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Rapid intracellular acidification is a plant defense response countered by the brown planthopper

Highlights

- Rapid intracellular acidification is a novel plant defense response
- Brown planthopper (BPH) delivers carbonic anhydrase (NICA) into rice for survival
- NICA counters intracellular acidification to suppress plant defense
- pH-responsive regulators of defense responses are predicted to exist in the rice-BPH interaction

Authors

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In brief

The brown planthopper (BPH) is the most devastating insect pest in rice. Jiang et al. discover that BPH secretes a salivary carbonic anhydrase (NICA) to regulate the intracellular pH of the rice cell to facilitate its feeding and survival on rice plants and that NICA counters host intracellular pH acidification to diminished defense responses.

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Rapid intracellular acidification is a plant defense response countered by the brown planthopper

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SUMMARY

The brown planthopper (BPH) is the most destructive insect pest in rice. Through a stylet, BPH secretes a plethora of salivary proteins into rice phloem cells as a crucial step of infestation. However, how various salivary proteins function in rice cells to promote insect infestation is poorly understood. Among them, one of the salivary proteins is predicted to be a carbonic anhydrase (*Nilaparvata lugens* carbonic anhydrase [NICA]). The survival rate of the *NICA*-RNA interference (RNAi) BPH insects was extremely low on rice, indicating a vital role of this salivary protein in BPH infestation. We generated *NICA* transgenic rice plants and found that *NICA* expressed in rice plants could restore the ability of *NICA*-RNAi BPH to survive on rice. Next, we produced rice plants expressing the ratiometric pH sensor pHusion and found that *NICA*-RNAi BPH induced rapid intracellular acidification of rice cells during feeding. Further analysis revealed that both *NICA*-RNAi BPH feeding and artificial lowering of intracellular pH activated plant defense responses and that NICA-mediated intracellular pH stabilization is linked to diminished defense responses, including reduced callose deposition at the phloem sieve plates and suppressed defense gene expression. Given the importance of pH homeostasis across the kingdoms of life, discovery of NICA-mediated intracellular pH modulation uncovered a new dimension in the interaction between plants and piercing/sucking insect pests. The crucial role of NICA for BPH infestation of rice suggests that NICA is a promising target for chemical or trans-kingdom RNAi-based inactivation for BPH control strategies in plants.

INTRODUCTION

The brown planthopper (BPH; *Nilaparvata lugens* Stål, Hemiptera, Delphacidae) is a monophagous insect pest of rice (*Oryza sativa* L.) found in all rice-growing Asian countries. The BPH sucks rice phloem sap via its stylet, causing leaf yellowing and wilting, stunted plant growth, reduced photosynthesis, and ultimately death of the rice plant.[1] During severe BPH outbreaks, tens of thousands of insects swarm on a rice field, resulting in the "hopperburn" phenomenon, which is characterized by large-scale wilting, yellowing, and lethal drying of rice plants.[2] Besides direct damage, the BPH may also indirectly damage rice plants by oviposition and transmitting viral disease agents.[3,4]

Application of chemical insecticides has been the main strategy for controlling BPH. Although it has the advantages of rapid killing of insects and low costs, use of insecticides leads to environmental pollution and resistance of the BPH to pesticides. In recent decades, breeding resistant rice varieties to control the BPH has attracted increasing attention. To date, more than 30 resistance genes have been found in the rice genome.[5] However, the BPH often quickly evolves new biological types that evade resistant rice genes. Therefore, additional methods of controlling the BPH need to be developed to complement the current control measures toward long-term solutions for achieving durable BPH resistance in rice. One method could be based on the disruption of key steps in the BPH's natural infestation process. However, development of such methods will require a

comprehensive understanding of the basic biology of BPH-rice interaction.

A critical step in the BPH's infestation of rice is the secreting of bioactive substances into the plant tissues through the stylet.[6–8] Specifically, during feeding, the BPH secretes both colloidal and watery saliva.[9,10] The main function of the colloidal saliva is to form a saliva sheath around the piercing-sucking mouthparts, stabilizing the overall feeding apparatus. The composition and function of watery saliva are more complex, and it contains salivary proteins that are believed, in most cases, to regulate various pathways in plant cells to enhance BPH feeding and survival in rice plants. For example, a salivary endo-β-1,4-glucanase (NIEG1) degrades plant celluloses to help the BPH's stylet reach the phloem[11]. NISEF1, an EF-hand (a motif that consists of an α-helix "E," a loop that may bind calcium, and a second α-helix "F") Ca^{2+}-binding protein, interferes with calcium signaling and H_2O_2 production during BPH feeding.[12] Salivary protein 7 is required for normal feeding behavior and for countering accumulation of a defense compound, tricin.[13] On the other hand, a BPH mucin-like protein (NIMLP) triggers defense responses in rice cells, including cell death, callose deposition, and upregulation of pathogen-responsive genes.[14,15]

Carbonic anhydrases (CAs) (Enzyme Commission 4.2.1.1) are zinc metalloenzymes that function as catalysts in the bidirectional conversion of CO_2 and water into bicarbonate and protons.[16] There are at least five distinct CA families (α-, β-, γ-, δ-, and ε-CAs), and three of them (α-, β-, and γ-CAs) are ubiquitously distributed among animal, plant, and bacterial species. The widespread distribution and adequate abundance of these CA families underline their evolutionary importance throughout the kingdoms of life. CAs participate in a wide range of biological processes, such as pH regulation, CO_2 homeostasis, stomatal aperture, and plant defense.[17–21] NICA belongs to the α-CA subfamily. Our previous study showed that *Nilaparvata lugens* carbonic anhydrase (*NlCA*) is expressed in BPH salivary glands.[6] Surprisingly, however, RNA interference (RNAi) of the *NlCA* transcript in BPH insects affects neither pH maintenance within the insect salivary gland, watery saliva or gut, nor insect feeding behavior or honeydew excretion on artificial diet, but greatly reduced survival of BPH on rice plants, suggesting a critical function *in planta* via an unknown mechanism.[6]

Here, we report that *NlCA*-RNAi BPH feeding results in rapid intracellular acidification of rice cells. We found that NICA is secreted into the rice tissues during BPH feeding and functions as an effector that stabilizes host cell intracellular pH, accompanied by suppression of defense responses. Thus, we have uncovered intracellular pH homeostasis as a previously uncharacterized battleground in plant-insect interactions.

RESULTS

NICA is detected in rice sheath tissues during BPH feeding

NICA was previously found to be highly expressed in salivary glands and present in the watery saliva of BPH fed on an artificial diet.[6] We conducted a more detailed characterization of *NlCA* expression in this study. RNA *in situ* hybridization showed that the expression level of *NlCA* was detectable throughout the principal glands (PGs) and accessory glands (AGs), but not in A-follicle of the PG (APG) (Figure 1A), which further raised the possibility that NICA may be one of the "effector proteins" secreted into the rice tissue during BPH feeding on rice plants. To test this possibility, we compared protein profiles in the leaf sheaths of Nipponbare rice plants before and after BPH feeding using liquid chromatography-mass spectrometry (LC-MS). We found 8 NICA-specific peptides in BPH-fed leaf sheath tissue (Figure 1B; Table S1), confirming that NICA is secreted into the host tissues during BPH feeding. As BPH is a phloem-feeding insect, its effector proteins, such as NlCA, presumably act in the phloem. We therefore collected the phloem exudate from Nipponbare rice leaf sheaths, with or without BPH feeding, for LC-MS analysis (Figure 1C). Six NlCA-specific peptides were detected in the phloem exudate of Nipponbare plants that had been fed on by BPH (Figure 1B), demonstrating that NICA is delivered into phloem by BPH.

Transgenic expression of *NlCA* in rice rescues the ability of *NlCA*-silenced BPH to feed and survive

To further clarify the site of function (i.e., in insect vs. in plant) of NICA in the BPH-rice interaction, we produced transgenic Nipponbare plants expressing *NlCA* (see STAR Methods). A total of 26 lines were produced, and 6 lines were found to robustly express the *NlCA* transcript (Figure S1A). *NlCA*-expressing plants exhibited no noticeable changes in appearance compared with Nipponbare plants (Figures S1B–S1D). *NlCA*-expressing plant lines were propagated to T3 generation, and three lines were subjected to further characterization, including BPH feeding. For BPH feeding assay, double-stranded RNA (dsRNA) of *NlCA* (ds*NlCA*) or the control green fluorescent protein gene (ds*GFP*) was injected into 3rd instar BPH nymphs to initiate RNAi of the *NlCA* transcript.[22] Quantitative real-time PCR analysis confirmed that the transcript levels of the *NlCA* gene were reduced by 99% and 97%, respectively, in tested individuals when compared with non-RNAi control and ds*GFP*-treated insects (Figure 2A). There were no significant differences in the survival rates between ds*GFP* and ds*NlCA* BPH when fed on the artificial diet, indicating that silencing *NlCA* expression has no obvious impact on the basic physiology of BPH (Figure 2B). However, we found that the survival rate of ds*NlCA* BPH was sharply decreased, starting at day 9 post infestation, to ~40% at day 14, whereas the control ds*GFP* BPH survived normally on wild-type (WT) Nipponbare plants (Figure 2C). This result is consistent with previous results conducted in the *japonica* cultivar Xiushui134 rice plants,[6] suggesting that the requirement of NICA for BPH survival is not specific to a specific rice genotype. Strikingly, *NlCA* transgenic plants almost fully restored the survival of *NlCA*-silenced BPH insects (Figure 2C; Figures S1E and S1F), demonstrating that *Nl*CA expressed in the host tissue (Figures 2D and 2E) can complement the infestation defect of *NlCA*-silenced BPH insects.

The conserved catalytic site amino acids of *Nl*CA are critical to its function in rice

NICA contains several conserved amino acid residues predicted to be at the catalytic site of CAs (Figure 2F). We asked whether some of these conserved active site residues are critical to the function of *Nl*CA in rice-BPH interaction. Accordingly, we expressed three different *NlCA* mutants in Nipponbare

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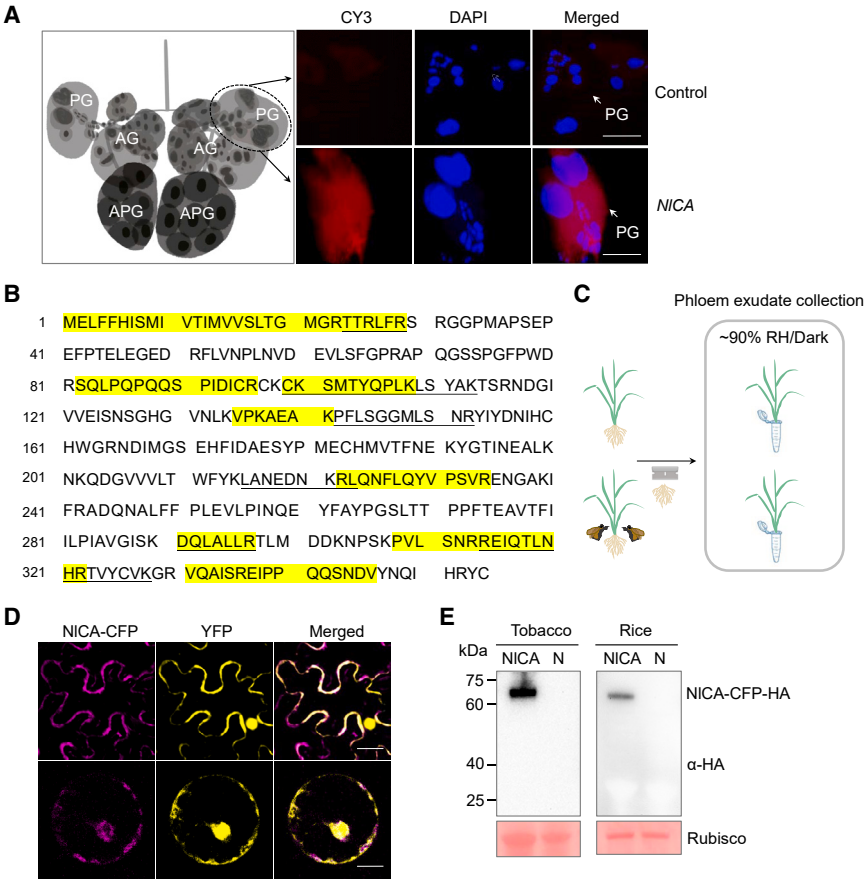

Figure 1. Initial characterization of *Nilaparvata lugens* carbonic anhydrase (NICA)

(A) *In situ* RNA hybridization of salivary glands in 5-instar BPHs. Left, a schematic diagram of BPH salivary glands, including the principal glands (PGs), the accessory glands (AGs), and the A-follicle of the principle gland (APG). Right, *NICA* expression was detectable in the PG (red) using antisense *NICA* sequence as a probe. Sense *NICA* probe was used as a negative control. Nuclei are stained blue by 4′,6-diamidino-2-phenylindole (DAPI). Scale bar, 50 μm.

(B) The amino acid sequence of NICA. The highlighted amino acid residues indicate the peptides detected in BPH-infested rice sheath tissue by LC-MS analysis. The underlined amino acid residues indicate the peptides detected in phloem exudate of BPH-infested rice by LC-MS analysis.

(C) A schematic diagram of the phloem exudate collection.

(D) NICA is colocalized with the YFP signals in *N. benthamiana* (upper row; scale bar, 10 μm) and rice cells (lower row; scale bar, 5 μm). YFP and NICA-CFP fusion proteins were co-expressed in *N. benthamiana* leaf cells for 48 h using the *Agrobacterium*-mediated transient expression method. YFP and NICA-CFP fusion proteins were co-expressed in rice protoplasts 16 h after the corresponding DNA constructs were introduced into rice protoplasts via polyethylene glycol-mediated transformation. Experiments were repeated three times with similar trends.

(E) NICA-CFP-hemagglutinin (HA) fusion protein levels are detected with anti-HA (Zenbio, 301113) in *N. benthamiana* leaves and rice protoplasts. Protein samples were extracted from NICA-CFP-HA-expressing *N. benthamiana* and rice protoplasts. Proteins from mock *N. benthamiana* leaves and rice protoplasts were as negative controls (N). Ponceau S staining of Rubisco shows protein loading control. See also Figure S5 and Table S1.

plants (Figures S1A and S1G). "*NICA-m1*" plants contain a *NICA* transgene in which His^{159} and His^{161} were mutated to Ala; "*NICA-m2*" plants contain a *NICA* transgene in which Glu^{182} and His^{184} were replaced by Ala; and "*NICA-m3*" plants carry a *NICA* transgene in which all seven conserved active site residues, His^{159}, His^{161}, Glu^{171}, Glu^{182}, His^{184}, Thr^{269}, and Phe^{279}, were changed to Ala. Like *NICA* transgenic plants, transgenic plants expressing these *NICA* mutants exhibited no noticeable changes in appearance (Figures S1B–S1D). We conducted BPH survival assay and found that in contrast to plants expressing WT *NICA*, the *NICA-m2* and *NICA-m3* plants could not fully rescue the infestation defect of *NICA*-silenced BPH insects. The *NICA-m1* plants, on the other hand, could recover the infestation defect of ds*NICA* BPH (Figures 2G and 2H; Figure S2). Thus, the conservative residues Glu182 and His184 and possibly some other residues at the predicted catalytic site are indispensable for the function of *NICA* inside the plant cell.

After showing the requirement of catalytic site residues for the function of NICA in rice, we next investigated the subcellular localization of NICA in both *Nicotiana benthamiana* leaf cells and rice leaf protoplasts transiently expressing cyan fluorescent protein (CFP) fused NICA:CFP. In both cases, NICA:CFP showed a nonuniform distribution of CFP signal in the cell. Further colocalization studies with YFP signal revealed that NICA was predominantly localized in the cytoplasm (Figure 1D). There was some nuclear localization of NICA-CFP in rice protoplasts. Intact NICA:CFP proteins were detected in these localization experiments (Figure 1E).

NICA counters rapid intracellular acidification during BPH feeding on rice

BPH feeding on rice plants can be divided into two phases, based on electropenetrography (EPG) waveforms.[23] The first phase involves BPH's stylet penetrating the plant through the cell walls and cell membranes of various epidermal and mesophyll cells until the stylet reaches the phloem sieve cells inside the vascular system. In Nipponbare, the time to reach the phloem sieve cells can be around 1.2 h before sustained phloem sap ingestion occurs at 3.8 h.[23] Our finding of the site of NICA action in the plant prompted us to test the hypothesis that NICA might modulate rice cell pH changes during BPH feeding. To directly test this hypothesis, we constructed a rice transgenic line expressing a cytoplasmic ratiometric pH sensor driven by the 35S promoter, cyto-pHusion. Cyto-pHusion consists of the tandem concatenation of enhanced green fluorescent protein (EGFP) and a monomeric red fluorescent protein 1 (mRFP1).[24] EGFP is highly sensitive to pH variation, with the brightest EGFP signal emitted at a pH of 7–8. EGFP fluorescence is gradually quenched at lower pH values and totally quenched at pH

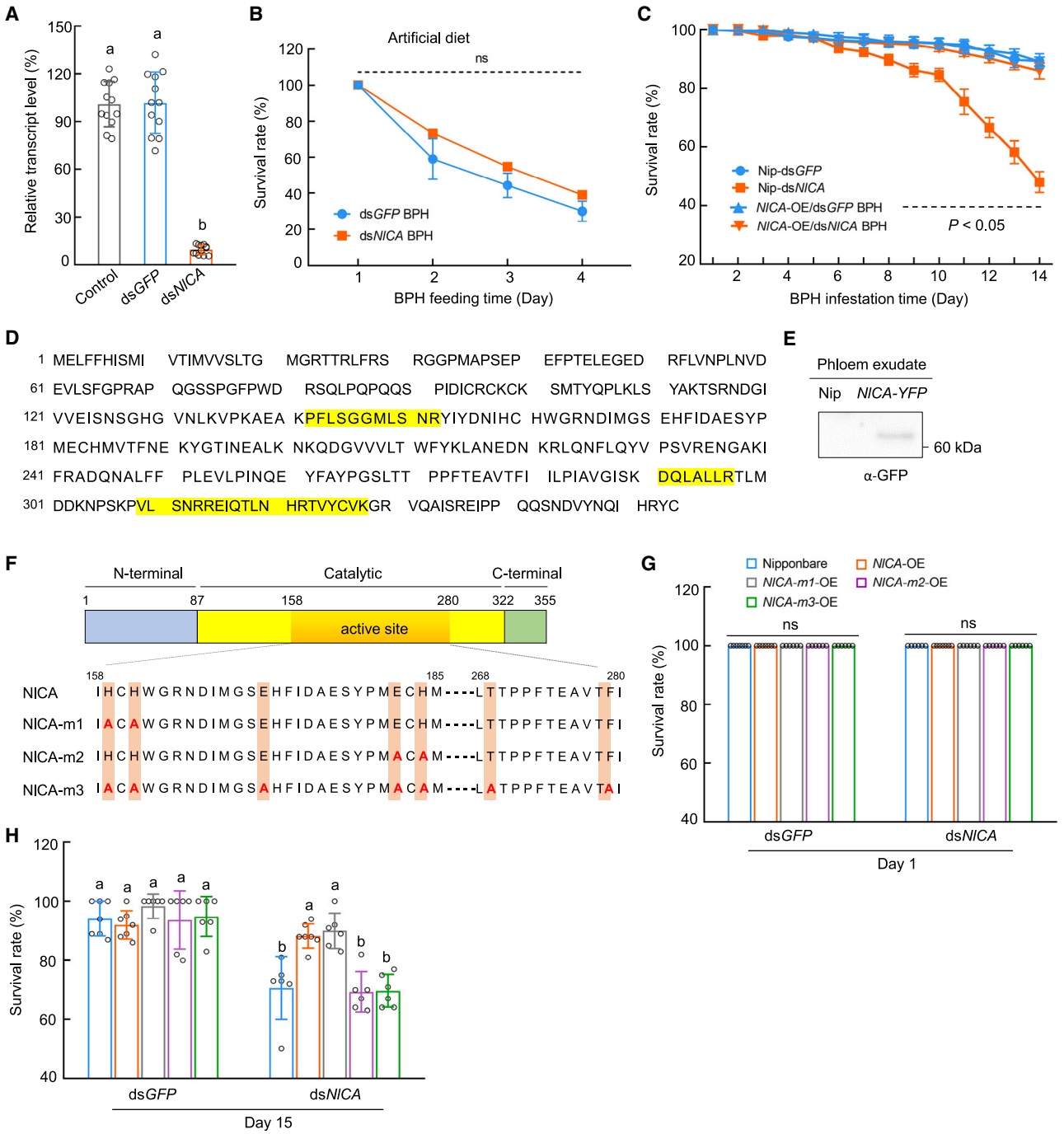

Figure 2. Effects of *NICA* RNAi on BPH survival on rice cultivar Nipponbare

(A) The *NICA* transcript levels in BPH at 3 days post injection of ds*GFP* or ds*NICA* were determined by quantitative real-time PCR (displayed as % of the *NICA* transcript abundance in control BPH). Values are displayed as mean ± SEM of 4 experimental replicates (two-way ANOVA; 3 biological replicates in each experiment and 30 individual insects pooled for each biological replicate).

(B) The daily survival rates of ds*NICA*-injected BPH insects feeding on artificial diet. Values are displayed as mean ± SEM of 3 biological replicates (1 biological replicate includes 50 individual BPH adults fed on artificial diet in a lucifugal plastic bottle).

(C) The daily survival rates of ds*NICA*-injected BPH insects feeding on Nipponbare (Nip) and *NICA*-expressing line 1. Values are displayed as mean ± SEM of 8 biological replicates (two-way ANOVA; 20 individual 2nd BPH nymphs per rice plant for each biological replicate). The black dashed line indicates significant differences ($p < 0.05$) in survival rates starting from day 9 between ds*NICA* BPH on Nip and other treatments.

(D) The amino acid sequence of NICA. The highlighted amino acid residues indicate the peptides detected in the phloem exudate of *NICA*-OE transgenic rice plants by LC-MS analysis.

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values < 5. In contrast, mRFP1 is insensitive to pH changes in the physiologically relevant range and serves as an internal reference. We placed 5th instar BPH nymphs on the leaf sheath of cyto-pHusion-expressing rice plants and recorded GFP and RFP signals of the feeding sites under microscopic observation at different time points with a 12-h test period. BPH can move around from one feeding site to another during the 12-h feeding experiments. The average feeding time at a feeding site is around 8 h. Therefore, the first 8 h of cellular responses best capture mostly synchronized rice-BPH interactions at a typical feeding cycle. As shown in Figures 3A–3D, ds*NlCA* BPH feeding induced a significant pH decrease in the phloem sieve elements/companion cells at 4 and 8 h (Figures 3C and 3D). The decreased ratio of EGFP: mRFP was caused by the quenched EGFP signals after ds*NlCA* BPH feeding, while the internal control, mRFP, kept at a stable level (Figures S3A–S3H). By contrast, ds*GFP* BPH feeding did not elicit an obvious intracellular pH change in the phloem sieve elements/companion cells during this period. These results uncovered a previously uncharacterized plant cellular response—rapid intracellular acidification—during the early stage of ds*NlCA* BPH feeding and demonstrated that BPH has evolved a critical virulence effector, NlCA, to counter this novel plant cellular response. Interestingly, we noted that the control ds*GFP* WT BPH feeding induced slight intracellular acidification at 12 h (Figure 3E). At this late time point of feeding, most BPHs would have left their initial feeding sites and have relocated to new feeding sites. In our analysis, we could not distinguish between old and new feeding sites. It is therefore likely that active BPH feeding (i.e., active NlCA injection) is needed to maintain intracellular pH homeostasis at all feeding sites.

Next, we conducted experiments to determine whether intracellular acidification is a highly localized cell-type-specific response or a spreading local response. We imaged and calculated the intracellular pH in the mesophyll cells and epidermal cells at the feeding site. We found that intracellular acidification occurred in the mesophyll cells and epidermal cells (Figures S3I–S3L) in response to feeding by ds*NlCA* BPH, albeit to a lesser degree compared with that in the sieve elements/companion cells. In contrast, rice plants fed on by ds*GFP* WT BPH responded with an initial slight intracellular acidification at 1.5 h but then consistently maintained a more alkalized intracellular pH in the mesophyll and epidermal cells compared with non-fed rice plants and ds*NlCA* BPH (Figures S3I–S3L).

Because extracellular pH change is associated with plant responses to biotic stress,[25,26] we speculated that rapid intracellular acidification of ds*NlCA* BPH feeding might induce defense gene expression. To test this possibility, Nipponbare plants infested by ds*GFP* and ds*NlCA* BPH were examined for defense gene expression. Commonly used marker genes (e.g., *OsNH1*, *OsNH2*, *OsWRKY45*, and *OsWRKY13*) in the studies of rice defense against BPH were selected.[27–30] We found that expression of those genes was significantly induced at a higher level by ds*NlCA* BPH infestation than by ds*GFP* BPH control at 4 and 8 h (Figures 3F–3I), which is consistent with the intracellular pH change at 4 and 8 h (Figures 3C and 3D). These results indicate that intercellular acidification is linked to rice defense activation.

Ectopic intracellular acidification causes defense gene expression in rice plants

Because plant defense responses are important for limiting BPH survival in rice,[28,31–33] we conducted experiments to determine whether intracellular acidification would trigger defense gene expression. Rice is normally grown in Yoshida medium, pH of 4.[34] To test whether medium pH changes can modulate defense responses in rice, we first grew rice seedlings in Yoshida medium until they reached the 5-leaf stage and then placed them in fresh Yoshida medium with pH adjusted to 2, 3, 4, 5, 6, and 7, respectively, for 48 h. Interestingly, drastically increased expression of defense response genes (e.g., *OsNH1*, *OsNH2*, *OsWRKY45*, and *OsPBZ1*) was observed in Nipponbare plants at an acidic pH of 2 (Figures S4A–S4H). The pHusion sensor plants exposed to external pH of 2 showed intracellular acidification that simulates pH changes observed during BPH feeding (Figures 3J–3K). Survival rates of BPH insects were lower in Nipponbare plants growing in media with a pH of 2 compared with those growing in media with a pH of 4 (Figure 3L). These results suggest that cellular acidification is sufficient to induce defense gene expression in rice and to reduce BPH's ability to survive on rice.

Rice defense responses are inhibited by NlCA

As NlCA stabilizes intracellular pH during WT BPH feeding (Figures 3A–3D), and both ds*NlCA* BPH feeding and ectopic intracellular acidification cause activation of defense gene expression (Figures 3F–3I; Figure S4), we next tested the hypothesis that a major function of NlCA-mediated stabilization of intracellular pH may be to prevent over-stimulation of downstream rice defense responses during feeding. Callose deposition in the phloem sieve tubes is a classical defense response that is associated with the feeding of piercing-sucking insects.[35] We examined this response using aniline blue to stain callose in the phloem sieve cells. Indeed, fewer and smaller callose depositions were found in the sieve plates of *NlCA*-expressing leaf sheaths compared with those found in the sieve plates of Nipponbare (Figures 4A–4H) at 72 h after BPH feeding, demonstrating that *Nl*CA suppresses callose deposition in sieve cell pates. Furthermore, ds*NlCA* BPH induced higher expression of callose biosynthesis genes, such as *OsGSL1*, *OsGSL3*, *OsGLS5*, and *OsGns5*, than the ds*GFP* control BPH; however, the induction of these genes was greatly compromised in *NlCA* transgenic

(E) Yellow fluorescent protein (YFP) fused NlCA protein (NlCA-YFP) is detectable in the phloem exudate of *NlCA*-OE transgenic plants by anti-GFP antibody (Abmart, 20004).
(F) Schematic display of the NlCA protein with the N terminus displayed in blue, the C terminus in green, and the central catalytic domain in yellow/orange. The amino acid (aa) sequence (158–280 aa) of the putative active site is showed. Amino acid substitutions in NlCA-m1, NlCA-m2, and NlCA-m3 are indicated in red.
(G and H) The survival rates of 20 ds*GFP* and ds*NlCA* BPH insects in Nip and *NlCA* transgenic plants after 1 day (G) and 15 days (H) post infestation. Values are displayed as mean ± SEM ($n \geq 6$ biological replicates; 20 individual 2nd BPH nymphs per rice plant for each biological replicate). ns indicates no significant difference between treatments (two-way ANOVA). Different letters indicate statistically significant differences analyzed by two-way ANOVA (Tukey test, $p < 0.05$). Experiments were repeated three times with similar trends. See also Figures S1 and S2.

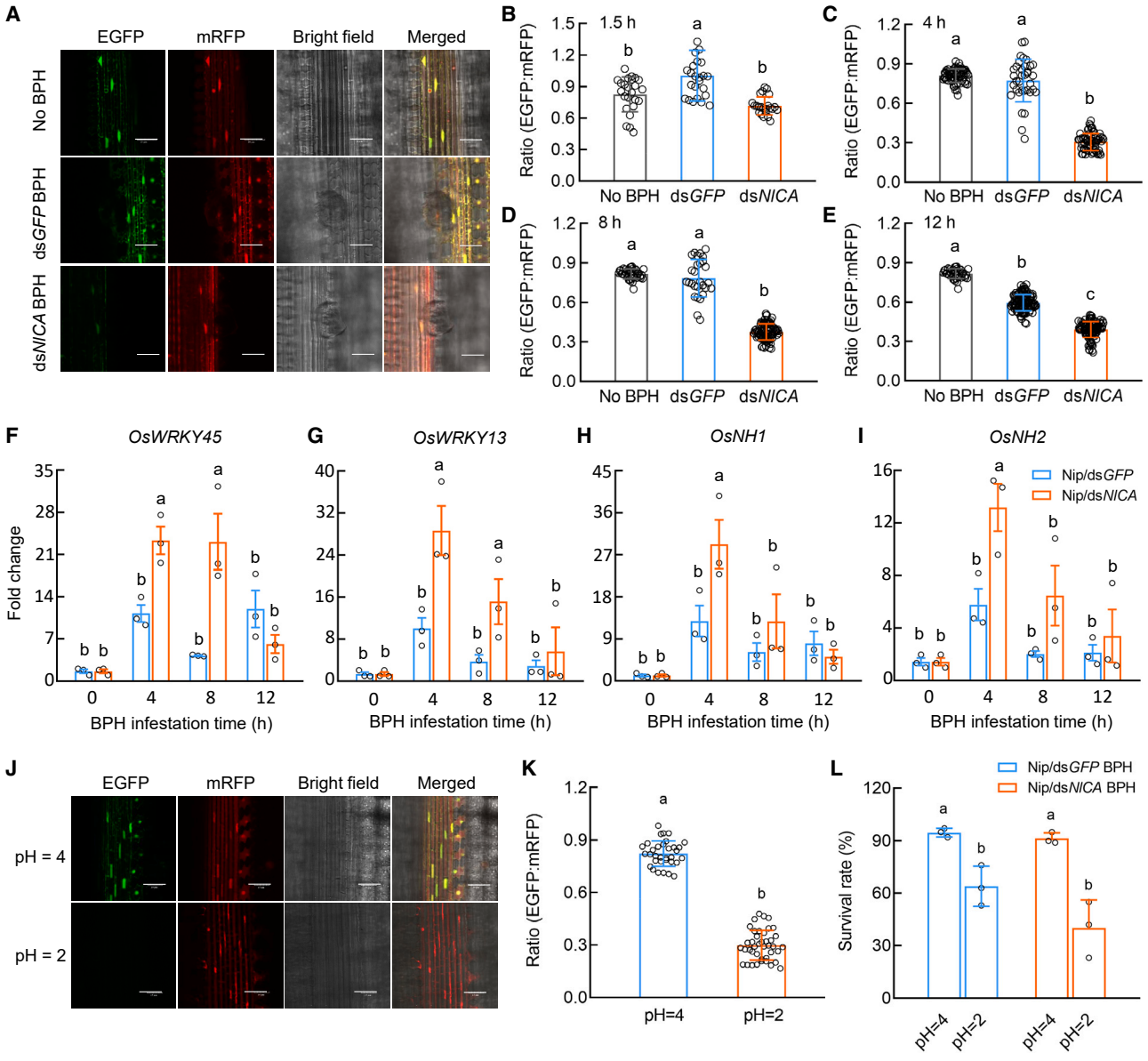


Figure 3. Role of NICA in maintaining intracellular pH of rice cells

(A) Confocal microscopic images at BPH feeding sites in Nipponbare plants expressing a ratiometric cytoplasmic pH sensor (cyto-pHusion) 4 h after placing 30 5th-instar BPH nymphs on each plant. A 40.0× objective was used to capture every feeding site on the tiled 1 × 1 cm section of rice leaf sheath. Confocal images from plants with no BPH feeding served as the control. Scale bar, 20 μm.

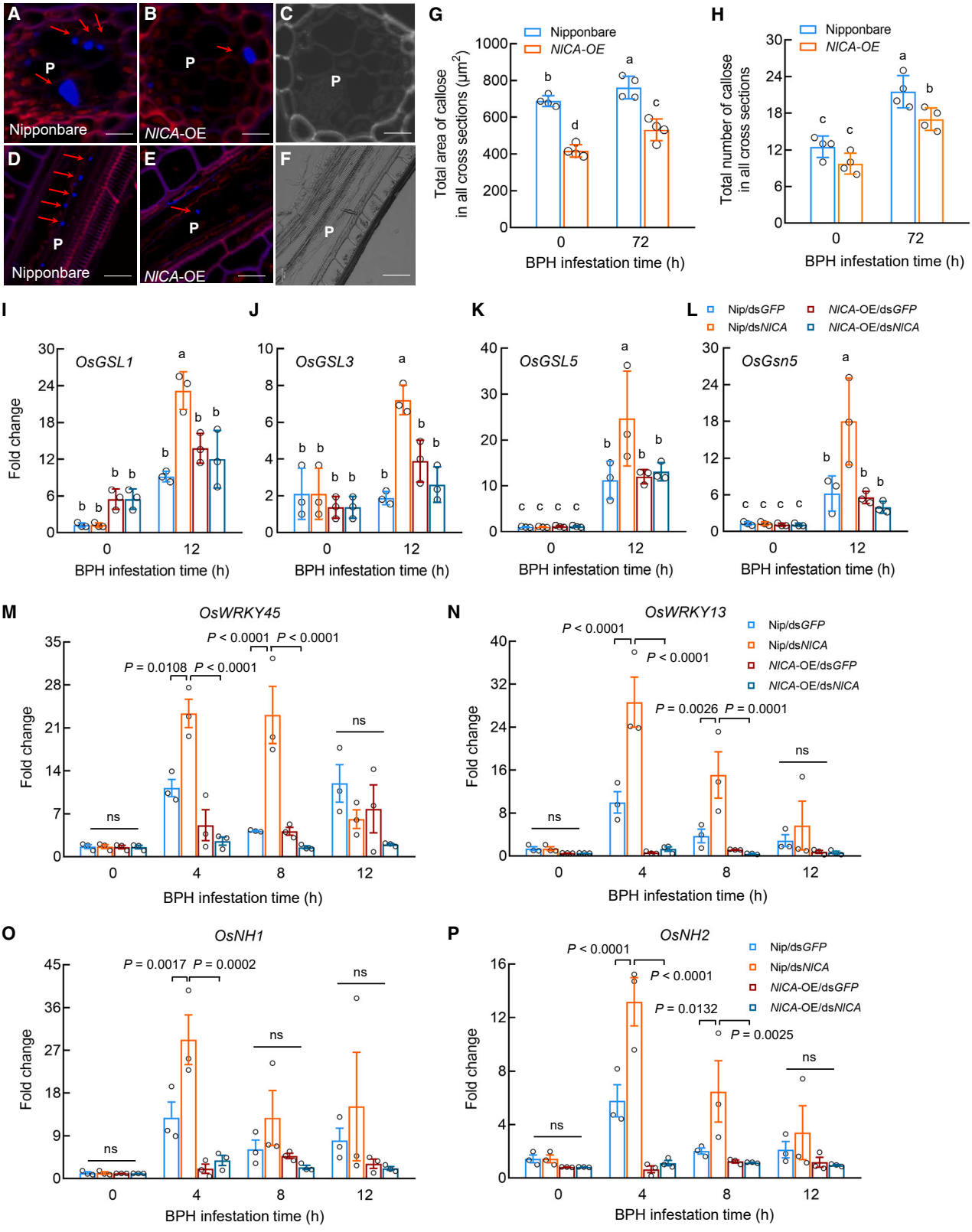
(B–E) EGFP:mRFP signal ratios at 1.5 (B), 4 (C), 8 (D), and 12 h (E) of ds*GFP* or ds*NICA* BPH treatment compared with no BPH control. EGFP was imaged at λ_{Ex} = 500 nm and λ_{Em} = 540 nm. mRFP was imaged at λ_{Ex} = 570 nm and λ_{Em} = 620 nm. Values are displayed as mean ± SEM ($n \geq 24$ circular areas of leaf sheath phloem with a diameter of 100 μm, with the feeding site at the center).

(F–I) Expression of defense response genes, *OsWRKY45* (F), *OsWRKY13* (G), *OsNH1* (H), and *OsNH2* (I), in Nipponbare plants infested by ds*GFP* or ds*NICA* BPH. Values are displayed as mean ± SEM of three biological replicates. Each biological replicate represents pooled leaf sheaths from three individual rice plants fed on by 20 5th instar BPH nymphs per plant.

(J) Confocal microscopic images of Nipponbare plants expressing a ratiometric cytoplasmic pH sensor (cyto-pHusion) at 12 h after being transferred to Yoshida medium at a pH of 2. Confocal images from plants grown in Yoshida medium with a pH of 4 served as the control. Scale bar, 15 μm.

(K) EGFP:mRFP signal ratios. EGFP was imaged at λ_{Ex} = 500 nm and λ_{Em} = 540 nm. mRFP was imaged at λ_{Ex} = 570 nm and λ_{Em} = 620 nm. Values are displayed as mean ± SEM ($n \geq 33$ calculation area per condition).

(L) The 7th-day survival rates of ds*GFP*- and ds*NICA*-injected BPH insects feeding on Nipponbare growing in media with different pHs. Values are displayed as mean ± SEM of 3 biological replicates (20 individual insects per biological replicate). Different letters indicate statistically significant differences analyzed by two-way ANOVA (Tukey test, $p < 0.05$). Experiments were repeated three times with similar trends. See also Figures S3 and S4.



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plants, with or without BPH treatment (Figures 4I–4L). We also measured the transcript levels of defense marker genes (e.g., *OsNH1, OsNH2, OsWRKY45*, and *OsWRKY13*) in Nipponbare and *NlCA*-expressing plants that had been fed on by ds*GFP* and ds*NlCA* BPH. As shown in Figures 4M–4P, induced expression of *OsNH1*, *OsNH2*, *OsWRKY13*, and *OsWRKY45* by ds*NlCA* BPH was suppressed in *NlCA* transgenic plants compared with those in Nipponbare plants. Collectively, these results showed that NlCA-mediated intracellular pH homeostasis is linked to downregulation of callose deposition in phloem sieve cells as well as defense gene expression in rice plants.

DISCUSSION

In this study, we provided evidence that intracellular acidification is a previously unrecognized plant defense response that occurs during BPH feeding on rice. This finding was facilitated by our attempt to understand the role of NlCA in rice-BPH interaction. We found that the *NlCA* transcript is detected mainly in the salivary glands (Figure 1A) and that the NlCA protein is found in rice tissues, including the phloem sap, that have been fed on by BPH (Figure 1B). *NlCA*-silenced (ds*NlCA*) insects survived very poorly on at least two independent cultivars of rice plants, Xiushui134[6] and Nipponbare (Figure 2), whereas *NlCA*-expressing rice plants can restore the normal survival of *NlCA*-silenced (ds*NlCA*) insects (Figure 2; Figure S1), suggesting that NlCA functions in plant cells. Using the cytoplasm pH sensor, we found that NlCA is required for BPH to maintain a normal plant cytoplasm pH during BPH feeding (Figures 3A–3D; Figure S3). Pathogen/insect-derived effectors can be powerful molecular probes for discovering novel plant regulators/responses to biotic attacks. Although other piercing/sucking herbivore-derived effectors, such as Btfer1, LsPDI1, LsSP1, Mp55, DNase II, and BISP, have been reported as defense-suppressive effectors,[36–41] our discovery of intracellular acidification as a plant defense response and our finding that BPH secretes NlCA as a counter-defense measure show host cell pH homeostasis as a novel battlefield that has not been revealed in any plant-biotic interactions.

Extracellular alkalinization of cultured plant cells has long been recognized as a canonical plant response to microbial elicitors as well as endogenous plant signals.[26] Extracellular alkalinization caused by plant endogenous RAPID ALKALINIZATION FACTOR (RALF) peptides, for example, is perceived by the FERONIA-family receptors.[42,43] There is evidence that this perception causes phosphorylation of plasma membrane (PM)-localized H(+)-ATPase 2, resulting in the inhibition of proton transport across the PM.[44] Notably, extracellular alkalinization has recently been shown to inhibit or promote growth- or immunity-associated cell-surface-receptor functions through specific pH-sensitive amino acid sensors.[26] In contrast, defense regulation by intracellular pH changes had escaped the discovery of researchers until this study. Our demonstration of a link between intracellular acidification and defense activation has laid a foundation for the future discovery of potentially diverse pH-sensitive intracellular regulators of defense responses, which could add a new dimension to the study of plant-biotic interactions.

Because cellular pH alterations could potentially affect multiple biomolecules and, hence, multiple cellular processes, future research should comprehensively define all cellular processes that are affected by intracellular pH acidification. In this study, we found that this pH change is linked to activation of callose deposition at phloem sieve cells and expression of defense response genes, such as *OsNH1*, *OsNH2*, *OsPBZ1*, and *OsWRKY45* (Figure 3; Figure S4). Conversely, NlCA-mediated intracellular pH stabilization dampens these defense responses (Figure 4). Callose deposition in phloem sieve cells, in particular, is a classical defense response to a variety of sucking/piercing insects and is thought to limit nutrient flow during insect feeding.[35,45–48] Together, these results suggest that a major effect of NlCA-mediated pH stabilization is to prevent overactivation of defense responses during BPH feeding.

The mechanism by which NlCA counters intracellular acidification is likely inherent in its reversible inter-conversion of carbon dioxide and water into carbonic acid, protons, and bicarbonate ions. CAs are universally present in all organisms (Figure S5); other piercing/sucking insects may use CAs or another mechanism to manipulate host intracellular pH as part of their infestation strategy. Indeed, CA has been reported as a protein component of saliva in rice green leafhopper, *Nephotettix cincticeps*,[49] and aphid *Myzus persicae*.[50] In the case of *M. persicae*, CA-II was shown to increase viral transmission via plant apoplastic-acidification-mediated acceleration of intracellular vesicular trafficking.[50] However, because NlCA plays a critical role in BPH's survival on rice plants per se (i.e., in the absence of viral infection), as shown in this study, it is likely that insect-secreted CAs play a primary role in facilitating insect survival by countering intracellular-acidification-associated

Figure 4. NlCA dampens callose deposition and defense gene expression

(A–F) Callose accumulation (bright blue fluorescence indicated by red arrows) on the sieve plates of Nipponbare leaf sheaths (A and D) and *NlCA*-OE leaf sheaths (B and E) plants at 72 h after BPH feeding. P, phloem. The pictures were taken under a Zeiss microscope. (A and B) Cross-sections. Scale bar, 5 μm. (D–E) Longitudinal sections. Scale bar, 10 μm. (C and F) The bright-field views of cross and longitudinal phloem sections, respectively.

(G) Total areas of callose deposition in BPH-infested leaf sheaths of Nipponbare and *NlCA*-OE. Each data point represents the total areas of callose deposition found in 300 cross-sections of each experiment. Values are displayed as mean ± SEM of four experiments.

(H) Total number of callose deposits in BPH-infested leaf sheaths of Nipponbare and *NlCA*-OE. Each data point represents the total number of callose deposits found in 300 cross-sections of each experiment. Values are displayed as mean ± SEM of four experiments.

(I–L) Relative expression levels of the callose synthase genes *OsGSL1* (I), *OsGSL3* (J), *OsGSL5* (K), and *OsGns5* (L) in response to BPH feeding. Values are displayed as mean ± SEM of 3 biological replicates. Each biological replicate represents pooled leaf sheath from 3 individual rice plants fed on by 20 5th instar BPH nymphs per plant.

(M–P) Expression of defense marker genes *OsWRKY45* (M), *OsWRKY13* (N), *OsNH1* (O), and *OsNH1* (P) in response to BPH feeding. Values are displayed as mean ± SEM of 3 biological replicates. Each biological replicate represents pooled leaf sheaths from 3 individual rice plants fed on by 20 5th instar BPH nymphs per plant. ns indicates no significant difference between treatments (two-way ANOVA). Different letters indicate statistically significant differences analyzed by two-way ANOVA (Tukey test, $p < 0.05$). Experiments were repeated three times with similar trends.

defense activation. Future research could examine whether CA-mediated increases in viral transmission may be affected by defense suppression.

Discovering the role of pH regulation during plant response to biotic and abiotic stresses and characterizing the impact of such pH alterations could be an important area for future research. Because maintaining proper external and internal pH is critical for all forms of life, prokaryotic and eukaryotic organisms alike have evolved mechanisms to achieve pH homeostasis. Facing fluctuating external pH, prokaryotes have evolved diverse mechanisms for sensing external pH. For instance, the bimodal sensing of pH is employed by *Bacillus subtilis* and *Escherichia coli*.[51] Fungi employ a conserved pathway, mediated by Rim101 and PacC, to sense external pH.[52] It has been reported that different subcellular compartments within the plant cell maintain different pH values, presumably as part of carrying out their unique physiological functions.[53] The demonstrated ability of NICA to counter stimulus-dependent pH changes in plant cells could make NICA a useful molecular tool to modulate and broadly understand the effects of pH stabilization on plant signaling and metabolic pathways in different cell types, organelles, and tissues in plants by, for example, targeting NICA expression in specific tissues, cells, or organelles. Additionally, the crucial role of NICA for BPH infestation of rice suggests that NICA is an important target for chemical or trans-kingdom RNAi-based inactivation for the development of novel BPH control strategies in plants.

RESOURCE AVAILABILITY

Lead contact

Further information or requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sheng Yang He (Shengyang.he@duke.edu).

Materials availability

All novel materials described in this paper will be made available upon request, subject to completion of an MTA.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- No original code is reported in this paper.
- Additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

S.Y.H., C.-X.Z., Y.J., and X.-Y.Z. conceptualized and designed this study. Y.J., X.-Y.Z., S.L., Y.-C.X., X.-M.L., Y.Y., Z.P., L.Z., J.-B.L., and H.-J.H. performed experiments and analyzed data. C.-X.Z. and S.Y.H. analyzed data. Y.J., X.-Y.Z., C.-X.Z., and S.Y.H. wrote the paper, and all authors approved the final article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-HA	Zenbio	Cat#301113; RRID: AB_3662580
Mouse monoclonal anti-GFP	Abmart	Cat#M20004; RRID: AB_2619674
Bacterial strains		
Escherichia coli DH5a	TransGen	CD201-01
Agrobacterium tumefaciens GV3101	N/A	N/A
Chemicals		
Phusion High-Fidelity DNA Polymerase	Thermo Scientific	Cat#F534
Gateway LR Clonase II Enzyme mix	Invitrogen	Cat#11791100
Gateway BP Clonase II Enzyme mix	Invitrogen	Cat#11789020
ProtoScript II Reverse Transcriptase	NEB	Cat#M0368
SYBR Green Mix	Roche	Cat#4887352001
Oligonucleotides		
NICA-overlap-F; NICA-overlap-R	ggggacaagtttgtacaaaaaagcaggcttc ATGGAGCTTTTCTTCCACATA; ggggaccactttgtacaagaaagctgggtc CTAACAGTATCTGTGTATTTGATTGTATC	Sangon Biotech
NICA-overlap-1-F; NICA-overlap-1-R	AACATTGCCTGTGCCTGGGGC AGAAATGATATAATGGGCAGC; CCCCAGGCACAGGCAATGTTG TCATAAATATAGCGATTTGACAG	Sangon Biotech
NICA-overlap-2-F; NICA-overlap-2-R	CGATGGCATGCGCCATGGTAAC TTTTAATGAAAAATACGGAACC; TACCATGGCGCATGCCATCGGA TACGATTCTGCATCGATG	Sangon Biotech
NICA-overlap-3-F; NICA-overlap-3-R	TGGGCAGCGCTCACTTCATCGA TGCAGAATCGTATCCGAT; GATGAAGTGAGCGCTGCCCAT TATATCATTTCTGCCCCAGT	Sangon Biotech
NICA-overlap-4-F; NICA-overlap-4-R	CAGTCACGGCCATCATTCTGCC AATTGCTGTTGGTATATCC; CAGAATGATGGCCGTGACTGG CTTCTGTAAAGGGTGGCGT	Sangon Biotech
NICA-overlap-5-F; NICA-overlap-5-R	CCAGGTTCTCTAGCAACGCCAC CCTTTACAGAAGCAGTCACG; GGCGTTGCTAGAGAACCTGGA TAAGCAAAGTATTCTTGGTTTATG	Sangon Biotech
NICA-qRT-F; NICA-qRT-R	CCTTGCTGCGTACATTGA; CGGTTCTGTGATTGAGTGT	Sangon Biotech
NI18S rRNA-qRT-F; NI18S rRNA-qRT-R	CGCTACTACCGATTGAA; GGAAACCTTGTTACGACTT	Sangon Biotech
OsACTIN-qRT -F; OsACTIN-qRT -R	TGGACAGGTTATCACCATTGGT; CCGCAGCTTCCATTCCTATG	Sangon Biotech
OsWRKY45-qRT -F; OsWRKY45-qRT -R	GGGAATTCGGTGGTCGTCAA; TGGATCTCCTTCTGCCCGTA	Sangon Biotech
OsPBZ1-qRT -F; OsPBZ1-qRT -R	GGGTGTGGGAAGCACATACA; CCTCGAGCACATCCGACTTT	Sangon Biotech
OsNH1-qRT -F; OsNH1-qRT -R	TGGCAGGTGAGAGTCTACGA; GCTACTCTTGCCTCCATCGG	Sangon Biotech

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
OsNH2-qRT -F; OsNH2-qRT -R	GTGGATACACGGCACTCCAT; CTCTGGCCATCAGCAGTCAA	Sangon Biotech
OsWRKY13-F; OsWRKY13-R	CAGTAGCTCCAAGGGGTGTC; CGAAGGAGTAGGTGACGAGC	Sangon Biotech
Software		
ImageJ	Schneider et al.[54]	https://imagej.nih.gov/ij/
GraphPad Prism version 8	San Diego, California USA	https://www.graphpad.com

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant materials and insects

Oryza sativa ssp. *Japonica* cv. Nipponbare was used as the wild type and for generating transgenic plants. The *Nilaparvata lugens* population used in this study was originally derived from a rice field on the Huajiachi Campus of Zhejiang University, Hangzhou (30° 16' N, 120° 11' E), China, in 2008, and artificially propagated for many generations until now. BPH insects were reared in environmentally controlled growth chambers on a susceptible cultivar rice variety Xiushui 134 at 27 ± 0.5°C and 50 ± 0.5% relative humidity under a 16-h light and 8-h dark photoperiod in a modified Yoshida solution (in which ammonium nitrate was replaced by 1.06 M carbamide) with pH of 4.

METHOD DETAILS

Transgenic rice

The *NlCA* coding sequence was amplified from BPH. Several *NlCA* mutants (*NlCA-m1*, *NlCA-m2*, and *NlCA-m3*) were made by site-directed mutagenesis. The *NlCA-m1*, *NlCA-m2* and *NlCA-m3* PCR products encoding proteins in which His^{159} and His^{161} were mutated to Ala, Glu^{182} and His^{184} were replaced by Ala, and all seven conserved active site residues, His^{159}, His^{161}, Glu^{171}, Glu^{182}, His^{184}, Thr^{269} and Phe^{279}, were changed to Ala, respectively. The WT and mutant *NlCA* PCR products were recombined into the intermediate vector pDonor207 using the BP recombination kit (Invitrogen), and then recombined into the destination vector pEarleygate104 using the LR recombination kit (Invitrogen) to generate P_{35S}*::NlCA-YFP* and P_{35S}::mutant *NlCA-YFP* expression vectors. The transgenic rice plants were generated by Agrobacterium-mediated transformation at BIOGLE GeneTech (Hangzhou Biogle Co., LTD.) where rice plants were reared in a greenhouse at 27 ± 0.5°C and 60 ± 0.5% relative humidity under a 16-h light and 8-h dark photoperiod.

In situ mRNA hybridization analysis

Fresh salivary tissues were isolated from 100 5^{th} instar BPH and washed 3 times before being placed in the 4% paraformaldehyde (PFA) fix solution (Servicebio, G1113) at 4°C overnight, dehydrated and embedded in paraffin. Ten μM-thick sections were cut on glass slides, dewaxed and digested by Protease K (Servicebio, G1205) at 37°C for 15 min. After three washes in phosphate buffered saline (PBS) buffer (Servicebio, G0020), the sections were incubated in pre-hybridization solution at 37°C for 1 h and then in hybridization solution containing the *NlCA* probe (1μM) at 42°C overnight. The PCR primer for generating the probe was 5'-GGTTCTGTGATTGAGTGTTTGGATCTCCCTGCG-3' with a single-end CY3 tag generated using the DIG RNA Labeling Kit (Roche Applied Science, Penzberg, Germany) based on the manufacturer's instructions. The slides were stained with DAPI for 8 min in the dark after being washed in saline sodium citrate (SSC) buffer (Servicebio, G3016-4) for three times (2 × SSC, 10 min; 1 × SSC, 2 × 5 min; 0.5 × SSC, 10 min). Sections hybridized with sense probe was used as a negative control and antisense probe was used to detect the target mRNA. All steps were performed at 37°C and sections were examined under a Nikon fluorescence microscope with a 40 × lens (330 nm–380 nm for DAPI, 510 nm–560 nm for CY3).

Rice sheath and phloem exudate sample preparation for LC-MS

Three independent sets of Nipponbare rice sheath protein samples (three biological replicates) were collected after feeding by 5^{th} instar nymphs as follows. Briefly, ten 6-leaf-stage rice plants were individually placed into a breathable transparent plastic bottle (5 cm in diameter, 30 cm in height) containing Yoshida medium, pH = 4, and then fed by ~500 fifth instar BPH for 72 h. Next, BPHs were gently shaken off leaves with intact stylets and the two outermost layers of leaf sheath surrounding the stem were cut into small pieces, rinsed with ultrapure water for three times and ground to powder in liquid nitrogen. Total protein was extracted in a buffer containing 60 mM Tris-HCl (pH = 7.6), 2.5% Glycerol, 6% SDS, 0.1 M DL-Dithiothreitol (DTT), 1x Protein inhibitor cocktail and 1 mM phenylmethanesulfonyl fluoride. Protein samples were concentrated using 3-kDa filtration units and shipped on dry ice to Shanghai Hoogen Biotech for shot-gun LC-MS analysis. Briefly, protein samples digested by trypsin for 16 h at 37°C. The hydrolysates were then loaded into Zorbax 300SB-C18 peptide traps (Agilent Technologies, Wilmington, DE) and analyzed by a Q Exactive

mass spectrometer (Thermo Fisher). The raw LC-MS data were searched against the BPH protein database (NCBI Accession No. PRJNA669454) using software MaxQuant 1.5.5.1. Peptides with a high confidence score was extracted, and the database search was conducted with false discovery rate (FDR) under 0.01.

For phloem exudate collection, 4-leaf-stage rice plants were used. Exudates were collected using the EDTA-facilitated exudation method as described by Gaupels et al.[55] and Du et al.[56] with some modifications. Briefly, the shoots of plants were cut just above the stem/root junction with a sharp razor blade while rootless shoots were submerged in a 20 mM EDTA solution (pH 8.0, established using KOH), and shaken for 15 seconds to remove compounds from damaged roots. Then 4-6 rootless plants were transferred to plastic tubes containing 1.5 mL fresh 20 mM EDTA solution (pH 8.0) to facilitate phloem sap exudation. Plants were placed in a dark, high humid chamber to reduce transpiration. The exudates were collected after 12 h and the samples were concentrated by centrifugation at 4°C in a 2 mL concentrator (10 kDa for NICA concentration; 30 kDa for NICA-YFP concentration) to a final volume of 20 μL (from a total of 40 plants) for Western blot or LC-MS analysis.

RNA interference (RNAi)

The nucleotide sequence (around 500 bp long) specific to the *NICA* coding region was cloned into the pMD 19-T vector (TAKARA). The double-stranded RNA was synthesized through PCR amplification by using the Mega script T7 High Yield RNA Transcription Kit (Vazyme, Nanjing, China), following the manufacturer's instructions. The procedure for gene knockdown by RNAi was described previously.[22] Briefly, the 2nd and 5th instar BPH nymphs in an insect rearing growth chamber were anaesthetized with carbon dioxide for ~10 s. Approximately 50–250 ng of dsRNA was injected into BPH mesothorax using FemtoJet (Eppendorf-Netheler-Hinz, Hamburg, Germany). The efficiency of RNA interference was assessed 48 h after injection. RNA from 30 nymphs was extracted using the RNAiso Plus kit (TaKaRa) according to the manufacturer's instructions as one biological replicate. The relative level of the *NICA* transcript was quantified using a CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with primers shown in key resources table. The Aequorea Victoria green fluorescent protein (GFP) gene sequence was used as the control template in RNAi experiments. Four biological replicates (30 individual insects pooled for each biological replicate) were conducted.

BPH survival test on artificial diet

The 5th instar BPH nymphs injected with ds*GFP* or ds*NICA* were immediately placed on one-week-old rice seedlings (1-leaf-stage) for recovery in the small breathable transparent plastic bottle for 3 days. Fifty normal-appearing BPH adults were gently transferred into a shaded black plastic bottle of 5 cm in diameter and 15 cm in height, with translucent double-layer parafilm loaded with fresh artificial diet. The first layer of parafilm closed to BPH was needled 40 holes by a 1 ml syringe and fresh artificial diet AADM, pH of 6.0, was filtered through a 0.22 micrometer needle-type bacterial filter (Sangon Biotech, F513142-0001). Fresh artificial diet was replaced every 24 hours. The second layer of parafilm wrapped the artificial diet to isolate the diet from bacteria in the air. Dead BPHs were separated from live BPHs daily and mucus adhering to the inner wall of the black plastic bottle was cleaned daily to eliminate possible impact on live BPHs. and the pH was changed from 6.8 to 6. There were three biological replicates for each treatment, and each biological replicate includes 50 BPH adults. Experiments were repeated three times with similar trends.

BPH survival test on rice

BPH nymphs with verified *NICA* knockdown by RNAi were placed into a small breathable transparent plastic bottle of 10 cm in diameter and 9 cm in height containing one-week-old rice seedlings (1-leaf-stage) for 12 h to recover. About 20 healthy-appearing 2nd instar nymphs were gently transferred into a longer breathable transparent plastic bottle of 5 cm in diameter and 30 cm in height containing four-week-old rice plants (4- to 5-leaf-stage) and observed for up to 15 days. The feeding space for BPH in each plastic bottle was around 200 cm^3 divided by two sponges of 5 cm in diameter and 2 cm in thickness. The number of surviving BPHs were counted daily on each plant. *dsGFP* BPH were used as control. The average of survival rate on each seedling was calculated.

For BPH survival test on normal and acidic media growing rice plants, the early 5th instar BPH nymphs inoculated ds*GFP* and ds*NICA* fed on one-week-old rice seedlings (1-leaf-stage) were recovered in the small breathable transparent plastic bottle for 24 hours. The Nipponbare rice plants (5-leaf stage) growing on normal liquid media (pH = 4) was transferred into fresh liquid media with pH of 4 and 2 for 24 hours, respectively, before conducting BPH survival test. There were five biological replicates for every treatment, and one biological replicate is one rice plant fed by 20 BPHs. Experiments were repeated three times with similar trends.

pH assay

Transgenic rice plants expressing the cyto-pHusion pH sensor were grown in the modified Yoshida medium as described above. Each 5-leaf-stage seedlings were fed by 30 5th instar BPH, which were starved in the breathable plastic tubes for 3 h before feeding. No BPH treatment was included as control. The microscopic images were taken from rice leaf sheaths that were fed by BPH for 1.5 h, 4 h, 8h and 12 h using an Olympus FV3000 with a 40.0 x objective. EGFP was imaged with λEm = 540 nm and the detection wavelength at 500–540 nm. mRFP was imaged with λEm = 570 nm and detection wavelength wat 570 to 620 nm. Mean fluorescent intensities of EGFP and mRFP from multiple images were quantified using Fiji Image J. Briefly, the EGFP and mRFP merged images were separated into the EGFP channel and the mRFP channel with a threshold of 1–4090 using Image J. Mean intensities of EGFP and mRFP fluorescence in a region of interest (ROI) were measured to calculate the EGFP: mRFP ratio. Each treatment includes > 24 circular areas of leaf sheath with a diameter of 100 μm each, including the feeding site at the center.

RNA isolation and quantitative real-time PCR (qRT-PCR)

The total RNA was extracted using the TRIzol reagent kit (Invitrogen), and then treated with Rnase-free Dnase I (Invitrogen) according to the manufacturer's protocol. The RNA was reverse-transcribed using a cDNA synthesis kit (TaKaRa) and one μg of each RNA samples was reverse transcribed. qRT-PCR was performed from cDNA using SYBR Green qPCR mix (TaKaRa, RR601A) following the manufacturer's instructions using the LightCycler 480 system (Roche Diagnostics). Fold changes were calculated using the ΔCt method. Each assay was replicated at least three times, each with three biological replicates (independent RNA preparations). The rice Actin gene (*LOC_Os03g50885*) and the BPH 18S rRNA (*LOC_111047943*) gene were used as a reference. Sequences of qRT-PCR primers are given in key resources table.

Callose deposition in rice sheath

For counting callose deposition, four-leaf-stage rice plants were infested with ~15 fifth instar BPH. The two outermost rice leaf sheaths were collected before and after BPH feeding for 72 h and fixed in FAA solution as described by Guo et al.,[28] dehydrated and embedded in paraffin. The sections were cut to 5 um thickness on glass slides, dewaxed and stained with 0.1 % aniline blue in 0.1 % 5 M K_2PHO_4 for 10 min, and examined under Zeiss confocal microscope with a 40× oil objective lens. The total area and number of callose deposition in the rice sieve pores were counted. Each treatment has at least 400 cross sections and 12 biological replicates.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistics were performed using GraphPad Prism version 8 for Windows, GraphPad Software, San Diego, California USA, https://www.graphpad.com. Statistical details relating to specific experiments can be found in relevant Figure legends. In all cases pair-wise comparisons were performed using Student's t-test, comparisons across multiple groups were performed using ANOVA with Tukey's HSD test for significance.

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Supplemental Information

Rapid intracellular acidification is a plant defense response countered by the brown planthopper

Yanjuan Jiang, Xiao-Ya Zhang, Shaoqin Li, Yu-Cheng Xie, Xu-Mei Luo, Yongping Yang, Zhengyan Pu, Li Zhang, Jia-Bao Lu, Hai-Jian Huang, Chuan-Xi Zhang, and Sheng Yang He

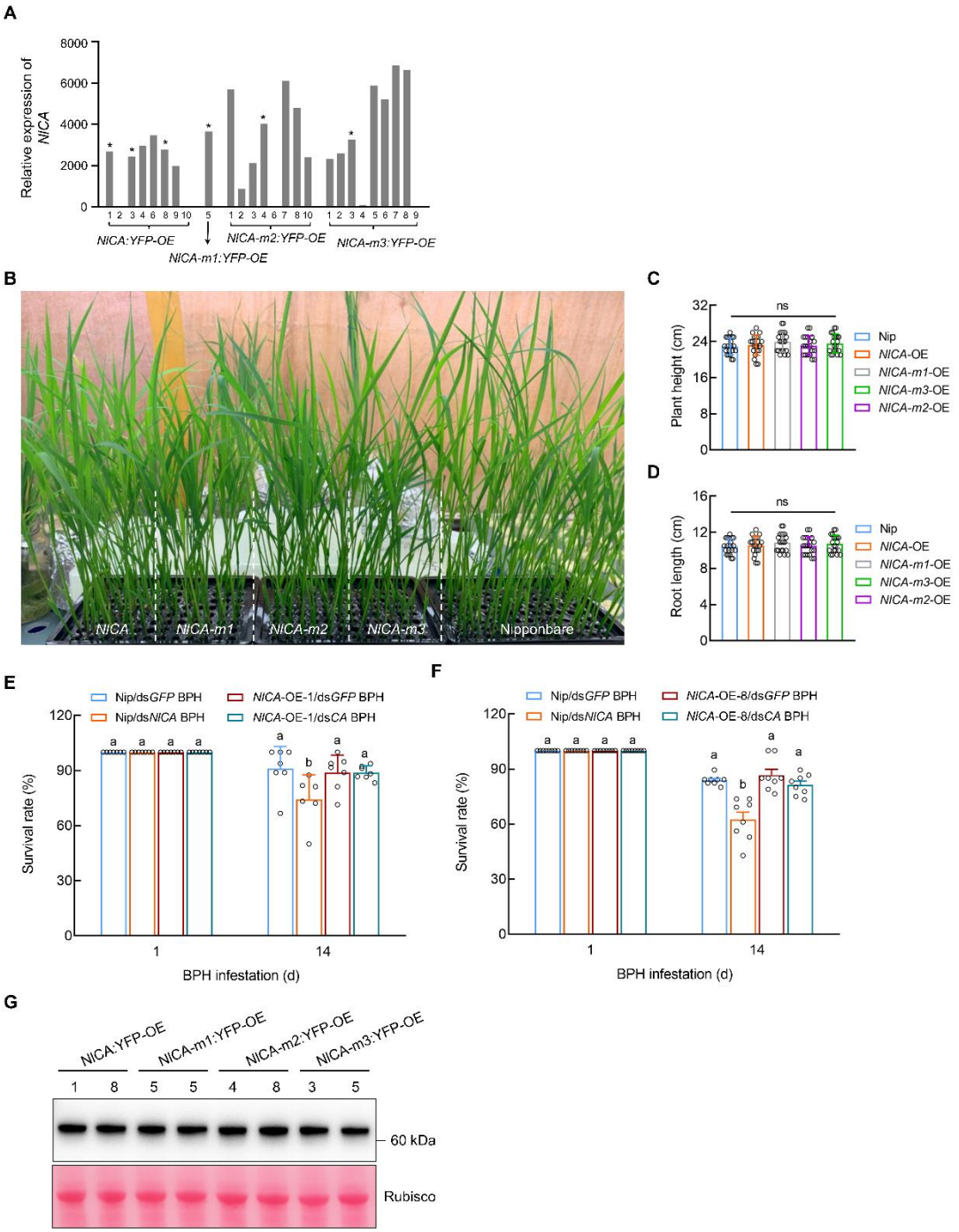

Figure S1. **Generation of *NICA-OE* and mutant *NICA-OE* plants and survival rates of BPH, related to Figure 2.** (A) qRT-PCR analysis of the *NICA* transcript levels in *NICA-OE, NICA-m1-OE, NICA-m2-OE NICA-m3-OE* plants. Plant lines labeled with an asterisk were selected for further BPH feeding assay. (B) 21-day-old of Nipponbare, *NICA-OE, NICA-m1-OE, NICA-m2-OE NICA-m3-OE* plants grown in the modified Yoshida medium. *NICA-OE, NICA-m1-OE, NICA-m2-OE NICA-m3-OE* plants displayed normal appearance compared with Nipponbare. (C and D) Plant height (C) and root length (D), respectively, in (B). Values are displayed as mean ± SD (n = 20 plants). ns indicate no significant statistically differences analyzed by one-way ANOVA (Tukey test). (E and F) The survival rates of ds*GFP* and ds*NICA* BPH insects on

Nipponbare and *NICA* transgenic plant line 3 (E) and line 8 (F) after 1-day and 14-days post-infestation. Values are displayed as mean ± SEM (n = 8 biological replicates; 20 individual insects per each biological replicate). (G) Protein samples were extracted from one-week-old seedlings of *NICA*-OE (lines 1 and 8) and *NICA*-mutant-OE (line 5 of NICA-m1, lines 4 and 8 of NICA-m2 and lines 3 and 5 of NICA-m3) plants. Abundance of fusion proteins were detected with anti-GFP (Abmart, 20004). Ponceau S staining of Rubisco confirmed equal loading. Experiments were repeated three times with similar trends.

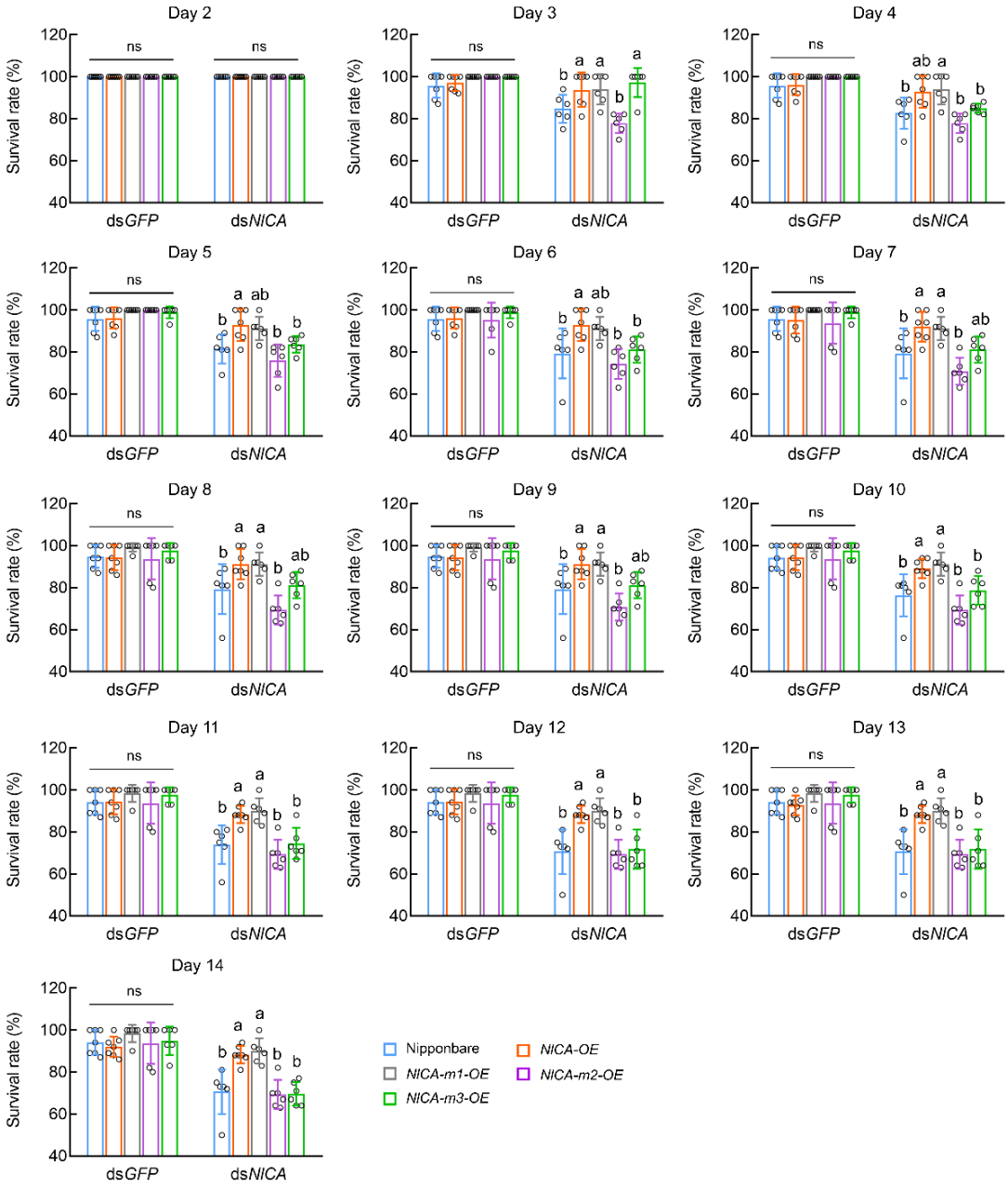

Figure S2. **The daily survival rates of ds*GFP* and ds*NICA* BPH insects on Nipponbare and *NICA* transgenic plants., related to Figure 2.** Values are represented as mean ± SEM (n > 6 biological replicates; 20 individual insects per each biological replicate). Different letters indicate statistically significant differences analyzed by two-way ANOVA (Tukey test, $P < 0.05$). Experiments were repeated three times with similar trends.

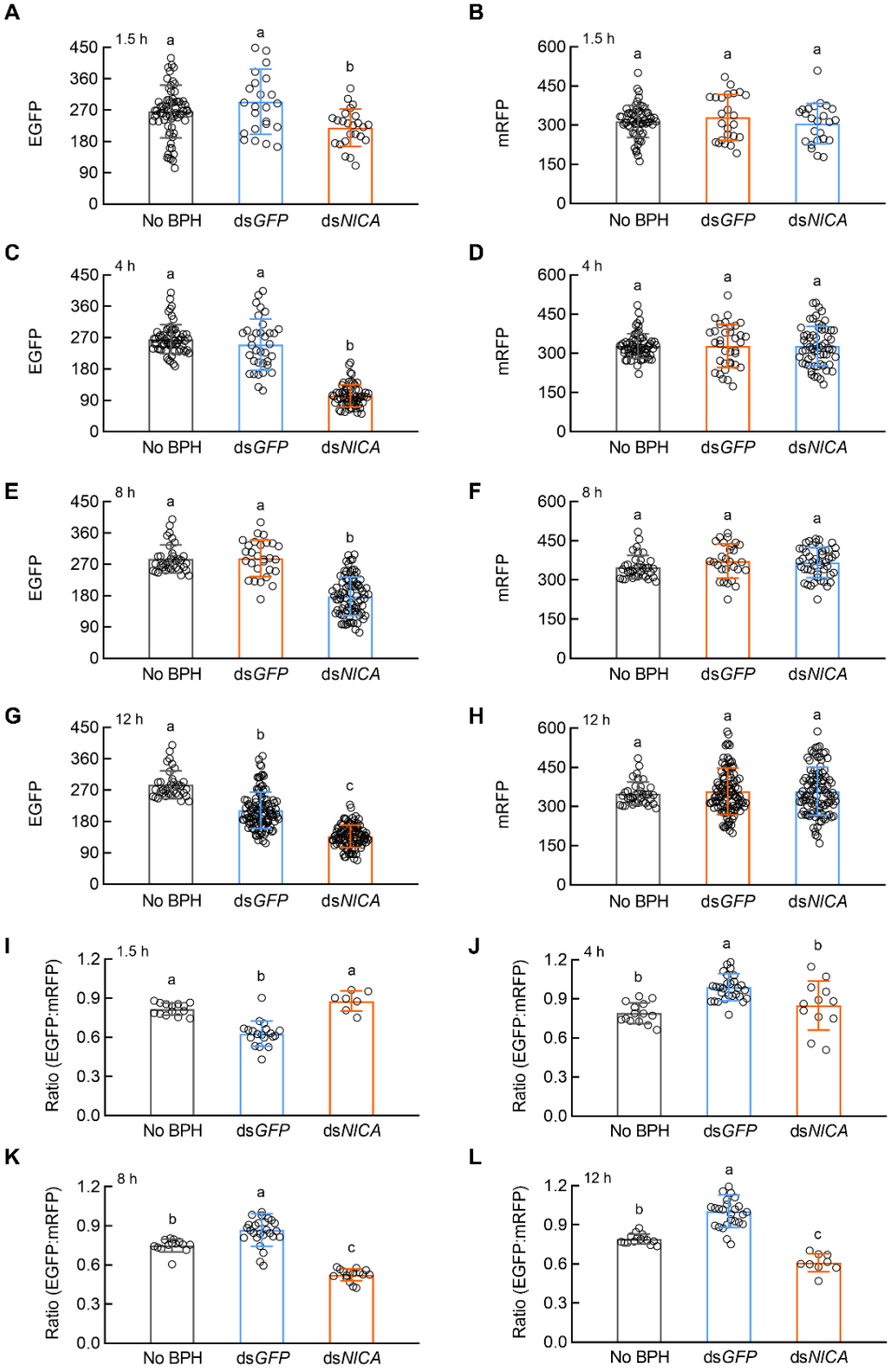

Figure S3. **Quantification of fluorescent signals in leaf sheaths of Nipponbare plants expressing the cyto-pHusion ratiometric pH sensor in response to BPH feeding, related to Figure 3.** (A, C, E, G) eGFP fluorescence intensity at 1.5, 4, 8 and 12 h after BPH feeding. (B, D, F, H) mRFP fluorescence intensity at 1.5, 4, 8 and 12 h after BPH feeding. Values are displayed as mean ± SEM (n > 14 feeding sites for each genotype). (I–L) Intracellular acidification of the mesophyll cells and epidermal cells at BPH feeding sites. EGFP:mRFP signal ratios at 1.5 h (I), 4 h (J), 8 h (K) and 12 h (L) of ds*GFP* or ds*NICA* BPH feeding compared with no BPH control. EGFP was imaged at λ_{Ex} = 500 nm and λ_{Em} = 540 nm. mRFP was imaged at λ_{Ex} = 570 nm and λ_{Em} = 620 nm. Values are displayed as mean ± SEM (n ≥ 16 circular areas of leaf sheath, each circular area had a diameter of 100 μm with the feeding site at the center).

Image processing and analysis was performed by Fiji. Different letters indicate statistically significant differences analyzed by one-way ANOVA (Tukey test, $P < 0.05$).

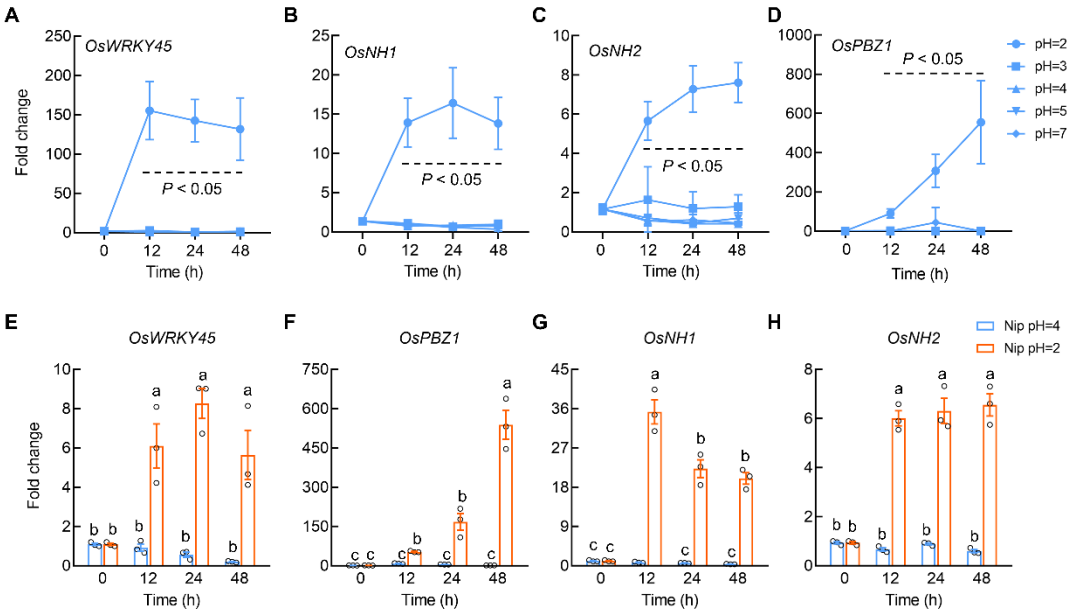

Figure S4. **Defense response genes are modulated by ectopic pH manipulation in rice, related to Figure 3.** (A-D) Expression levels of defense response genes, *OsWRKY45* (A), *OsNH1* (B), *OsNH2* (C) and *OsPBZ1* (D), are induced after acidification of Yoshida medium in which WT Nipponbare plants were grown. Rice plants were first grown in Yoshida media with pH of 4 to 5-leaf-stage and were then placed into fresh Yoshida medium with different pH for 48 h. RNA samples collected from the stem tissues for RT-qPCR in the indicated time. (E-H) Expression levels of defense response genes, *OsWRKY45* (E), *OsPBZ1* (F), *OsNH1* (G) and *OsNH2* (H), are induced after acidification of Yoshida medium in which WT Nipponbare plants were grown. Rice plants were first grown in Yoshida media with pH of 4 to 5-leaf-stage and were then placed into fresh Yoshida medium with pH of 4 or pH of 2 for 48 h. RNA samples collected from the stem tissues for RT-qPCR in the indicated time. Values represent mean ± SEM (Two-way ANOVA, n = 4). Different letters indicate statistically significant differences analyzed by two-way ANOVA (Tukey test, $P < 0.05$). Experiments were repeated three times with similar trends.

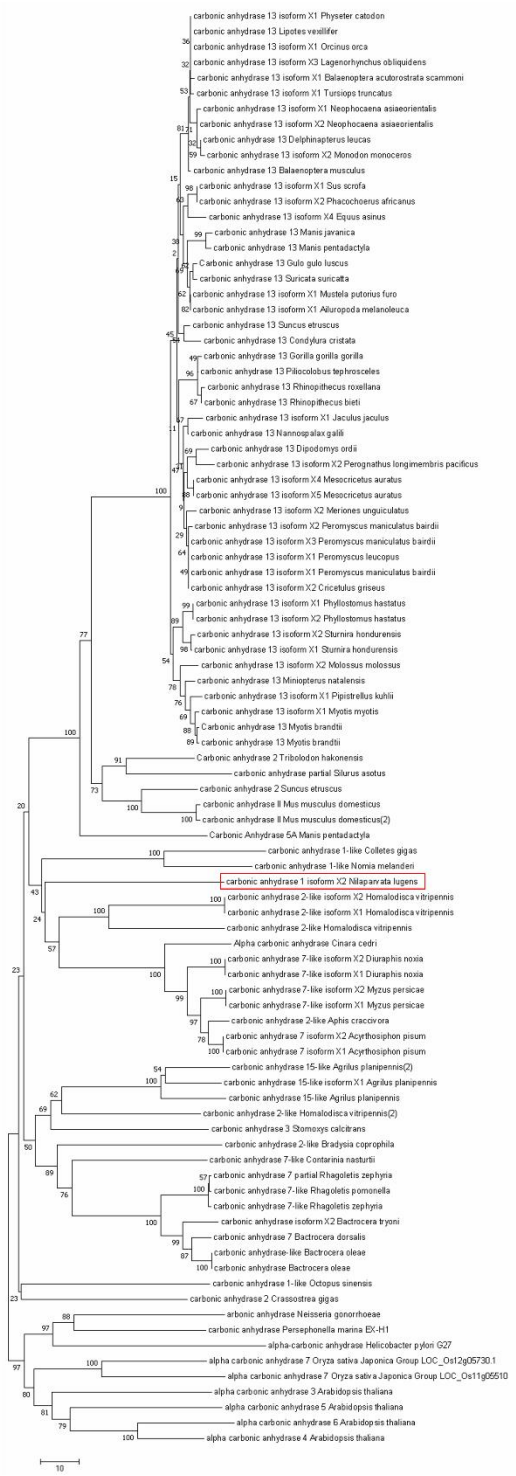

Figure S5. Phylogenic analysis of carbonic anhydrase proteins from representative bacteria, insect, mammal, and plant species, related to Figure 1. The protein sequences were obtained from NCBI (www.ncbi.nlm.nih.gov), TAIR (http://www.arabidopsis.org/) and Rice Genome Annotation Project (http://rice.uga.edu/index.shtml). Red rectangle denotes NICA. Sequence alignment was performed using Clustal W, and the phylogenetic tree was generated by MEGA 7.0 using the Neighbor–Joining method. Bootstrap values from 1,000 replicates were used to assess the robustness of the tree. The scale indicates the average number of substitutions per site.

Gene ID	Description	Accession number	References
chr11.0266.t1	*Nilaparvata lugens* uncharacterized protein (LOC11106027)	XM_022347943.1	This study
chr06.1357.t1	*Nilaparvata lugens* serpin B5 (LOC111051915)	XM_022338506.1	This study
chr07.0208.t1	*Nilaparvata lugens* 46 kDa FK506-binding nuclear protein (LOC111043757)	XM_022328800.1	This study
chr05.0798.t1	*Nilaparvata lugens* carbonic anhydrase 1-like isoform X3, NlCA	XP_022198625.1	S1
chr02.2197.t1	*Nilaparvata lugens* peritrophin-like protein	KU365934.1	S1
chr03.1919.t2	*Nilaparvata lugens* clone Nl3 carboxylesterase precursor	MF278673.1	S2
chr13.0231.t1	*Nilaparvata lugens* clone Nl48 hypothetical protein	MF278715.1	S2
chr04.0145.t1	*Nilaparvata lugens* salivary sheath protein (Nl33/NlShp)	KT764972.1, MF278701.1	S1
chr12.0438.t1	*Nilaparvata lugens* salivap-3/BISP (LOC111051577)	KU365937.1	S1, S3
chr05.1218.t1	*Nilaparvata lugens* trypsin-25	KJ512136.1	This study
chrX.1425.t1	*Nilaparvata lugens* phosphate carrier protein, mitochondrial-like (LOC111061302)	XM_022348996.1	This study
chr10.0094.t1	*Nilaparvata lugens* annexin A2-like protein, Nl66	XM_022342235.1, MF278732.1	S2
chr03.0845.t1	*Nilaparvata lugens* profilin (LOC111049006), NlSFP_unconfirmed_comp27457 seminal fluid protein	XM_022334999.1, KU932337.1	This study
chr07.0115.t1	*Nilaparvata lugens* Nl1 EF-hand motif protein (LOC111055487)	MF278671.1, XM_022342702.1	S2
chr11.0447.t1	*Nilaparvata lugens* four and a half LIM domains protein 2 (LOC111051634), prickle-like protein 3 (LOC111053000)	XM_022338188.1, XM_022339830.1	This study
chr11.0142.t1	*Nilaparvata lugens* uncharacterized (LOC111052897/LOC111052895)	XM_022339709.1, XM_022339707.1	This study
chr10.0196.t3	*Nilaparvata lugens* myosin heavy chain, muscle (LOC111049488)	XM_022335714.1	This study
chr05.0533.t1	*Nilaparvata lugens* programmed cell death protein 4 (LOC111050730)	XM_022337080.1	This study
chr03.0491.t4	*Nilaparvata lugens* peptidyl-prolyl cis-trans isomerase 6-like (LOC111043670), secreted seminal fluid protein	XM_022328683.1, KU932224.1	This study
chr13.0283.t1	*Nilaparvata lugens* cathepsin B-like cysteine proteinase 4 (LOC111048658)	XM_022334589.1	This study

chr09.0212.t1	*Nilaparvata lugens* uncharacterized (LOC111055944), Arthropod defensin	XM_022343249.1	This study
chr08.1008.t1	*Nilaparvata lugens* mRNA for lipophorin precursor (NlSP4), apolipophorins (LOC111049867)	AB465596.1, XM_022336048.1	This study
chr04.1268.t1	*Nilaparvata lugens* uncharacterized (LOC111064634), Chitin binding domain	XM_022352394.1	This study
chr04.0029.t1	*Nilaparvata lugens* histone H1-like (LOC111057020)	XM_022344439.1	This study
chr12.0536.t1	*Nilaparvata lugens* uncharacterized (LOC111050237)	XM_022336529.1	This study
chr06.0287.t3	*Nilaparvata lugens* heat shock 70 kDa protein (NlSP1), NlSFP_secreted_comp36780 seminal fluid protein	KU932222.1, XM_022337451.1	This study
chr04.1212.t1	*Nilaparvata lugens* putative inorganic phosphate cotransporter (LOC111044010)	XM_022329061.1	This study
chr09.0164.t1	*Nilaparvata lugens* trypsin-9, trypsin-like protease	AJ316142.1, KJ512120.1	This study
chr12.0385.t1	*Nilaparvata lugens* voltage-dependent anion-selective channel-like (LOC111059470)	XM_022347131.1	This study
chr05.0502.t1	*Nilaparvata lugens* apolipoprotein D-like (LOC111046805)	XM_022332442.1	This study
chrX.1656.t1	*Nilaparvata lugens* uncharacterized (LOC111053686)，ATP synthase subunit gamma, mitochondrial	XM_022340604.1	This study
chr07.0132.t1	*Nilaparvata lugens* uncharacterized (LOC111054816)	XM_022341916.1	This study
chr02.0357.t1	*Nilaparvata lugens* ATP-citrate synthase-like (LOC111048221)	XM_022334091.1, XM_022334090.1	This study
chr09.0427.t1	*Nilaparvata lugens* kallikrein-6-like (LOC111044315), uncharacterized LOC111044314	XM_022329413.1，XM_022329414.1	This study

Table S1 List of detected BPH-secreted salivary proteins, related to Figure 1. Putative “effector proteins” are highlighted in blue.

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S1. Huang, H.J., Liu, W.C., Huang, X.H., Zhou, X., Zhuo, J.C., and Zhang, C.X. (2016). Screening and Functional Analyses of *Nilaparvata lugens* Salivary Proteome. J. Proteome Res. *15*, 1883–1896.

S2. Rao, W., Zheng, X.H., Liu, B.F., Guo, Q., Guo, J.P., Wu, Y., Shangguan, X.X., Wang, H.Y.,

Wu, D., Wang, Z.Z. *et al*. (2019). Secretome analysis and in planta expression of salivary proteins identify candidate effectors from the brown planthopper, *Nilaparvata lugens*. Mol. Plant Microbe Interact. *32*, 227–239.

S3. Guo, J., Wang, H., Guan, W., Guo, Q., Wang, J., Yang, J., Peng, Y., Shan, J., Gao, M., Shi, S., *et al*. (2023). A tripartite rheostat controls self-regulated host plant resistance to insects. Nature *618*:799–807.