BRHIS1 suppresses rice innate immunity through binding to monoubiquitinated H2A and H2B variants

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Abstract

In the absence of pathogen attack, organisms usually suppress immune responses to reduce the negative effects of disease resistance. Monoubiquitination of histone variants at specific gene loci is crucial for gene expression, but its involvement in the regulation of plant immunity remains unclear. Here, we show that a rice SWI/SNF2 ATPase gene *BRHIS1* is downregulated in response to the rice blast fungal pathogen or to the defense-priming-inducing compound BIT (1,2-benzisothiazol-3(2h)-one,1, 1-dioxide). The BRHIS1-containing complex represses the expression of some disease defense-related genes, including the pathogenesis-related gene *OsPBZc* and the leucine-rich-repeat (LRR) receptor-like protein kinase gene *OsSIRK1*. This is achieved through BRHIS1 recruitment to the promoter regions of target genes through specific interaction with monoubiquitinated histone variants H2B.7 and H2A.Xa/H2A.Xb/H2A.3, in the absence of pathogen attack or BIT treatment. Our results show that rice disease defense genes are initially organized in an expression-ready state by specific monoubiquitination of H2A and H2B variants deposited on their promoter regions, but are kept suppressed by the BRHIS1 complex, facilitating the prompt initiation of innate immune responses in response to infection through the stringent regulation of BRHIS1.

Introduction

Due to sessility, plants rely on a complex, sophisticated, innate immune system to fight pathogen assaults. The activation of inducible defenses may bring about costs that can negatively affect fitness; therefore, plant immune system is usually suppressed or minimally expressed until induced in response to pathogen attack [1,2]. Like immunity in invertebrate animals, the plant immune system enables the primary pathogen infection to induce lifelong enhanced resistance to the secondary infection. This common immune memory correlates with the so-called cellular priming that renders more rapid and robust responses to secondary attacks to primed cells than to non-primed cells [3,4]. Defense priming can be induced by pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively), damage-associated molecular patterns (DAMPs), pathogen effectors, wound stimuli, or treatments with some natural or synthetic compounds. This process has been recently proven pivotal to diverse types of systemic plant immunity [3,5–8], including systemic acquired resistance (SAR) [8–10], induced systemic resistance (ISR) [5–7,11], the resistance provided by symbiotic fungi [12], β-aminobutyric acid-induced resistance (BABA-IR) [13], and wound-induced resistance [5–7,14], and thus enables its promising application in sustainable modern pest management in the field since some priming-inducing compounds have been used as pesticides on the basis of their known plant health- and yield-increasing effects [3,15].

However, until recently the underlying molecular mechanism of cellular defense priming remains largely unclear. A widely acceptable hypothesis proposes that some dormant cellular signaling components, such as mitogen-activated protein kinases, are activated during defense priming by exposure to secondary biotic or abiotic stresses [3,4]. Additionally, the emerging data have also linked this induced plant immunity to epigenetic modifications, such as histone H3 and H4 acetylation, H3K4 methylation, and H2A.Z—an H2A variant—replacement [16–18]. Monoubiquitination of histone variants, however, has not yet been correlated with plant disease resistance despite its critical role in gene activation [19,20].

Belonging to the DNA-dependent ATPase family, SWI/SNF2 proteins are responsible for chromatin modification and gene activation [21,22], suggesting that the epigenetic regulation by SNF2 proteins may play important roles in defense priming.

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So far, four members of this SNF2 family in *Arabidopsis*, Photoperiod-Independent Early flowering 1 (PIE1), Splayed (SYD), Brahma (BRM), and Decrease in DNA Methylation 1 (DDM1), have been found to function as chromatin remodellers in the epigenetic control of disease resistance [23]. PIE1 [17,18] and BRM [24] are involved in the constitutive repression of SAR, while SYD is associated with the activation of some genes in the JA (jasmonate)/ET (ethylene) signaling pathway and with the resistance against *Botrytis cinerea* [25]. DDM1 seems to play a role in maintenance of the stability of nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins [26–28]. Except for the confirmed role of PIE1 in H2A.Z deposition on the promoters of SA (salicylic acid)-responsive genes [17], the epigenetic molecular mechanisms of the other three SNF2 proteins (SYD, BRM, DDM1) in plant defense are not well understood.

Rice (*Oryza sativa* L.) is the most important staple food for more than half of the world's population. Rice blast disease, caused by the fungus *Magnaporthe oryzae*, annually decreases rice yields by ~13–30% [29,30]. Probenazole (3-allyloxy-1,2-benzisothiazole-1,1-dioxide, PBZ) is an effective agrochemical widely used to control rice blast disease [31,32]. Previous studies have reported that salicylic acid (SA) acts as a defense signal in the PBZ-induced resistance in adult rice at the 8-leaf stage, but not in young plants at the 4-leaf stage [32], which implies that the PBZ-induced resistance in young plants is SA-independent.

Here, we show that an SNF2 ATPase gene, named *BRHIS1*, is downregulated by 1,2-benzisothiazol-3(2h)-one,1,1-dioxide (BIT), an active metabolite of PBZ, or by the infection of *M. oryzae*, in rice seedlings. BRHIS1, interacting with OsTINP1 (a rice homolog of human TGF-β-inducible nuclear protein 1 and yeast NSA2 involved in ribosome biogenesis and cell cycle regulation and proliferation [33]), is recruited to the promoter regions of certain antioxidative stress-related and disease defense-related genes, but not to those of SA marker genes, via specific interaction with some monoubiquitinized histone variants deposited on the BRHIS1-targeted promoter regions. This interaction constantly restricts the defense gene expression to a basal level. Upon BIT treatment or pathogen attack, the BRHIS1 suppression is relieved to induce defense priming. Our data suggest the critical role of BRHIS1 in the SA-independent disease resistance of rice.

Results

BRHIS1 encodes a putative RING finger SNF2 ATPase

To elucidate the mechanism of SA-independent defense priming in rice, we performed full-length cDNA suppression subtractive hybridization to identify genes responsive to the defense-priming-inducing compound, BIT [34], using rice seedlings (21-day-old, 4-leaf stage) treated with 0.2 mM BIT by spraying. This approach identified a BIT-downregulated gene Os08g0180300, encoding a putative SWI/SNF2 class ATPase of superfamily 2 helicase [35] (Fig 1A and B). Besides the SWI/SNF2 domain, this protein also contains a RING finger domain known to mediate protein–protein interactions [22]. There are 39 putative SNF2 family genes in the rice genome [36], but their functions are unclear. As we demonstrate below, Os08g0180300 functions in
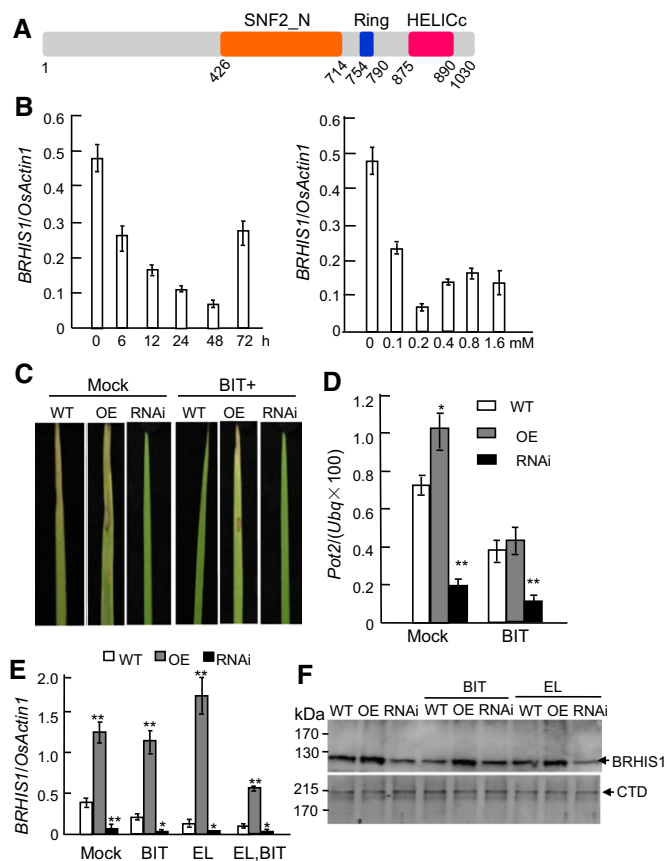

Figure 1.   BIT-downregulated RING finger SWI/SNF2 ATPase BRHIS1 is a negative regulator in rice blast resistance.

A   Functional domains of BRHIS1. HELICc, helicase superfamily conserved C-terminal domain. Numbers indicate amino acid positions.

B   Time course of *BRHIS1* expression in 21-day-old seedlings of wild-type (*japonica* rice cultivar ZH11) treated with 0.2 mM BIT (left panel), and dose-dependent (right panel) expression of *BRHIS1* after BIT treatment for 48 h.

C   Blast resistance phenotypes of the leaves of ZH11 (WT), *BRHIS1*-OE (OE), and *BRHIS1*-RNAi (RNAi) 21-day-old seedlings, 6 days after inoculation with incompatible *M. oryzae* EL0917 (EL). BIT (0.2 mM) and mock (water) were applied to the plants 24 h before fungus inoculation.

D   Growth rates of EL0917 in plants at 6 day post-inoculation were represented as the infection ratios of fungal DNA (*Pot2*) to rice host *Ubiquitin* DNA (*Ubq*) and were quantified by qPCR.

E, F   BRHIS1 mRNA (E) and protein (F) levels in ZH11, the *BRHIS1*-OE, and the *BRHIS1*-RNAi plants with or without (mock) the 24-h BIT treatment and/or pathogen inoculation (EL) were determined by qRT–PCR and immunoblotting, respectively. Relative expression values represent the mRNA level of *BRHIS1* normalized to that of *OsActin1*. RNA polymerase II carboxy-terminal domain (CTD) repeat protein was used as a loading control.

Data information: 300 seedlings were pooled for each group. Error bars indicate SD (*n* = 3), and the significant difference from WT was determined by paired two-tailed *t*-tests (**P* < 0.05; ***P* < 0.01).

priming some antioxidative stress-related and disease defense-related genes via direct interaction with several H2A and H2B variants. Therefore, we refer to it as *BIT-responsive Histone-interacting SNF2 ATPase 1* (*BRHIS1*) herein. BRHIS1 is constitutively expressed and localized in the nucleus (Fig EV1A and B),

consistent with its presumed functions in chromatin remodeling (see below).

BRHIS1 functions as a suppressor of rice blast resistance

To characterize the function of *BRHIS1*, knockdown and overexpression rice lines were obtained by RNA interference (RNAi) and the ubiquitin promoter (*Pubi*) driven *BRHIS1* cDNA. Since *BRHIS1* is BIT-responsive, we explored its potential roles in disease defense priming by assessing the responses of the *BRHIS1*-RNAi, the *BRHIS1*-OE, and the wild-type (*japonica* cultivar Zhonghua11, ZH11) plants to blast fungus. When these 21-day-old seedlings were challenged with the rice blast fungus *Magnaporthe oryzae* EL0917, the *BRHIS1*-RNAi plants exhibited the strongest resistance to the pathogen, whereas the *BRHIS1*-OE plants were mostly susceptible, with or without the BIT (0.2 mM, 24 h) pretreatment (Figs 1C and EV2A). To measure fungal growth in the inoculated plants, we used a DNA-based real-time PCR to quantify *M. oryzae* EL0917 with two primer sets specific to *M. oryzae Pot2* [37] and rice *ubiquitin*, respectively. For DNA samples from the infected leaves, the real-time PCR analysis of the infection ratio [*MgPot2*/(*Osubiquitin* × 100)] was coincident with their phenotype analysis (Fig 1D). In addition, increased resistance to EL0917 correlated with suppressed expression of *BRHIS1* mRNA and the protein (Fig 1E and F). Taken together, these results suggest that *BRHIS1* is a negative regulator of resistance to rice blast pathogen.

BRHIS1 regulates the expression of certain defense-related genes

Induction of peroxidase (POD) correlates with disease resistance [38,39]. To determine whether the enhanced resistance of the *BRHIS1*-RNAi plants is associated with the constitutive activation of peroxidase, the expression of two peroxidase genes, *OsPrx41* (NM_001056594.1) and *OsPOXgX9* (D16442.1), and POD activities, were investigated. The results showed that both the *POD* gene expression and POD activities were upregulated in the *BRHIS1*-RNAi seedlings, indicating that the downregulation of *BRHIS1* constitutively activates defense responses (Fig 2A). However, neither *BRHIS1*-RNAi nor *BRHIS*-OE affected the expression of three SA marker genes, *OsNPR1* [40], *OsWRKY03* [41], and *OsWRKY71* [42] (Fig EV2B), which suggests that the BRHIS1-mediated disease defense priming is SA-independent.

Next, we performed whole transcriptome sequencing (RNA-seq) to screen BRHIS1 target genes using 21-day-old seedlings of the *BRHIS1*-RNAi and the WT, ZH11. Thirty upregulated (over twofold change) and 33 downregulated (over twofold change) disease defense-related genes were identified in the *BRHIS1*-RNAi compared with ZH11 (Table EV1, GenBank accession number: SAMN03771629). Two upregulated plant–pathogen interaction-related genes, *OsPBZc* (Os12g0555200) and *OsSIRK1* (Os09g0356000), were selected for further analysis. Our qRT–PCR indicated that both *OsPBZc* and *OsSIRK1* were upregulated in the BIT-treated or fungus-infected ZH11, as well as in the *BRHIS1*-RNAi (Fig 2B). Further qRT–PCR analysis also revealed that none of the other four *OsPBZc* homologs within the *OsPBZc* cluster, SA-responsive *PBZ1* (Os12g0555500), *OsPBZ14* (Os12g0555000), *OsPBZ15* (Os12g0555100), and *OsPBZ19* (Os12g0555300), were
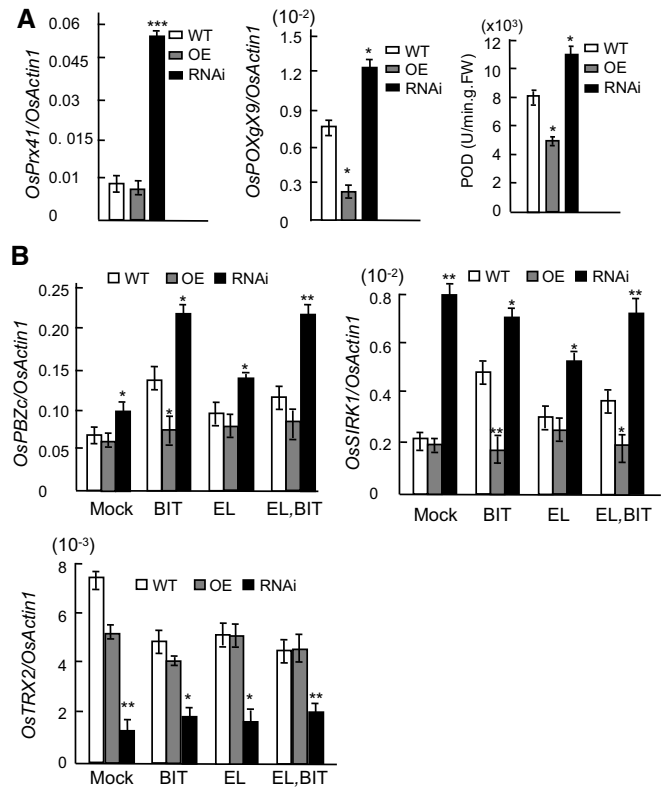

Figure 2. BRHIS1 suppresses defense responses marked by pathogen-attack-induced oxidative burst.

A   POD activities and *POD* mRNA levels in the wild-type (ZH11), *BRHIS1*-OE, and *BRHIS1*-RNAi plants.

B   Expression profiles of two differentially expressed disease defense-related genes, *OsPBZc* (Os12g0555200), and *OsSIRK1* (Os09g0356000), and a differentially expressed antioxidant gene *OsTRX2* (Os01g0913000) identified by RNA-seq, in ZH11, *BRHIS1*-OE, and *BRHIS1*-RNAi plants with or without (mock) the 24-h BIT treatment and/or *M. oryzae* EL0917 (EL) inoculation.

Data information: Relative expression values represent mRNA levels of the genes normalized to that of *OsActin1*. 300 seedlings were pooled for each group. Error bars indicate SD ($n = 3$), and the significant difference from WT was determined by paired two-tailed *t*-tests (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

BRHIS1-targeted and responsive to the BIT treatment and the pathogen infection (Fig EV2C). In addition, seven other differentially expressed defense-related genes were also validated by qRT–PCR (Fig EV2D).

Dependence of the molecular identity of BRHIS1 as a putative SNF2 ATPase suggests that BRHIS1 may act directly on transcription of its target genes as a chromatin-remodeling factor. We further used deep-sequencing immunoprecipitation (ChIP-seq) to identify global BRHIS1 locations in the rice genome (ZH11). This ChIP-seq detected about 400 gene loci, including 37 disease resistance-related genes (Fig EV3A, Table EV2, NCBI accession number: SAMN03771629) that contain *OsPBZc* and *OsSIRK1* but no SA marker genes. This observation was confirmed by our chromatin immunoprecipitation (ChIP) assays (Figs 3A and EV3B). Our ChIP assays also indicated no BRHIS1 occupancy at the promoter regions of *PBZ1*, *OsPBZ14*, *OsPBZ15*, and *OsPBZ19* (Fig EV3B). These findings conform to our RNA-seq data and
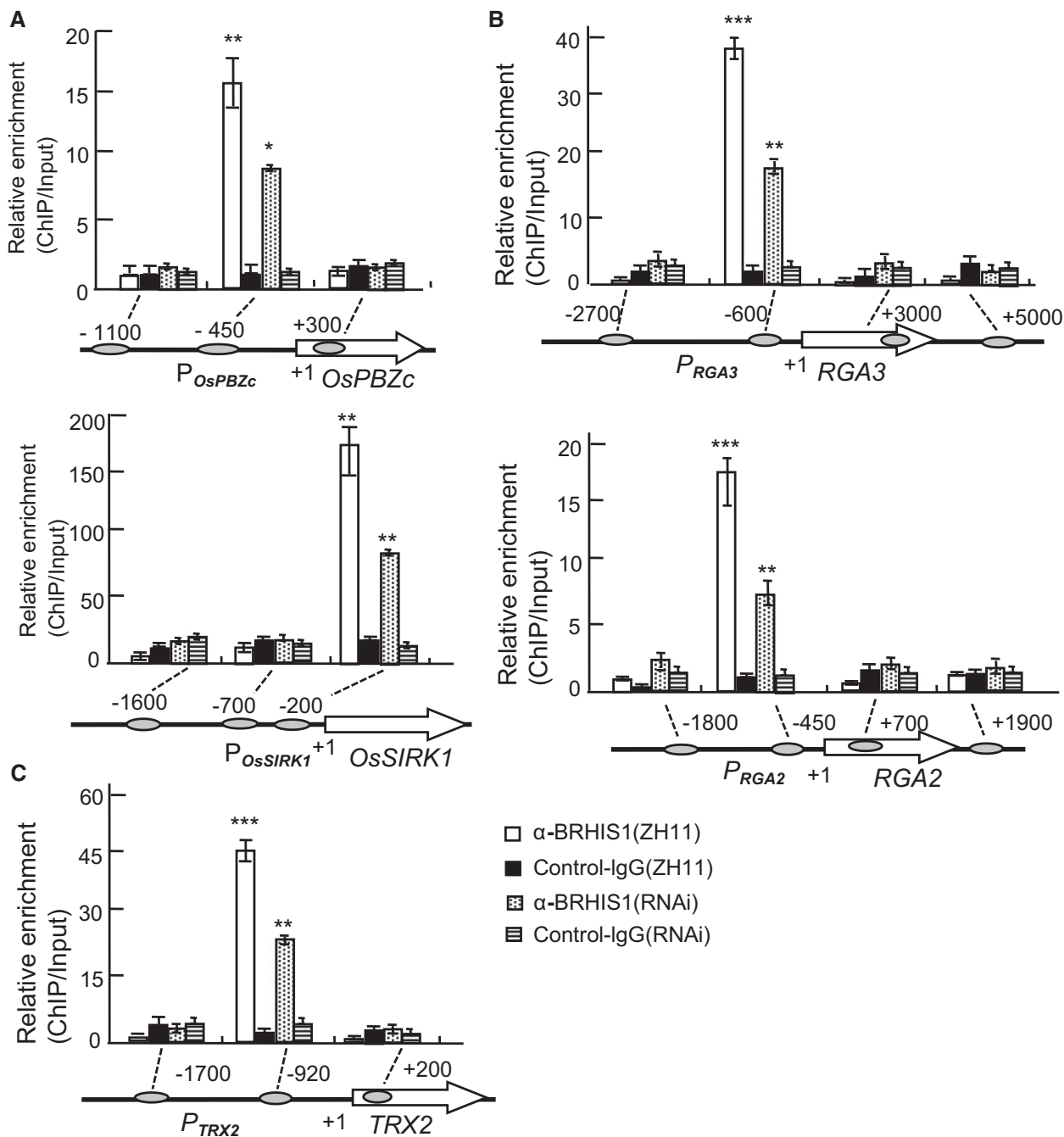
Figure 3. BRHIS1 is recruited to the promoter regions of certain disease defense-related and antioxidative stress-related genes.

A BRHIS1 ChIP analysis of the *OsPBZc* and *OsSIRK1* promoters in ZH11.

B Two BRHIS1 ChIP-seq positive disease resistance-related gene analogs, *RGA2* and *RGA3*, were also validated by ChIP-qPCR.

C BRHIS1 ChIP analysis of the *OsTRX2* promoter in ZH11.

Data information: Normal rabbit control IgG was used as a negative control. *BRHIS1*-RNAi plants were used as a specificity control for the ChIP performed. Relative enrichment was represented as the normalized ratio of the ChIP DNA to the input genomic DNA at the site. 300 seedlings were pooled for each group. Data that came from three independent experiments were averaged. Error bars indicate SD, and the significant difference determined by paired two-tailed *t*-tests is shown (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

further support our concept that the BRHIS1-involved blast resistance of young rice plants is SA-independent. Distribution of BRHIS1 locations along the *OsPBZc* and *OsSIRK1* promoters was

also determined (Fig EV3C), consistent with the results of our ChIP-qPCR (Fig 3A). To further confirm the reliability of the ChIP-seq data, BRHIS1 locations in the promoter regions of two other

disease resistance-related gene analogs, *RGA2* (Os12g0489800) and *RGA3* (Os04g0111900), were validated by ChIP-qPCR (Fig 3B).

The combination of ChIP-seq and RNA-seq analyses helped identify 13 BRHIS1-complex-bound target genes (Table EV3). Of these, besides *OsPBZc* and *OsSIRK1*, a thioredoxin-encoding gene *OsTRX2* (Os01g0913000) was also involved. Given that pathogen infection-triggered oxidative stress may suppress thioredoxin, a key antioxidant, we examined its expression profile by qRT–PCR and validated BRHIS1 recruitment to its promoter by ChIP-qPCR. As we expected, the qRT–PCR data indicated downregulation of *OsTRX2* by *M. oryzae* infection and *BRHIS1* silence (Fig 2C), and the ChIP assays validated BRHIS1 occupancy at the *OsTRX2* promoter (Fig 3C), strongly supporting our notion that suppressing BRHIS1 primes defense responses marked by oxidative burst.

BRHIS1 specifically interacts with OsTINP1 and certain histone H2B and H2A variants

We observed that BRHIS1 did not directly bind to the DNA sequences of the *OsPBZc* promoter per se, in our yeast one-hybrid assays (Fig EV4). This observation suggests that BRHIS1 might interact with some sequence-specific transcriptional activator(s)/repressor(s), or some factor(s) that are specifically located on the promoter regions. To further understand the molecular mechanism of BRHIS1 regulation, we conducted a yeast two-hybrid (Y2H) screening using BRHIS1 as bait and thus identified two BRHIS1-interacting proteins, the histone H2B variant H2B.7 (encoded by Os01g0152900) and the putative transforming growth factor beta inducible nuclear protein OsTINP1 (encoded by Os07g0673100). The *in vivo* interactions of BRHIS1 with H2B.7 and OsTINP1 in the nucleus were confirmed by bimolecular fluorescence complementation (BiFC) in rice protoplasts (Fig 4A). OsTINP1 is a homolog of human TINP1 and yeast NSA2. TINP1 and NSA2 belong to the ribosome S8e superfamily and are involved in ribosome biogenesis and cell cycle regulation and proliferation [33], but the function of OsTINP1 in rice is unknown. *OsTINP1* is constitutively expressed, as does *BRHIS1* (Fig EV5A), implying the involvement of OsTINP1 in the BRHIS1-containing complex.

Given the critical role of histone modification in transcriptional regulation, we drew attention to H2B.7 to unveil the correlation between BRHIS1-mediated chromatin-remodeling events and blast resistance. Database searches identified 11 H2B variants in rice (Fig EV5B). H2B.7 and three other H2B variants, H2B.3 (encoded by Os01g0152300), H2B.5 (encoded by Os01g0153300), and H2B.9 (encoded by Os05g0574300), share a highly conserved H2B domain wherein there are only three sequence variations (Fig EV5C). To determine whether the BRHIS1-involved interaction is specific to H2B.7 or is a general property of H2B, we created a variety of mutants that have mutations on these three variations (Fig EV5C), and tested the interactions between BRHIS1 and all these H2B variants and their mutants. Y2H assay indicated that the mutations in H2B.7 (H2B.7-A, H2B.7-AA) impaired or prevented its interaction with BRHIS1 and that other H2B variants, as well as their mutants, also failed to interact with BRHIS1 (Fig 4B). We further demonstrated this specific BRHIS1-H2B.7

interaction *in vivo* by co-immunoprecipitation (Co-IP) analysis. As shown in Fig 4C, immunoprecipitated by anti-BRHIS1 was the FLAG-tagged H2B.7, but not the Myc-tagged H2B.3, in rice protoplasts co-transfected with an *H2B.7*-FLAG and an *H2B.3*-Myc fusion constructs.

To further explore the possibility that another histone protein H2A may also interact with BRHIS1, we tested the possible interaction between BRHIS1 and all the ten H2A variants identified by database searches (Fig EV5B). Y2H assay revealed that three H2A variants, H2A.3, H2A.Xa, and H2A.Xb, interacted with BRHIS1 (Fig 4B). Indeed, in the subsequent Co-IP analysis where rice protoplasts were co-transfected with an *H2A.1*-Myc and an *H2A.3*-FLAG, an *H2A.Xa*-FLAG, or an *H2A.Xb*-FLAG fusion constructs, the specific *in vivo* interactions between BRHIS1 and H2A.3, H2A.Xa, or H2A.Xb were confirmed (Fig 4C).

The BRHIS1-interacting histone variants are in monoubiquitinated form

Given the critical roles of histone monoubiquitination in gene activation, we performed Co-IP assays to investigate the ubiquitination state of the BRHIS1-interacting histones. In all the tested materials (ZH11, BIT-treated ZH11, and *BRHIS1*-RNAi), the immunoprecipitated H2B (~29 kDa) and H2A (~23 kDa) by anti-BRHIS1 can be detected by both anti-H2B and anti-monoubiquitinated-H2B (uH2B), and both anti-H2A and anti-monoubiquitinated-H2A (uH2A), respectively, indicating that the BRHIS1-interacting histones are in a monoubiquitinated state (Fig 5). Since the specific interactions of BRHIS1-H2B.7, BRHIS1-H2A.3, BRHIS1-H2A.Xa, and BRHIS1-H2A.Xb were shown by Y2H and Co-IP analysis done with rice protoplasts, it can be inferred that the coimmunoprecipitated H2B is no other than the H2B.7 and that the coimmunoprecipitated H2A is no other than the H2A.3/H2A.Xa/H2A.Xb. Furthermore, a ChIP assay using a commercially available anti-H2A.X that specifically recognizes rice H2A.3 and H2A.Xb (Fig EV5D) revealed the specific occupancy of H2A.3/H2A.Xb at the *PBZc* promoter within the *OsPBZc* cluster (Fig 6A), consistent with the BRHIS1 ChIP result (Fig EV3A). These data suggest the histone-mediated selective promoter targeting of the BRHIS1 complex in transcriptional regulation.

Further ChIP analysis using anti-H2A.X and anti-uH2B showed that the deposition of monoubiquitinated H2A.3/H2A.Xb and H2B.7 on both the *OsPBZc* and *OsSIRK1* promoter regions were significantly increased by BIT treatment (Fig 6B), suggesting the monoubiquitinated-histone-variants-modulated gene expression in BIT-induced resistance. However, based on our ChIP analysis using antibodies against H3 lysine-4/lysine-9 dimethylation (H3K4/K9me2) and H3 lysine-4/lysine-9 trimethylation (H3K4/K9me3), we found that H2B monoubiquitination-triggered H3 methylation associated with transcriptionally active chromatin [43] was unaffected in both the *OsPBZc* and *OsSIRK1* promoter regions in BIT-treated ZH11 plants (Fig 6B). This finding implicates the independence of on-site H3 methylation in monoubiquitinated-H2B.7-activated gene expression.

The increased H2A.X- and uH2B-based ChIP enrichment in the *OsPBZc* and *OsSIRK1* promoter regions in response to BIT raised a possibility that expression of these histone genes might be affected by BIT signaling. Indeed, qRT–PCR analysis of certain histone
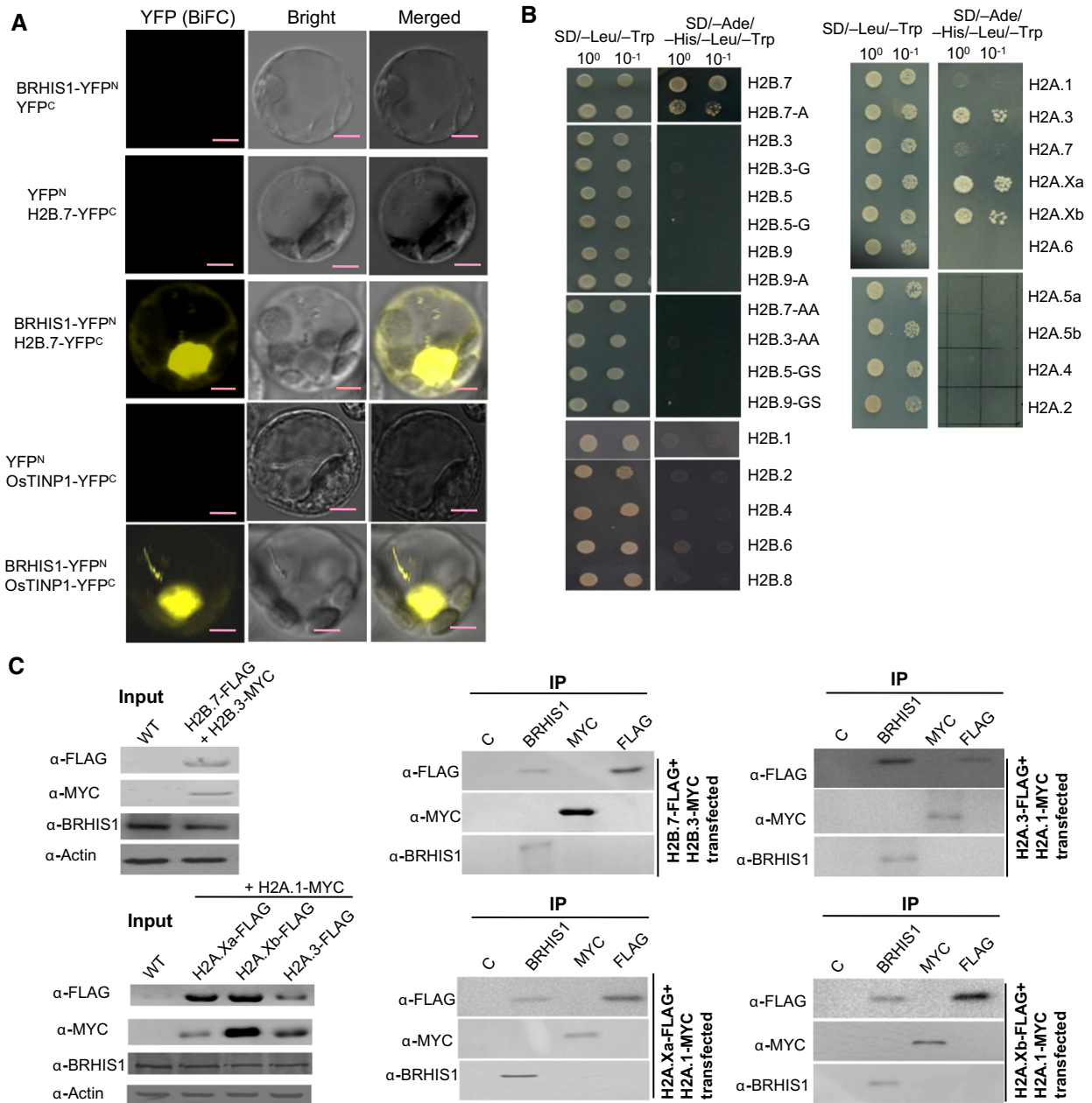
Figure 4. BRHIS1 specifically interacts with OsTINP1 and certain histone H2A and H2B variants.

A BiFC assays in rice mesophyll protoplasts confirmed the *in vivo* BRHIS1-H2B.7 and BRHIS1-OsTINP1 interactions. Scale bars, 5 μm. Representative images from three independent experiments are shown.

B Yeast two-hybrid (Y2H) assays that tested the possible interactions between BRHIS1 and H2B (left panel) or H2A variants (right panel). 10^0 and 10^{-1} indicate the relative dilutions of yeast cells for Y2H. H2B.7-A, H2B.3-G, H2B.5-G, H2B.9-A, H2B.7-AA, H2B.3-AA, H2B.5-GS, and H2B.9-GS are mutants with mutations within the conserved H2B domain. Representative images from three independent experiments are shown.

C Co-IP analysis in rice mesophyll protoplasts transiently co-transfected with various constructs expressing FLAG- or MYC-tagged H2A and H2B variants. Immunoprecipitates of BRHIS1, FLAG, MYC, or normal control Rabbit IgG (C) were immunoblotted with anti-FLAG, anti-MYC, or anti-BRHIS1. The protein levels of BRHIS1 and FLAG-tagged- or MYC-tagged H2A or H2B variants in the transfected cells are also shown by Western blot analysis. 100 seedlings were pooled for each group. Representative images from two independent experiments are shown.

variant genes showed that the transcript levels of *H2A.Xa* and *H2B.7*, but not those of the other histone variant genes, were upregulated in BIT-treated plants (Fig 6C), indicating the BIT-responsive character of *H2A.Xa* and *H2B.7*.

Discussion

Although known as "sensitization" as early as 1933 [3], defense priming is still an enigma to date. Here, we report that rice employs
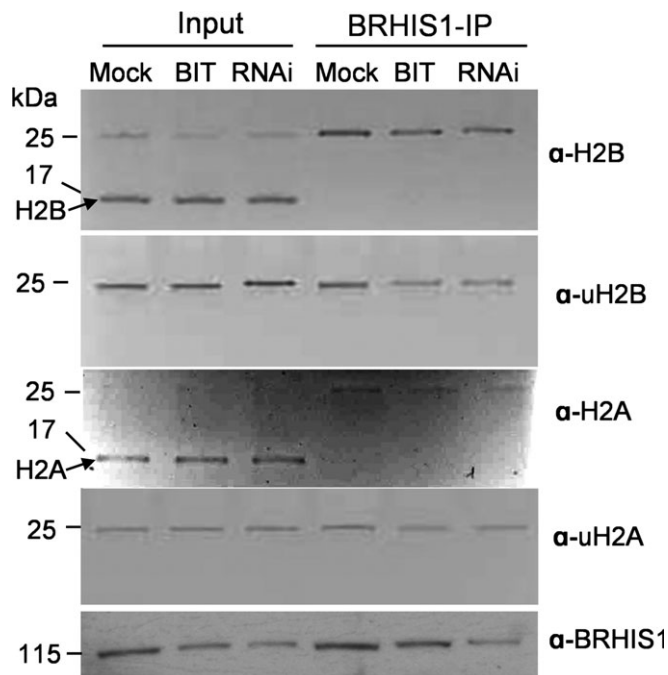
Figure 5. The BRHIS1-interacting histone variants are in a monoubiquitinated state.

Co-IP analysis in *BRHIS1*-RNAi plants (mock-treated) and ZH11 plants with or without (mock) the 24-h BIT treatment. BRHIS1 immunoprecipitates were immunoblotted with antibodies against H2B, monoubiquitinated H2B (uH2B), H2A, or monoubiquitinated H2A (uH2A). Anti-BRHIS1 was used to control for levels. Representative images from three independent experiments are shown.

an SA-independent, specific H2A and H2B monoubiquitination-regulated, disease defense priming negatively operated by BRHIS1, a previously unrecognized SNF2 DNA-dependent ATPase, strongly supporting the hypothesis that epigenetic change sustains long-lasting immune memory in plants.

BRHIS1 and the BRHIS1-interacting monoubiquitinated H2A and H2B variants are transcriptional regulators in age-related innate immunity

SA signaling pathway has long been considered to play a central role in plant defense against pathogens. However, out results show that SA-responsive genes, such as *PBZ1*, *OsNPR1*, *OsWRKY03*, and *OsWRKY71*, are unresponsive to BRHIS1-mediated blast resistance of rice seedlings, consistent with the published data that SA acts as a defense signal only in adult rice plants [32]. These data taken together indicate that the regulation of induced immune responses in rice is age-dependent. We have found that with the help of monoubiquitinated H2B.7 and H2A.3/H2A.Xa/H2A.Xb, BRHIS1 is specifically targeted to given disease defense-related gene loci implicated in blast resistance of rice seedlings. Moreover, BRHIS1-influenced monoubiquitination of H2B.7 and H2A.3/H2A.Xa/H2A.Xb promotes exclusive disease defense-related gene expression in response to pathogen infection in rice seedlings. Our mechanism for monoubiquitinated-histone-involved transcriptional regulation by BRHIS1 provides a model for how innate immunity is age dependently regulated in plants.

Specific interaction of BRHIS1 with certain H2B and H2A variants underlies the selective promoter targeting of BRHIS1

H2A and H2B consist of various variants that may have distinct biological functions [44]. Database searches reveal that rice has 10 H2A and 11 H2B variants, but their unique functions are totally unknown, except for the conserved role of H2A.X phosphorylation in meiotic double-strand break formation [45]. Here, we show that the transcriptional activation of certain defense-related genes by BRHIS1 is mediated by four rice histone variants, H2B.7, H2A.Xa, H2A.Xb, and H2A.3, thus unveiling a previously unappreciated link between monoubiquitination of histone variants and plant innate immunity. Distinct from the H2A variant H2A.Z reported to play a key role in SAR [17,18], the BRHIS1-interacting histone variants function in monoubiquitinated form. Of note, H2A.Z deposition suppresses [17,18], while H2B.7 and H2A.3/H2A.Xa/H2A.Xb monoubiquitinations activate, expression of defense-related genes. In addition, these monoubiquitinated histone variants provide the selective promoter targeting for the BRHIS1 complex, based on our ChIP analysis, which suggests that specific modified histone variants confer the loci selectivity on SWI/SNF complexes along with specific transcriptional activators/repressors [46,47]. Undoubtedly, monoubiquitinated H2B.7 and H2A.3/H2A.Xa/H2A.Xb may have different roles than BRHIS1 recruiting. Given reduced BRHIS1 binding with increasing monoubiquitination of H2B.7 and H2A.3/H2A.Xa/H2A.Xb, we deduce that these histone monoubiquitinations could also get involved in the control of BRHIS1 activities.

A potential cross talk between monoubiquitinated H2A and H2B variants at defense gene loci in BRHIS1-mediated transcriptional regulation

In a broad sense, the roles of H2A and H2B ubiquitinations are deemed distinct [19], and the relationship between H2A and H2B during transcription initiation at the same locus still remains unknown [19]. The co-regulation of *OsPBZc* and *OsSIRK1* expression by monoubiquitinated H2A and H2B variants provides a potential cross talk between monoubiquitinated H2A and H2B at these sites, as well as their parallel positive effects on transcriptional activation of given defense-related genes.

A model for BRHIS1-mediated disease defense responses

Here, we propose a BRHIS1-mediated disease defense-response model in young rice plants (Fig 7). Under normal growth conditions, monoubiquitinated histone variants, H2A.3/H2A.Xa/H2A.Xb and H2B.7, are deposited on the promoter regions of certain disease defense genes, such as *OsPBZc* and *OsSIRK1*. However, for the purpose of minimizing the negative effects of disease resistance, BRHIS1 binds, as a complex, to monoubiquitinated H2A.3/H2A.Xa/H2A.Xb and H2B.7 to blunt their further monoubiquitination and thus constantly suppresses the expression of its target genes (Fig 7A). Upon the perception of signals from BIT or pathogen assaults, *H2A.Xa* and *H2B.7* expression is upregulated, whereas *BRHIS1* expression is reduced, thereby leading to more deposition of monoubiquitinated H2A.3/H2A.Xa/H2A.Xb and H2B.7, and to the concurrent relief of BRHIS1-complex binding, on the local chromatin. Meanwhile, BRHIS1 activities could be
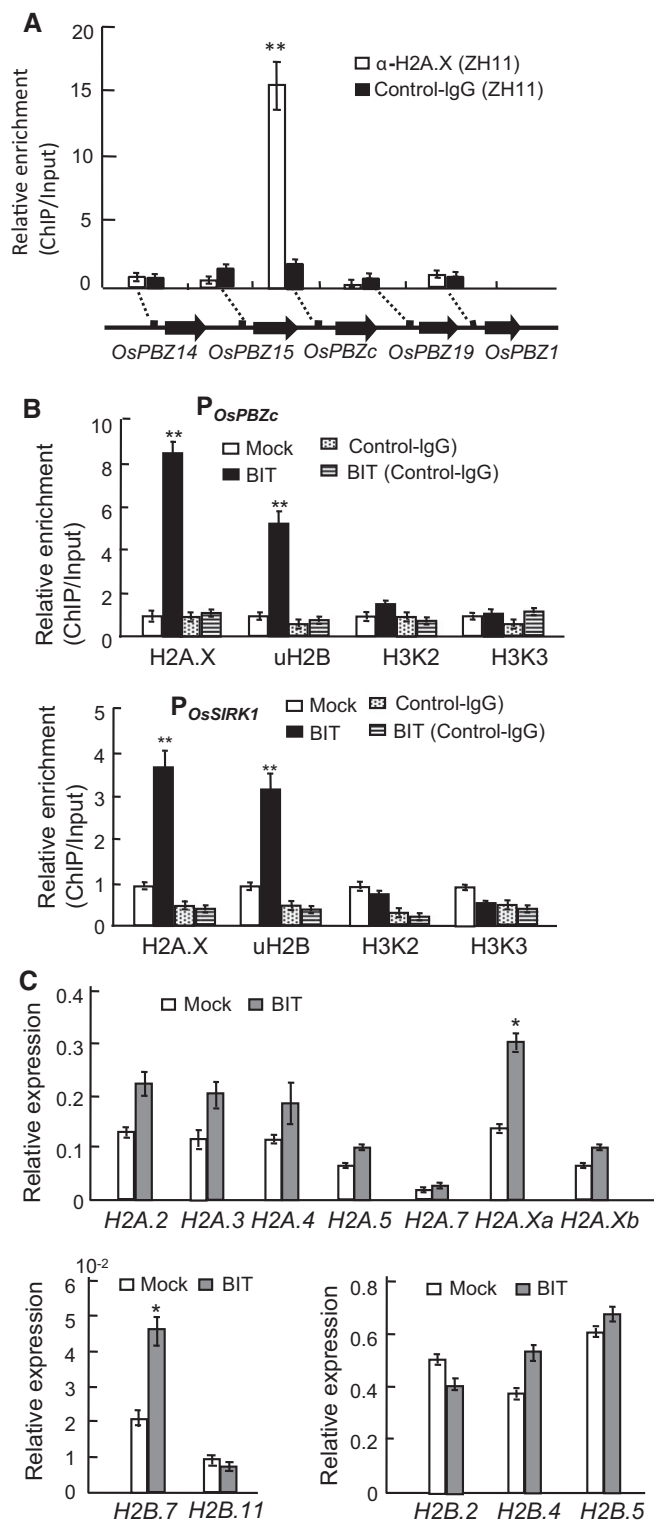
facilitating rapid modulation of defense gene expression for induced immune responses.

Overall, our data support a novel epigenetic control model for SA-independent disease defense responses wherein BRHIS1 negatively regulates disease defense-relevant chromatin-remodeling events for defense priming.

Materials and Methods

Reagents and antibodies

BIT powder was purchased from Sigma-Aldrich. Anti-BRHIS1 rabbit IgG was raised and purified by Invitrogen with a synthetic peptide corresponding to the N-terminal sequence of BRHIS1, RFPSRSSFGTDNKR. Anti-monoubiquitinated H2B (clone 56), anti-H3K4/K9me2 (07-1843), anti-H3K4/K9me3 (07-992), anti-H2A (ABE327), and anti-monoubiquitinated H2A (clone E6C5) were from Millipore. Anti-RNA polymerase II CTD repeat YSPTSPS (ab5408), anti-H2B (ab1790), anti-H2A.X (ab11175), and anti-MYC (ab9106) were from Abcam. Anti-FLAG (F3165 Mz) was from Sigma-Aldrich.

Full-length cDNA suppression subtractive hybridization (FL-SSH)

The construction and differential screening of the subtracted cDNA library by FL-SSH were described previously [34].

Plant growth and conidial inoculation

Seedlings of *japonica* rice ZH11 and the transgenic plants were grown in a growth chamber under 14-h-light long-day conditions at 30/25°C day/night cycles. For BIT treatment, BIT solution was sprayed over the 3-week-old rice seedlings grown in growth chambers. Pathogen inoculation with rice blast fungus was performed as described before [48]. *Magnaporthe oryzae* race EL0917, which is virulent for ZH11, was grown on rice polish agar medium for about 10 days at 25°C in the dark. *Magnaporthe oryzae* spore formation was then induced under blue light for 2–3 days. Spore suspension (1×10^5–5×10^5 conidia per ml) was sprayed onto the rice plants. After inoculation, the plants were incubated at 25°C with saturated humidity for 20 h, and then transferred to a moist vinyl tunnel at 25–30°C. Infection rate of *M. oryzae* was determined by qPCR as described elsewhere [37,49]. The sequence used for quantifying rice DNA is a single-copy sequence from the

further inhibited with increasing histone monoubiquitination within a possible positive feedback loop. All these steps taken together eventually facilitate and ensure the prompt activation of defense gene expression in disease defense priming (Fig 7B). Our working model suggests that plants may establish an expression-ready state at some poised promoters of defense genes, thus
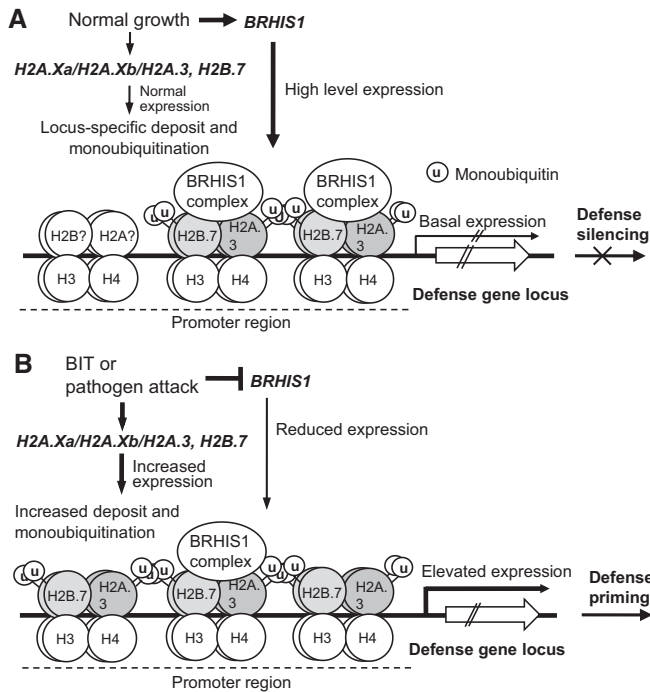
A

B

Figure 7. Working model of rice disease defense priming associated with BRHIS1-mediated chromatin remodeling.

Simplified chromatin structure of a disease defense-related gene locus, where H2A.3 is used as an example, is shown.

A   Under normal growth conditions, *BRHIS1* is expressed at a relatively high level, and the BRHIS1 complex, containing OsTINP1 and other possible interactors as well, is recruited to the promoter regions where it targets through binding to most of, if not all, the monoubiquitinated H2B.7 and H2A.3/H2A.Xa/H2A.Xb. This BRHIS1-complex binding obstructs further monoubiquitinations and thus constantly restricts promoter activities to a basal level.

B   Upon pathogen attack or BIT treatment, H2A.Xa and H2B.7 expression is upregulated, while BRHIS1 expression is decreased, thus leading to more deposition of H2A.3/H2A.Xa/H2A.Xb and H2B.7, and to the relief of the BRHIS1-complex binding, on the chromatin. The increasing monoubiquitination of these histone variants could also further inhibit BRHIS1 activities. This process significantly improves promoter activities and consequently induces strong expression of certain disease defense genes.

rice *OsUbiquitin* gene. The *MoPot2* transposon sequence used for quantifying *M. oryzae* DNA has about 100 copies in the fungus genome. The infection ratio (*Pot2/OsUbi* × 100) was calculated from the determined numbers of the target sequences of *MoPot2* and *OsUbiquitin* in each sample. The data for each line were collected from total 144 DNA samples taken from three independent inoculation tests.

Transcriptome sequencing

Total RNAs of ZH11 and *BRHIS1*-RNAi line were isolated with TRIzol (Invitrogen) according to the manufacturer's instructions. Then the cDNA libraries for digital gene expression were prepared according to the Illumina sequencing protocol. A virtual library was prepared containing all the possible CATG+17 base-length sequences of the reference gene sequences (ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/ annotation_dbs/pseudomolecules/

version_7.0/). A total of 4,888,951 clean tags from ZH11 and 5,611,798 clean tags from *BRHIS1*-RNAi line were obtained, and mapped to the rice genomic reference sequence (from the *japonica* cultivar Nipponbare). The number of unambiguous clean tags for each gene was calculated and then normalized to TPM (number of transcripts per million clean tags) [50] (NCBI accession number: SAMN03771629).

ChIP and ChIP-sequencing

ChIP was performed according to Fiil *et al* [51]. Lysed nuclei were sonicated to be in the size range of 0.3–1.5 kb. Supernatant was incubated with anti-BRHIS1, anti-H2A.X, anti-uH2B, anti-H3K4/K9me2, or anti-H3K4/K9me3 antibodies coupled to protein-A agarose beads overnight at 4°C. Part of the eluted DNA was tagged and sequenced (ChIP-seq) by the Illumina sequencing system. Relative enrichment of ChIP DNA to input genomic DNA on the selected sites was estimated by qPCR as described [52]. For the ChIP-seq, we merged the reads with overlap before mapping to the reference genome (MSU 7.0 http://rice.plantbiology.msu.edu/), and 6,156,890 (99.59%) reads were merged to a total of 6,182,222 reads pair. Based on the map result of BWA-MEM align method (version: 0.7.4. http://bio-bwa.sourceforge.net/), we filtered the false-positive peak region of mean depth < 10×. After that, we identified disease resistance-related genes with gene annotation, gene ontology (GO), and KEGG pathway analyses. We ran the ChIP-seq data analysis using the online software HOMER (http://homer.salk.edu/homer/ngs/index.html). To obtain the graphical distribution of the BRHIS1 ChIP-seq reads for each sample over the TSS, we calculated the average number of reads on each position from 3,000 bp upstream to 1,000 bp downstream of the TSS of all genes (according to GenBank), normalizing by the total number of reads for each ChIP-Seq experiment. All the ChIP-seq data were collected from three independent experiments (NCBI accession number: SAMN03771629).

Co-IP analysis

The procedure for Co-IP was essentially based on a published protocol [51]. Nuclear fractions isolated from rice seedling leaves were incubated with anti-BRHIS1, anti-FLAG, or anti-MYC antibody coupled to protein-A agarose beads (GE Healthcare) overnight at 4°C. The beads were washed three times with IP-B buffer, and bound proteins were eluted with buffer containing 2% SDS and 0.1% β-mercaptoethanol. The samples were analyzed by SDS–PAGE and immunoblotting.

Confocal microscopy

For subcellular localization analysis, the CFP fusion of the full-length BRHIS1, P35S::*BRHIS1-CFP*, was made in a pUC18-based vector driven by the *CaMV 35S* promoter. *TDR* (Tapetum Degeneration Retardation [53]) cDNA coding sequences were amplified using the primer pairs TDRRFP-T5F (5′-ATGGGAAGAGGAGACCACCTGCT-3′)/TDRRFP-T5R (5′-ATCAAACGCGAGGTAATGCAGGT-3′) and cloned into a pUC18-based vector containing an *RFP* gene driven by the *CaMV 35S* promoter. CFP fluorescence was excited by 458 nm and visualized with a confocal scanning microscope fitted with a 40× water immersion objective (7 DUO; Zeiss).

Y2H screen

The full-length coding sequence of *BRHIS1* was cloned into the pGBKT7 vector (Clontech) and transformed into the yeast strain Y187 as bait. The rice cDNA library was constructed from 3-week-old rice seedlings of ZH11. The Y2H screen was performed according to the user's manual of a Matchmaker Library Construction & Screening Kit (Clontech). The site-directed mutagenesis for H2B.7 and the other H2B variants was carried out by using a QuikChange Site-Directed Mutagenesis kit (Stratagene) with the pGADT7 constructs containing H2B.7 or the other H2B variants as templates.

BiFC assays

For BIFC assay, the constructs, P35S::*BRHIS1-YFP^N*, P35S::*H2B.7-YFP^C*, and P35S::*OsTINP1-YFP^C*, were created and used to transiently transfect rice mesophyll protoplasts from ZH11 with the polyethyleneglycol (PEG)-calcium method. The yellow fluorescent protein (YFP) reconstructed was excited by 514 nm and visualized at 527 nm with a confocal scanning microscope (7 DUO; Zeiss).

Protein analysis

Total protein was extracted from rice seedling leaves, rice protoplasts, or yeast cells in 2× SDS–PAGE buffer for Western blots. Subsequently, separated proteins were transferred onto PVDF membranes (Millipore). After overnight incubation with the primary antibodies in TBS containing 1% BSA and 0.1% Tween-20 at 4°C, membranes were then rinsed with TBS containing 0.1% Tween-20 (TBST), followed by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature. After TBST washes, Amersham ECL Prime (GE Healthcare) was used to visualize signals on the membranes by ChemiDoc XRS+ (Bio-Rad).

Data availability

The RNA-seq and ChIP-seq data from this publication have been submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) and assigned the identifier SAMN03771629.

Statistics

About 300 seedlings were pooled for each sample. All the data came from three independent experiments. Value represented the average ± the standard deviation of the average (SD). Significant difference was determined by paired two-tailed Student's *t*-tests. $P < 0.05$ was considered significant.

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Author contributions
QZ and YL conceived and designed the experiments. XL, QZ, YJ, and ZJ performed the experiments. XL and QZ analyzed the data. XL, QZ, and YL wrote the paper.

Conflict of interest
The authors declare that they have no conflict of interest.

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