Intracellular acidification is a novel plant defense response countered by the brown planthopper for survival in rice

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Abstract

The brown planthopper (BPH; *Nilaparvata lugens*) is the most destructive insect pest in rice. Through a stylet, BPH secrets 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 (NlCA). The survival rate of the *NlCA*-RNA interference (RNAi) BPH insects was extremely low on rice, indicating a vital role of this salivary protein in BPH infestation. We generated *NlCA* transgenic rice plants and found that *NlCA* expressed in rice plants could restore the ability of *NlCA*-RNAi BPH to survive on rice. Next, we produced rice plants expressing the ratiometric pH sensor pHusion and found that *NlCA*-RNAi BPH induced rapid intracellular acidification of rice cells during feeding. Further analysis revealed that both *NlCA*-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 piecing/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.

Significance Statement

Insect pests pose a serious biotic threat to crop production worldwide. Understanding how insect pests attack plants could inspire innovative pest control measures to enhance global food security. The brown planthopper (BPH; *Nilaparvata lugens*) is the most devastative insect pest in rice. In this study, we discovered 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-mediated intracellular pH stabilization is linked to diminished defense responses. These findings not only uncovered that intracellular pH homeostasis is a previously uncharacterized battleground in plant-piecing/sucking insect interactions, but also open a door to potentially using NICA as a valuable molecular tool to gain insights into the broad impact of intracellular pH homeostasis on plant cell physiology.

Main Text

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 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 damages, the BPH may also indirectly damage rice plants by oviposition and transmitting viral disease agents (3, 4).

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

A critical step in BPH infestation of rice is secreting bioactive substances into the plant tissues through the stylet (6-8). Specifically, during feeding, 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 is 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, NlEG1, a salivary endo-b-1,4-glucanase, degrades plant celluloses to help the BPH's stylet reach to the phloem (11). NlSEF1, an EF-hand 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, mucin-like protein (NlMLP) triggers defense responses in rice cells, including cell death, callose deposition and up-regulation of pathogen-responsive genes (14, 15).

Carbonic anhydrases (CAs) (EC 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). NlCA belongs to the α-CA subfamily. Our previous study showed that *NlCA* expressed in BPH salivary glands (6). Surprisingly, however, RNA interference (RNAi) of the *NlCA* transcript in BPH insects affects neither pH maintenance within the salivary gland, watery saliva or gut, nor insect feeding behavior or honeydew excretion, 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 NlCA is secreted into the rice tissues and functions as an effector that stabilizes host cell intracellular pH, accompanied by suppression of defense responses, during BPH feeding. Thus, we have uncovered intracellular pH homeostasis is a previously uncharacterized battleground in plant-insect interactions.

Results

NlCA is detected in rice sheath tissues during BPH feeding. NlCA was previously found to be highly expressed in salivary glands and present in the watery saliva of BPH fed on 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 principal gland (APG) (Fig. 1*A*), which further raised the possibility that NlCA 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

chromatograph-mass spectrometer (LC-MS). We found 8 NICA-specific peptides in BPH-fed leaf sheath tissue (Fig. 1*B; SI Appendix,* Fig. S1), confirming that NlCA is secreted into the host tissues during BPH feeding.

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 NlCA in the BPH-rice interaction, we produced transgenic Nipponbare plants expressing *NlCA* (see Methods). A total of 26 lines were produced and 6 lines were found to robustly express the *NlCA* transcript. *NlCA*-expressing plants exhibited no noticeable changes in appearance compared to Nipponbare plants (*SI Appendix,* Fig. S2). *NlCA*-expressing plant lines were propagated to T3 generation, and 3 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). The sequence of *NlCA* used for RNAi treatment is expected to be specific to BPH (*SI Appendix,* Fig. S3). 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 2*A*). There were no significant differences in 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 (Fig. 2*B*). 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 Nipponbare plants (Fig. 2*C*). 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 (Fig. 2*C*; *SI Appendix,* Fig. S4), demonstrating that *Nl*CA stably expressed in the host tissue 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. NlCA contains several conserved amino acid residues predicted to be at the catalytic site of carbonic anhydrases (Fig. 2*D*). We asked if some of these conserved active site residues are critical to the function of *Nl*CA in the rice-BPH interaction. Accordingly, we expressed three different *NlCA* mutants in Nipponbare plants. "*NlCA-m1*" plants contain a *NlCA* transgene in which His^{159} and His^{161} were mutated to Ala; "*NlCA-m2*" plants contain a *NlCA* transgene in which Glu^{182} and His^{184} were replaced by Ala; and "*NlCA-m3*" plants carry a *NlCA* 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 *NlCA* transgenic plants, transgenic plants expressing these *NlCA*-mutants

exhibited no noticeable changes in appearance (*SI Appendix,* Fig. S2). We conducted BPH survival assay and found that, in contrast to plants expressing wild-type *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 (Fig. 2*E* and *F*; *SI Appendix,* Fig. S5). Thus, several conservative residues at the predicted catalytic site are indispensable for the function of *NI*CA inside the plant cell.

After having demonstrated 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 *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 colocalized in the cytoplasm but not in the nucleus (Fig. 1*C; SI Appendix,* Fig. S6).

NICA counters rapid intracellular acidification during BPH feeding of rice. BPH feeding on rice plants can be divided into two phases based on the electropenetrography (EPG) waveforms (23). The first phase involves BPH's stylet penetrates 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 expressed a cytoplasmic ratiometric pH sensor, 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 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. As shown in the Fig. 3*B-E*, control ds*GFP* BPH feeding did not elicit an obvious intracellular pH change in rice cells during this period. In contrast, ds*NICA* BPH feeding induced a significant pH decrease at 4 h and 8 h (Fig. 3*C* and *D*). The decreased ratio of EGFP: mRFP was caused by the quenched EGFP signal after ds*NICA* BPH feeding, while the average signal intensity of the internal control, mRFP, kept at a stable level (*SI Appendix,* Fig. S7). These results uncovered a previously uncharacterized plant cellular response, rapid intracellular acidification,

during the early stage of ds*NICA* BPH feeding and demonstrated that BPH has evolved a critical virulence effector, NlCA, to counter this novel plant cellular response.

Intracellular acidification is linked to defense gene expression in rice plants. Plant defense responses are important for limiting BPH survival in rice (25-28), Although extracellular pH change has long been known as a classical plant defense response to biotic stress (29, 30), our discovery of intracellular acidification during dsNICA BPH feeding raises the intriguing possibility that intracellular pH change may be a previously missed defense response. Indeed, we found that expression of defense response genes (e.g., *OsNH1*, *OsNH2*, *OsWRKY45*, *OsWRKY13*) was significantly induced at a higher level by ds*NlCA* BPH infestation than by ds*GFP* BPH control at 4 h and 8 h (Fig. 3*F-I*), which is consistent with the intracellular pH change at 4 h and 8 h (Fig. 3 *C-D*). These results provide evidence that intercellular acidification is linked to rice defense activation. Next, we conducted experiments to determine if ectopic intracellular acidification, in the absence of BPH infestation, would trigger defense gene expression. Rice is normally grown in Yoshida medium, pH of 4 (31). To test if 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 to fresh Yoshida medium with pH adjusted to 2, 3, 4, 5, 6 and 7, respectively, for 48 hours. Interestingly, drastically increased expression of defense response genes (e.g., *OsNH1*, *OsNH2*, *OsWRKY45*, *OsPBZ1*) was observed in Nipponbare plants at acidic pH of 2 (*SI Appendix,* Figs. S8, S9). The pHusion sensor plants exposed to external pH of 2 showed significant intracellular acidification (Fig. 3*J-K*). Survival rates of BPH insects were lower in Nipponbare plants growing in media with pH of 2, compared to those growing in media with pH of 4 (Fig. 3*L*). 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 NICA stabilizes intracellular pH during WT BPH feeding (Fig. 3*A-D*) and both ds*NlCA* BPH feeding and ectopic intracellular acidification cause activation of defense gene expression (Fig 3*F-I*; *SI Appendix,* Fig. S8-9), we next tested the hypothesis that a major function of NICA-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 feeding of piercing-sucking insects (32). We examined this response using aniline blue to stain callose in the phloem sieve cells. Indeed, we found that fewer and smaller callose deposition was found in the sieve plates of *NlCA*-expressing leaf sheaths compared to those found in the sieve plates of Nipponbare fed by BPH (Fig. 4*A-F*), demonstrating that *Nl*CA suppresses callose deposition in sieve cell pates. Interestingly, the transcript levels of callose biosynthesis genes, such as *OsGSL1*, *OsGSL3*, *OsGSL5* and *OsGns5,* were expressed at lower levels in *NlCA*

transgenic plants compared to Nipponbare plants (Fig. 4*G-J*). We also measured the transcript levels of defense marker genes (e.g., *OsNH1, OsNH2, OsWRKY45* and *OsWRKY13*) in Nipponbare and *NlCA*-expressing plants fed by ds*GFP* and ds*NlCA* BPH. As shown in Fig. 4*K-N*, induced expression of *OsNH1, OsNH2, OsWRKY13* and *OsWRKY45* by ds*NlCA* BPH was suppressed in *NlCA* transgenic plants compared to those in Nipponbare plants. Collectively, these results showed that NICA-mediated intracellular acidification 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 NICA in the rice-BPH interaction. We found that the *NlCA* transcript is detected mainly in the salivary glands (Fig. 1*A*) and that the NICA protein is found in rice tissues fed by BPH (Fig. 1*B*). *NlCA*-silenced (ds*NlCA*) insect survived very poorly on at least two independent cultivars of rice plants, Xiushui134 and Nipponbare ((6), Fig. 2), whereas *NlCA* expressing rice plants can restore the normal survival of *NlCA*-silenced (ds*NlCA*) insects (Fig. 2; *SI Appendix,* Fig. S4), 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 (Fig. 3*A-D*). Pathogen/insect-derived effectors can be powerful molecular probes to discovering novel plant regulators/responses to biotic attacks. Although several piercing/sucking herbivores derived effectors, such as Btfer1, LsPDI1, LsSP1, Mp55, DNase II and BISP, have been reported as defense-modulating effectors (33-38), the discovery of intracellular acidification as a previously uncharacterized plant defense response is unprecedented in plant-biotic interactions.

Our demonstration of a link between intracellular acidification and defense activation has laid a foundation for future discovery of potentially diverse pH-sensitive intracellular regulators of defense responses, which could add a new dimension in the study of plant-biotic interactions. Of note, extracellular alkalinization of culture 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, are perceived by the FERONIA-family receptors (39, 40). There is evidence that this perception causes phosphorylation of PM-localized H(+)-ATPase 2, resulting in the inhibition of proton transport across the PM (41). 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, intracellular pH change as a plant

defense response has so far escaped the discovery by researchers until this study. Future research might investigate if intracellular acidification in the BPH-rice interaction is linked to extracellular alkalinization. However, ectopic extracellular acidification of rice growth medium causes intracellular acidification and activates defense response genes (*SI Appendix,* Figs. S8, S9), which is opposite to what would be expected if intracellular acidification-associated defense activation requires extracellular alkalinization. Thus, it appears that intracellular acidification can activate plant defense independently of extracellular alkalinization.

CAs are universally present in all organisms (*SI Appendix,* Fig. S10); other piecing/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* (46) and aphid *Myzus persicae* (42). In the case of *M. persicae,* CA-II was shown to increase viral transmission via plant apoplastic acidification-mediated acceleration of intracellular vesicular trafficking (42). However, because NICA 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 more likely that insect-secreted CAs constitute play a primary role in facilitating insect survival by countering intracellular acidification-associated defense activation. In fact, future research should examine if CA-mediated increase in viral transmission may be partly an indirect consequence of defense suppression, which was not examined in the previous study (47).

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 the 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* (43). Fungi employ a conserved pathway, mediated by Rim101 and PacC, to sense external pH (44). 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 (45). 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* (Fig. 3; and *SI Appendix,* Figs. S8, S9). In reverse, NICA-mediated intracellular pH stabilization dampens these defense responses (Fig. 4). Callose deposition in phloem sieve cells, in particular, is a classical defense response to a variety of sucking/piecing insects and is thought to limit nutrient flow during insect feeding (32, 46-50). Nevertheless, 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. 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.

Materials and Methods

Experimental materials and methods, including *in situ* mRNA hybridization, LC-MS, transgenic rice construction, RNA interference of BPH, BPH survival test, pH assay and callose deposition staining, can be found in *SI Appendix, Supplementary Materials and Methods.*

Data Availability.

All data are presented within this paper.

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Figures

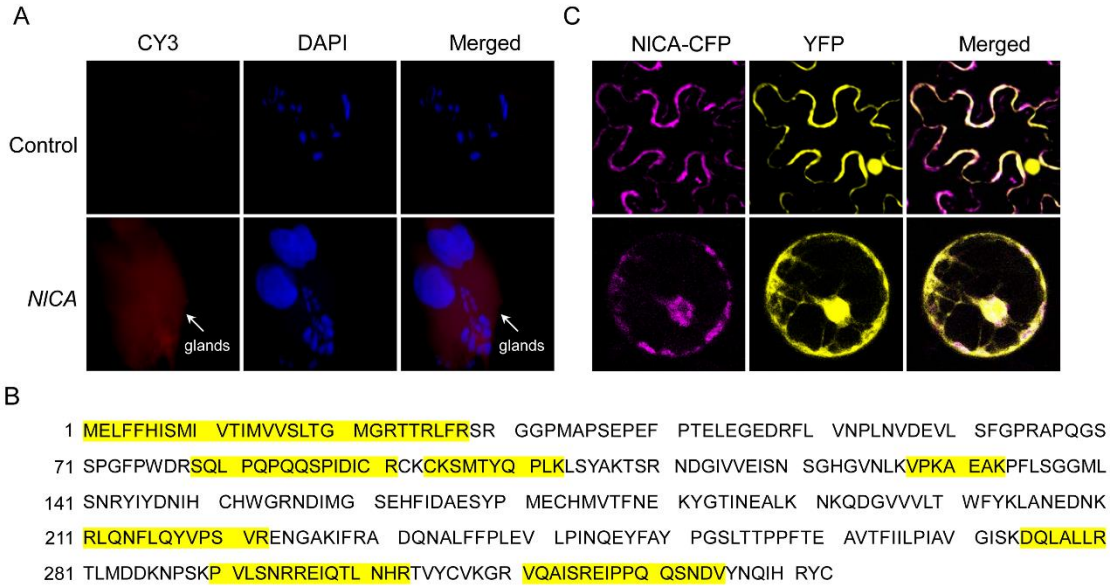

Fig. 1. Initial characterization of *Nilaparvata lugens* carbonic anhydrase (NlCA). (*A*) *in situ* RNA hybridisation of salivary glands in 5-instar BPHs (lower row) using antisense NlCA sequence as a probe. Sense *NlCA* probe was used as a negative control (upper row). White arrows indicate glands. Nuclei are stained blue by 4',6-diamidino-2-phenylindole (DAPI). (*B*) The amino acid sequence of NlCA. The highlighted amino acid residues indicate the peptides detected in BPH-infested rice sheath tissue by LC-MS analysis. (*C*) NlCA is colocalized with the YFP signals in *N. benthamiana* (upper row) and rice cells (lower row). YFP and NlCA-CFP fusion proteins were co-expressed in *N. benthamiana* leaf cells for 48 h using the *Agrobacterium*-mediated transient expression method. YFP and NlCA-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.

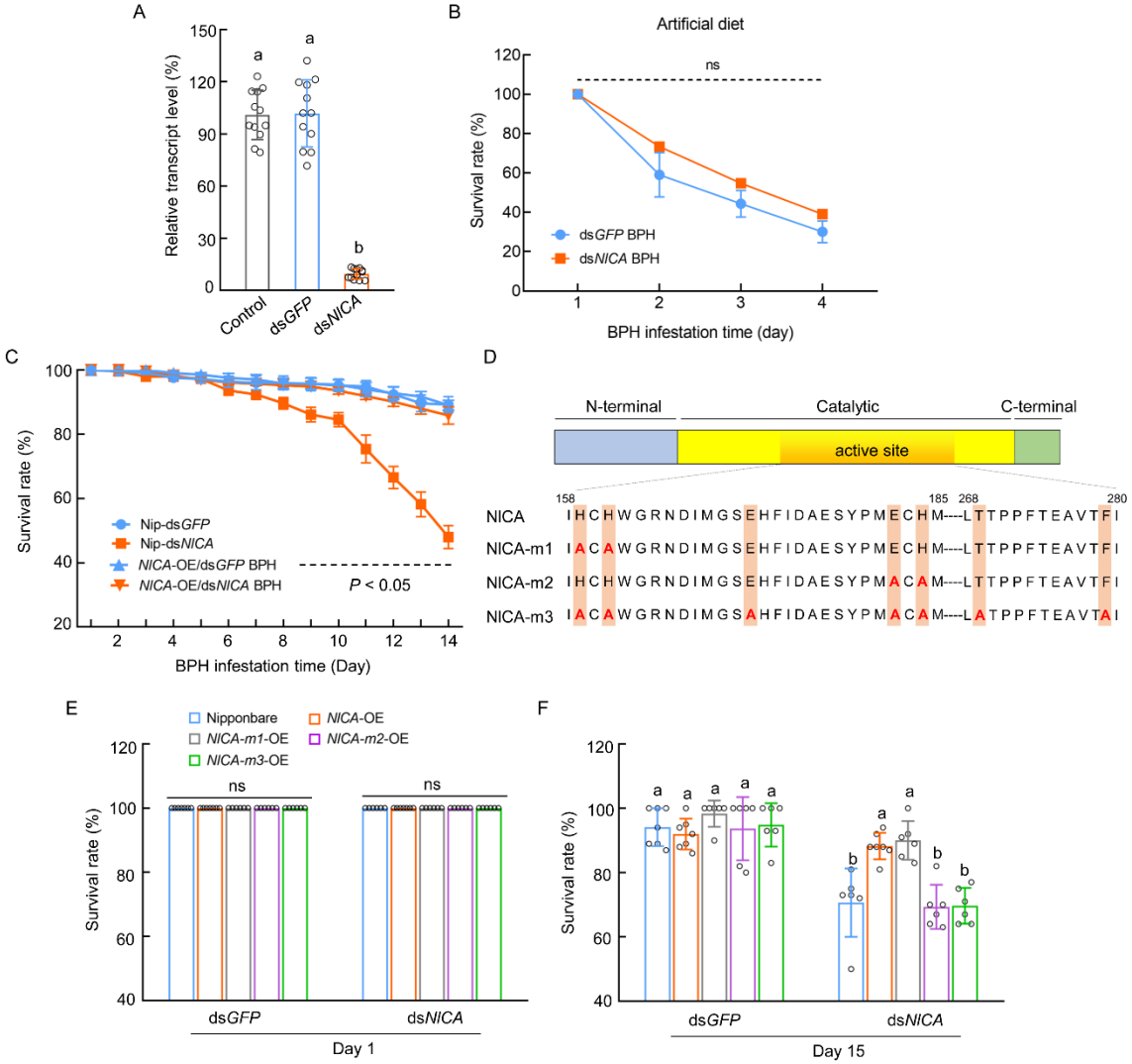

Fig. 2. Effects of *NICA* RNAi on BPH survival on rice cultivar Nipponbare. (*A*) The *NICA* transcript levels in BPH at 3 dpi post-injection of ds*GFP* or ds*NICA* were determined by qRT-PCR (displayed as % of the *NICA* transcript abundance in control BPH). Values are displayed as mean ± SEM of 4 experimental 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 (one 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 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 start day 9 between ds*NICA* BPH on Nipponbare (Nip) and other treatments. (*D*) Schematic display of

the NlCA protein with the N-terminus displayed in bule, the C-terminus in green and the central catalytic domain in yellow/orange. The amino acid 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. (*E-F*) The survival rates of 20 ds*GFP* and ds*NlCA* BPH insects in Nipponbare and *NICA* transgenic plants after 1- and 15-days post-infestation. Values are displayed as mean ± SEM (n ≥ 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.

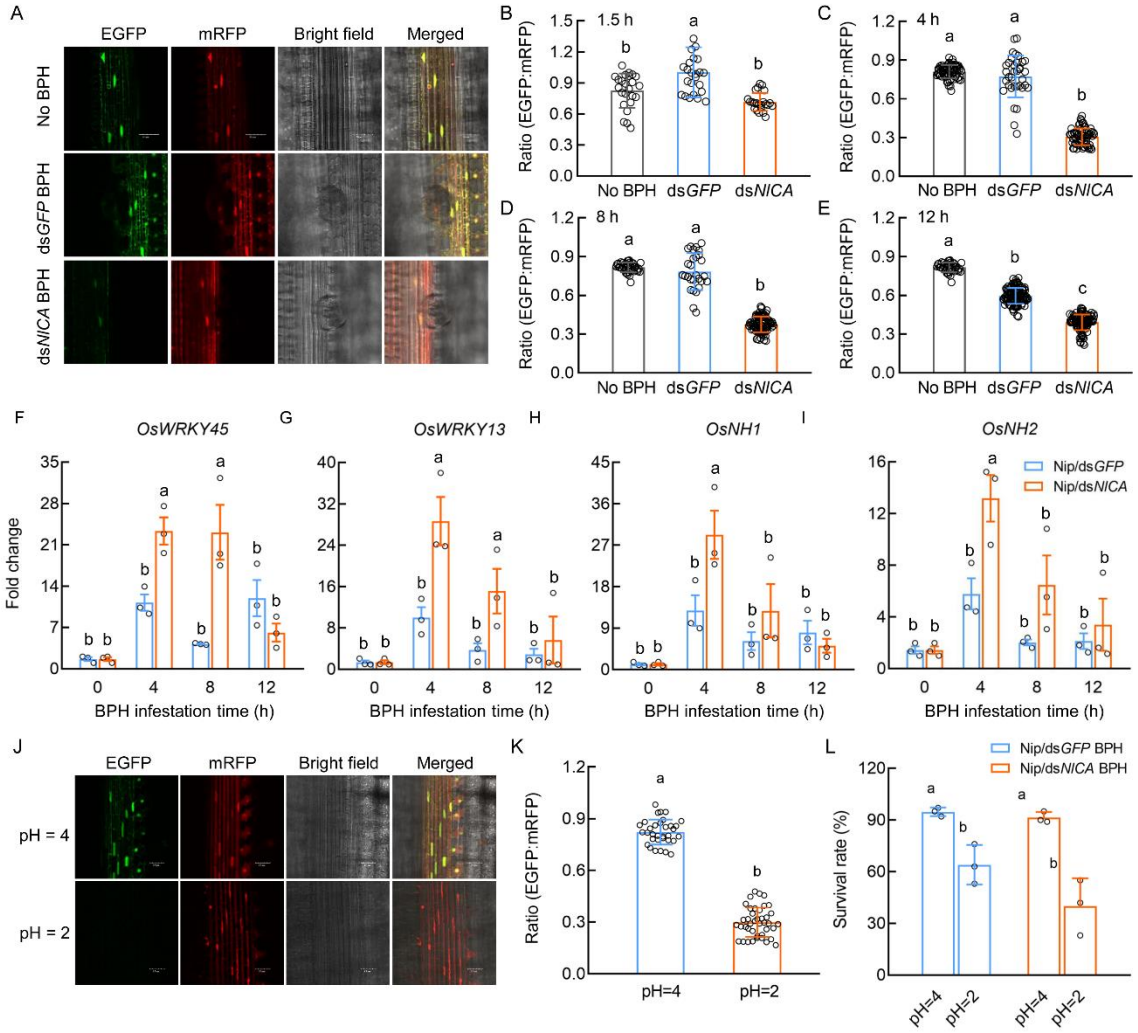

Figure 3. Role of NlCA in maintaining intracellular pH of rice cells. (*A*) Confocal microscopic images at BPH feeding sites in Nipponbare plants expressing a ratiometric cytoplasmic pH sensor (Cyto-pHusion) 4 h after placing 30 5th-instar BPH nymphs on each plant. Confocal images from plants with no BPH feeding served as the control. (*B-E*) EGFP:mRFP signal ratios at 1.5 h (B), 4 h (C), 8 h (D) and 12 h (E) of ds*GFP* or ds*NlCA* 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 ≥ 24 circular areas of leaf sheath, each circular area had a diameter of 100 μm with the feeding site at the center). (*F-I*) Expression of defense response genes, *OsWRKY45*, *OsWRKY13*, *OsNH1* and *OsNH2*, in Nipponbare plants infested by ds*GFP* or ds*NlCA* BPH. Values are displayed as mean ± SEM of three biological replicates. Each biological replicate represents pooled leaf sheaths from three individual rice plants fed 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 pH of 2. Confocal images from plants grown in Yoshida medium with pH of 4 served as the control. (*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 ≥ 33 calculation area per condition). (*L*) The 7th-day survival rates of ds*GFP*- and ds*NlCA*-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 each biological replicate). Different letters indicate statistically significant differences analyzed by two-way ANOVA (Tukey test, $P < 0.05$). Experiments were repeated at least three times with similar trends in this figure.

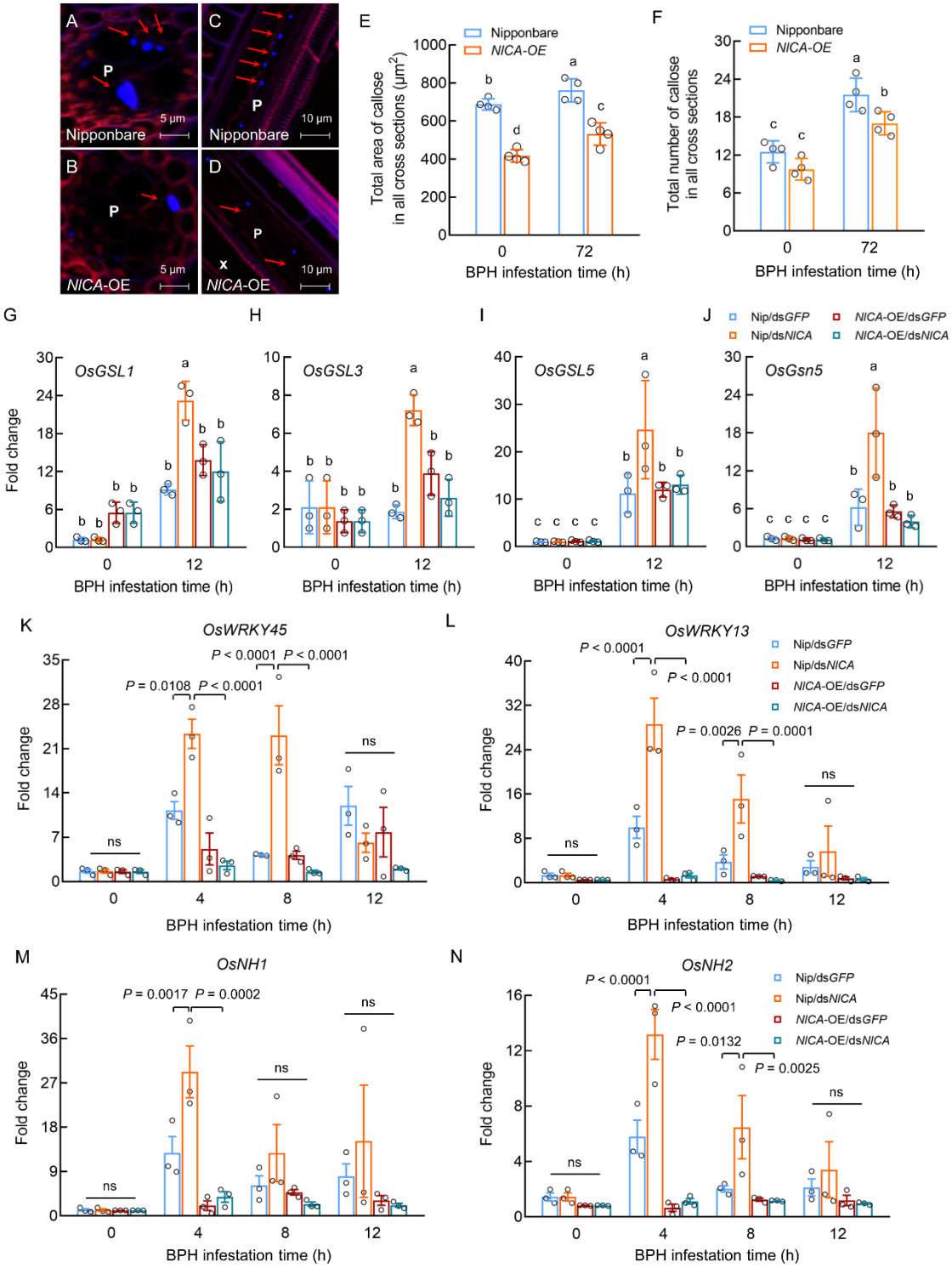

Figure 4. NICA dampens callose deposition and defense gene expression. (*A-D*) Callose accumulation (bright blue fluorescence indicated by red arrows) on the sieve plates of Nipponbare leaf sheaths (*A* and *C*) and *NICA-OE* leaf sheaths (*B* and *D*) plants before and 72 h

after BPH feeding. P, phloem; X, xylem. The pictures were taken under a Zeiss Microscope. (*A*-*B*) Cross-sections. (*C*-*D*) Longitudinal sections. (*E*) 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. (*F*) 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. (*G*-*J*) Relative expression levels of the callose synthase gene *OsGSL1* (*G*), *OsGSL3* (*H*), *OsGSL5* (*I*) and *OsGns5* (*J*) in response to BPH feeding. Values are displayed as mean ± SEM of 3 biological replicates. Each biological replicate represents pooled leaf sheath form three individual rice plants fed by 20 5th instar BPH nymphs per plant. (*K*-*N*) Expression of defense marker gene *OsWRKY45* (*K*), *OsWRKY13* (*L*), *OsNH1* (*M*) and *OsNH1* (*N*) in response to BPH feeding. Values are displayed as mean ± SEM of 3 biological replicates. Each biological replicate represents pooled leaf sheaths from three individual rice plants fed 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.