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 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 (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 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-piercing/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, NIEG1, a salivary endo-b-1,4-glucanase, degrades plant cellulosics to help the BPH’s stylet reach to the phloem (11). NISEF1, an EF-hand Ca\(^{2+}\)-binding protein, interferes with calcium signaling and H\(_2\)O\(_2\) 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 (NIMLP) 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 (\(\alpha\)-, \(\beta\)-, \(\gamma\)-, \(\delta\)-, and \(\varepsilon\)-CAs) and three of them (\(\alpha\)-, \(\beta\)-, and \(\gamma\)-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 \(\alpha\)-CA subfamily. Our previous study showed that NICA expressed in BPH salivary glands (6).

Surprisingly, however, RNA interference (RNAi) of the NICA 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 NICA-RNAi BPH feeding results in rapid intracellular acidification of rice cells. We found that NICA 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**

**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 artificial diet (6). We conducted a more detailed characterization of NICA expression in this study. RNA in situ hybridization showed that the expression level of NICA was detectable throughout the principal glands (PGs) and accessory glands (AGs), but not in A-follicle of the principal gland (APG) (Fig. 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
chromatograph-mass spectrometer (LC-MS). We found 8 NICA-specific peptides in BPH-fed leaf sheath tissue (Fig. 1B; SI Appendix, Fig. S1), confirming that NICA is secreted into the host tissues during BPH feeding.

**Transgenic expression of NICA in rice rescues the ability of NICA-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 NICA (see Methods). A total of 26 lines were produced and 6 lines were found to robustly express the NICA transcript. NICA-expressing plants exhibited no noticeable changes in appearance compared to Nipponbare plants (SI Appendix, Fig. S2). NICA-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 NICA (dsNICA) or the control green fluorescent protein gene (dsGFP) was injected into 3rd instar BPH nymphs to initiate RNAi of the NICA transcript (22). The sequence of NICA 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 NICA gene were reduced by 99% and 97%, respectively, in tested individuals when compared with non-RNAi control and dsGFP-treated insects (Figure 2A). There were no significant differences in survival rates between dsGFP and dsNICA BPH when fed on the artificial diet, indicating that silencing NICA expression has no obvious impact on the basic physiology of BPH (Fig. 2B). However, we found that the survival rate of dsNICA BPH was sharply decreased, starting at day 9 post-infestation, to ~40% at day 14, whereas the control dsGFP BPH survived normally on wild-type Nipponbare plants (Fig. 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, NICA transgenic plants almost fully restored the survival of NICA-silenced BPH insects (Fig. 2C; SI Appendix, Fig. S4), demonstrating that NICA stably expressed in the host tissue can complement the infestation defect of NICA-silenced BPH insects.

**The conserved catalytic site amino acids of NICA are critical to its function in rice.** NICA contains several conserved amino acid residues predicted to be at the catalytic site of carbonic anhydrases (Fig. 2D). We asked if some of these conserved active site residues are critical to the function of NICA in the rice-BPH interaction. Accordingly, we expressed three different NICA mutants in Nipponbare plants. “NICA-m1” plants contain a NICA transgene in which His159 and His161 were mutated to Ala; “NICA-m2” plants contain a NICA transgene in which Glu182 and His184 were replaced by Ala; and “NICA-m3” plants carry a NICA transgene in which all seven conserved active site residues, His159, His161, Glu171, Glu182, His184, Thr269 and Phe279, were changed to Ala. Like NICA transgenic plants, transgenic plants expressing these NICA-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 dsNICA BPH (Fig. 2E and F; SI Appendix, Fig. S5). Thus, several conservative residues at the predicted catalytic site are indispensable for the function of NICA 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. 1C; 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. 3B-E, control dsGFP BPH feeding did not elicit an obvious intracellular pH change in rice cells during this period. In contrast, dsNICA BPH feeding induced a significant pH decrease at 4 h and 8 h (Fig. 3C and D). The decreased ratio of EGFP: mRFP was caused by the quenched EGFP signal after dsNICA 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 dsNICA BPH feeding and demonstrated that BPH has evolved a critical virulence effector, NICA, 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 dsNICA BPH infestation than by dsGFP BPH control at 4 h and 8 h (Fig. 3F-I), which is consistent with the intracellular pH change at 4 h and 8 h (Fig. 3 C-D). These results provide evidence that intracellular 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. 3J-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. 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 NICA.** As NICA stabilizes intracellular pH during WT BPH feeding (Fig. 3A-D) and both dsNICA BPH feeding and ectopic intracellular acidification cause activation of defense gene expression (Fig 3F-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 NICA-expressing leaf sheaths compared to those found in the sieve plates of Nipponbare fed by BPH (Fig. 4A-F), demonstrating that NICA 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 NICA
transgenic plants compared to Nipponbare plants (Fig. 4G-J). We also measured the transcript levels of defense marker genes (e.g., OsNH1, OsNH2, OsWRKY45 and OsWRKY13) in Nipponbare and NICA-expressing plants fed by dsGFP and dsNICA BPH. As shown in Fig. 4K-N, induced expression of OsNH1, OsNH2, OsWRKY13 and OsWRKY45 by dsNICA BPH was suppressed in NICA 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 NICA transcript is detected mainly in the salivary glands (Fig. 1A) and that the NICA protein is found in rice tissues fed by BPH (Fig. 1B). NICA-silenced (dsNICA) insect survived very poorly on at least two independent cultivars of rice plants, Xiushuai34 and Nipponbare ((6), Fig. 2), whereas NICA expressing rice plants can restore the normal survival of NICA-silenced (dsNICA) insects (Fig. 2; SI Appendix, Fig. S4), suggesting that NICA functions in plant cells. Using the cytoplasm pH sensor, we found that NICA is required for BPH to maintain a normal plant cytoplasm pH during BPH feeding (Fig. 3A-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 alkalization. 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 alkalization. Thus, it appears that intracellular acidification can activate plant defense independently of extracellular alkalization.

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/piercing 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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References


Fig. 1. Initial characterization of *Nilaparvata lugens* carbonic anhydrase (NICA). (A) *in situ* RNA hybridisation of salivary glands in 5-instar BPHs (lower row) using antisense NICA sequence as a probe. Sense NICA 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 NICA. The highlighted amino acid residues indicate the peptides detected in BPH-infested rice sheath tissue by LC-MS analysis. (C) NICA is colocalized with the YFP signals in *N. benthamiana* (upper row) and rice cells (lower row). 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.
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 dsGFP or dsNICA 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 dsNICA-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 dsNICA-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 dsNICA BPH on Nipponbare (Nip) and other treatments. (D) Schematic display of
the NICA protein with the N-terminus displayed in blue, 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 NICA-m1, NICA-m2 and NICA-m3 are indicated in red.

(E-F) The survival rates of 20 dsGFP and dsNICA 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 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. 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 dsGFP or dsNICA BPH treatment compared with no BPH control. EGFP was imaged at \( \lambda_{\text{Ex}} = 500 \, \text{nm} \) and \( \lambda_{\text{Em}} = 540 \, \text{nm} \). mRFP was imaged at \( \lambda_{\text{Ex}} = 570 \, \text{nm} \) and \( \lambda_{\text{Em}} = 620 \, \text{nm} \). Values are displayed as mean \( \pm \) SEM (n ≥ 24 circular areas of leaf sheath, each circular area had a diameter of 100 \( \mu \text{m} \) with the feeding site at the center). (F-I) Expression of defense response genes, OsWRKY45, OsWRKY13, OsNH1 and OsNH2, in Nipponbare plants infested by dsGFP or dsNICA BPH. Values are displayed as mean \( \pm \) 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 $\lambda_{\text{Ex}} = 500$ nm and $\lambda_{\text{Em}} = 540$ nm. mRFP was imaged at $\lambda_{\text{Ex}} = 570$ nm and $\lambda_{\text{Em}} = 620$ nm. Values are displayed as mean ± SEM (n ≥ 33 calculation area per condition). (L) The 7th-day survival rates of dsGFP- and dsNICA-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.