. migratoria*) were obtained from Ensembl Genomes databases. Amino acid sequence alignment of the six PDIs and the 12 CSPs was performed using ClustalX 2.1 (Larkin et al. 2007).

Salivary gland collection and Illumina sequencing.

Salivary glands were dissected from BPH adult females on ice following a previously described procedure (Shangguan et al. 2018). Approximately 100 salivary glands for each of the five BPH populations were collected. Total RNA of salivary glands was isolated using the RNAiso Plus kit (TaKaRa) according to the manufacturer's protocol. The RNA samples were shipped on dry ice to Beijing Genomics Institute, and subsequently sequenced on the Illumina Hiseq 2000 platform.

Prediction of the BPH secretome and protein clustering.

The unigenes were aligned using BLASTX (E-value < 0.00001) to the protein databases Nr, Swiss-Prot, and KEGG. The BLAST results were used to extract coding sequences (CDSs) of unigenes. The CDSs of unigenes with no BLAST hits were predicted by ESTScan (Iseli et al. 1999). Signal peptide prediction was carried out using SignalP 4.1 (Petersen et al. 2011). Subsequently, transmembrane domains were predicted using THMM v2.0 (Krogh et al. 2001). Proteins

Fig. 8. Pipeline for the identification of brown planthopper (BPH) candidate effectors. **A**, Bioinformatics pipeline for the prediction of secreted proteins following salivary gland transcriptome sequencings. **B**, Secretome analysis for the identification of candidate effectors. **C**, Selection criteria applied to candidate effectors to identify most-likely functional effectors. **D**, Experimental methodology to verify the functionality of effectors.

that had a signal peptide and a 0-to-1 transmembrane domain (the transmembrane domain being in the signal peptide) were considered as secreted proteins. The secretome was then submitted to generate protein families using OrthoMCL v2.0 (Li et al. 2003) with standard parameters.

Cloning of candidate BPH effectors and plasmid construction.

To clone the candidate BPH effectors, primers were designed for the corresponding ORFs. Sequences were amplified from cDNA prepared from 10 whole insect bodies of BPH biotype I, then ligated into a pMD18-T vector. For construction of the PVX vectors, two fragment versions (ORF+SP and ORF-SP) were amplified and ligated into a pGR107 vector. For construction of the Gateway entry clones, primers were designed to amplify genes without a stop codon. PCR products were inserted into pDONR207 via BP reactions (Gateway BP clonase enzyme mix; Invitrogen), then cloned into the plant expression vectors pEarleyGate100 and pEarleyGate101 which contain the CaMV 35S promoter via LR reactions (Gateway LR clonase enzyme mix; Invitrogen). The resulting pEarleyGate101 constructs were used for subcellular localization of candidate effectors in rice protoplasts and *N. benthamiana*. The resulting pEarleyGate100 constructs were used for callose deposition assays, cell death assays of candidate effectors, and analysis of deletion mutants and effector families in *Nicotiana benthamiana*. For *NI32* and structural analysis of *NI28*, we used the PVX vector. Deletion mutants of *NI43* were produced by overlapping PCR. Details of constructs and primers (including primers used for expression analysis of defense-related genes) are listed in Supplementary Table S8.

Transient expression in *N. benthamiana*.

Constructs were introduced into *Agrobacterium tumefaciens* GV3101 by electroporation. Transformed GV3101 strains were grown at 28°C for 24 h. The cultured *Agrobacterium* cells were harvested by centrifugation, resuspended in infiltration buffer (10 mM MgCl₂, 10 mM morpholineethanesulfonic acid, and 150 mM acetosyringone) to an optical density at 600 nm of 0.6, and incubated at 28°C for 1 to 3 h before infiltration. *A. tumefaciens* cell suspensions were infiltrated using a needleless syringe into the leaves of *N. benthamiana* plants grown in a greenhouse for 4 to 6 weeks.

Protein extraction and immunoblot analysis.

At 48 h following agroinfiltration in *N. benthamiana* leaves, total protein extracts were prepared by homogenizing 400 mg of leaf tissue in 1 ml of radio immunoprecipitation assay buffer (Beyotime) in the presence of 0.1 mM protease inhibitor phenylmethylsulfonyl fluoride (Beyotime). Proteins samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. Immunoblot analysis was performed as previously described (Hu et al. 2011). Protein expression was detected using anti-HA tag antibodies.

Callose deposition assays in *N. benthamiana*.

Callose deposition assays in *N. benthamiana* were performed as previously described (Naessens et al. 2015), with some modifications. Leaf discs were mounted in 80% glycerol and observed under a laser-scanning confocal microscope (Olympus Fluoview FV1000). Images were analyzed using the ImageJ software.

RNA isolation and RT-qPCR.

Different tissues, including salivary glands, midguts, fat bodies, and the remaining parts, were dissected from BPH

adults under a stereomicroscope. For BPH RNA extraction, total RNA of whole insect bodies and different tissues was isolated using an RNAiso Plus kit (TaKaRa) according to the manufacturer's instructions. Total RNA was isolated from *N. benthamiana* leaves expressing various constructs using an EASYspin tissue/cell RNA rapid extraction kit (Yuanpinghao Biotech). cDNA was synthesized using a PrimeScript RT reagent kit (TaKaRa). For RT-qPCR assays, GFP was infiltrated as a control. Expression levels of target genes were normalized to the housekeeping gene β -actin. Relative expression ratios were calculated using the Pfaffl method (Pfaffl 2001). RT-qPCR was performed using a Bio-Rad CFX96 Real-Time System (Applied Biosystems).

Cell death assays and subcellular localization in rice protoplasts.

Transient expression in rice protoplasts was carried out as previously described (Chen et al. 2006). The firefly luciferase gene (LUC) was used as a reporter that reflected protoplast viability. Constructs of candidate effectors under the control of the maize ubiquitin promoter were cotransformed alongside the LUC gene into rice protoplasts by polyethylene-glycol-mediated transformation (Chen et al. 2006). Luciferase activity was detected 40 h following transformation using a luciferase assay system (Promega Corp.), and the reduction in LUC signal was compared with that in GFP-transformed protoplasts.

For subcellular localization, the pCXUN-effector-YFP constructs were cotransformed with the cyan fluorescent protein-nuclear marker bZIP63 (Walter et al. 2004) in rice protoplasts. At 16 to 24 h following transformation, protoplasts were observed using a laser-scanning confocal microscope (Olympus Fluoview FV1000).

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