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A bHLH transcription activator regulates defense signaling by nucleo-cytosolic trafficking in rice

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Research Article

Abstract Crosstalk between plant hormone signaling pathways is vital for controlling the immune response during pathogen invasion. Salicylic acid (SA) and jasmonic acid (JA) often play important but antagonistic roles in the immune responses of higher plants. Here, we identify a basic helix-loop-helix transcription activator, OsbHLH6, which confers disease resistance in rice by regulating SA and JA signaling via nucleo-cytosolic trafficking in rice (*Oryza sativa*). *OsbHLH6* expression was upregulated during *Magnaporthe oryzae* infection. Transgenic rice plants overexpressing *OsbHLH6* display increased JA responsive gene expression and enhanced disease susceptibility to the pathogen. Nucleus-localized OsbHLH6 activates JA signaling and suppresses SA signaling; however, the SA regulator OsNPR1 (Nonexpressor of PR genes 1) sequesters OsbHLH6 in the cytosol to alleviate its effect. Our data suggest that OsbHLH6 controls disease resistance by dynamically regulating SA and JA signaling.

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INTRODUCTION

Phytohormones are involved in all aspects of higher plant biology, from growth and development to their responses to environmental stresses (Verma et al. 2016; Berens et al. 2017). Plants often sacrifice growth to adapt to and survive under biotic and abiotic stresses, which is usually implemented by modifying the hormone signaling pathways (Karasov et al. 2017). Different types of pathogens can require plants to activate different signaling pathways, including salicylic acid (SA), jasmonic acid (JA), and ethylene signaling. SA and JA are well-characterized "defense hormones"; SA is known to be effective in defending against biotrophic pathogens, whereas JA is often involved in plant responses to necrotrophic pathogens and herbivorous insects (Caarls et al. 2015). The mechanisms by which ethylene functions in the response of *Arabidopsis thaliana* to pathogens are still under debate (Yang et al. 2017); however, this hormone has been shown to contribute to blast disease resistance in rice (Helliwell et al. 2016; Yang et al. 2017). Other hormones, such as abscisic acid (ABA) and gibberellins (GA), are also reported to be involved in the plant immune response (Robert-Seilaniantz et al. 2011; Pieterse et al. 2012; Berens et al. 2017).

Salicylic acid plays essential roles in the plant immune response. Both pathogen-associated molecular patterns (PAMPs) and pathogen effectors when they are recognized by plants can activate SA signaling (Dempsey and Klessig 2012; Fu and Dong 2013), and exogenous application of SA can enhance plant disease resistance to a wide variety of pathogens, especially biotrophic

pathogens. NPR1 (nonexpressor of PR genes 1) is a master regulator of SA signaling that normally exists as an inactive oligomer in the cytosol (Mou et al. 2003; Tada et al. 2008). An upregulation of SA signaling induces a redox change in the cytosol that causes the intermolecular disulfide bonds of the oligomer to be reduced (Tada et al. 2008), freeing monomeric NPR1 to be translocated into the nucleus and bind the TGA transcription factors (TFs), which belong to bZIP transcription factor family and bind the TGACGT motifs of targeted promoters, to activate SA-responsive gene expression in *Arabidopsis*. In rice, OsNPR1 shares its role in the regulation of SA-responsive genes with OsWRKY45 (Shimono et al. 2012; De Vleesschauwer et al. 2014), the overexpression of which confers disease resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *M. oryzae* (Shimono et al. 2012).

The F-box protein CORONATINE INSENSITIVE 1 (COI1) and the JASMONATE ZIM-DOMAIN (JAZ) repressor proteins play a central role in JA recognition and the regulation of JA-responsive gene transcription (Kazan and Manners 2013; Gimenez-Ibanez et al. 2016). JAZ repressor proteins associate with JA-responsive TFs, such as MYC2 and OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF domain protein 59 (ORA59), to repress their function in the nucleus. In the presence of JA, COI1 binds the JAZ proteins, leading to their proteasomal degradation. Subsequently, their targeted TFs are released to activate JA-responsive gene transcription. MYC2 is one of the master downstream regulators of JA signaling (Kazan and Manners 2013). In rice, overexpression of *OsMYC2* caused the early expression of JA-responsive genes and resulted in bacterial blight resistance (Uji et al. 2016).

Jasmonic acid and SA signaling often act antagonistically in both dicot and monocot species (De Vleesschauwer et al. 2014). SA can affect JA signaling at the transcriptional level or cause the post-translational modification of the TFs and co-regulators of the JA pathway (Caarls et al. 2015). NPR1 has been demonstrated to be a key regulator in SA signaling (Dong, 2004); overexpression of *OsNPR1* leads to strong activation of SA -responsive genes and concomitant suppression of JA signaling (Yuan et al. 2007). Rice plants overexpressing *OsNPR1* have an enhanced disease resistance to *M. oryzae* and *Xoo* (Chern et al. 2005), but are more susceptible to herbivorous insects (Li et al. 2013). The cytosolic localization of NPR1 is important for the SA-mediated antagonism of JA-responsive gene expression in *Arabidopsis* (Spoel et al. 2003); however, the mechanism by which it restricts JA signaling remains elusive. Interestingly, in animal cells, SA or aspirin can induce the transcription factor NF-κB in the cytosol by associating with IκB (with structure similarity to NPR1) (Kopp and Ghosh 1994). Similar to plants, the nuclear localization of NF-κB is necessary for the production of prostaglandins (structural analogs of JA).

Unlike biotrophic and necrotrophic pathogens, which typically activate either SA or JA signaling, respectively, hemibiotrophic pathogens induce dynamic hormone-level changes in higher plants. *Magnaporthe oryzae*, the rice blast fungus, can suppress SA signaling at the early infection stages, likely by upregulating ABA-mediated responses (Cao et al. 2016); however, cytokinin signaling is significantly activated at a later infection stage, leading to the metabolic substance efflux from host cytosol in the infection region. The accumulation of nutrients in the infection region presumably enables the pathogen to assimilate host metabolites for their own benefit. Khang et al. (2010) proposed that *M. oryzae* maintains a continuous biotrophic phase in newly infected cells, but leaves the early infected cells in a necrotrophic phase (Khang et al. 2010), which theoretically would lead to a complex hormone signaling in rice. Future research should therefore aim to elucidate the mechanisms by which plants fine-tune their hormone signaling pathways to respond to the dynamic changes during hemibiotrophic pathogen infections.

Rice is a staple crop for half of the world's population (Liu et al. 2014); however, rice blast disease causes severe yield losses every year. We recently reported the dynamic changes in plant hormonal signaling during *M. oryzae* infection (Cao et al. 2016; Yang et al. 2017); however, the mechanism responsible for this hormone network rewiring is yet to be elucidated. In this study, we characterize a basic helix-loop-helix (bHLH) transcription factor, OsbHLH6, which activates JA signaling in rice when localized to the nucleus. Following infection by *M. oryzae*, the SA signaling regulator OsNPR1 sequesters OsbHLH6 in the cytosol, which activates SA signaling but represses JA signaling. We therefore propose that bHLH transcription factor(s) may regulate the SA/JA antagonism in rice and/or other monocot plants. The nucleo-cytosolic trafficking of this transcription activator likely results

in the dynamic changes in SA/JA signaling during *M. oryzae* infection and the activation of the immune response in rice.

RESULTS

OsbHLH6 decreases blast disease resistance

Previously, we showed that 24 h post inoculation (hpi) is a critical time point during *M. oryzae* infection (Cao et al. 2016; Yang et al. 2017). At this time point, the fungal hyphae are mainly found in the primarily infected cells and are just beginning to spread to the neighboring cells (Cao et al. 2016). Accordingly, the maximum differences in the expression of the differentially expressed genes (DEGs) between infected and non-infected plants are often observed at this stage. Here, we identified 2,352 DEGs in *M. oryzae*-infected rice plants at 24 hpi. We particularly focused on the 103 DEGs encoding TFs at 24 hpi, which included members of the ERF, WRKY, MYB, NAC, and bHLH families (Figure S1A). Among these TFs, the bHLH family genes were especially interesting because their fold changes ranged from – 4.53 to 8.35 (log2) relative to the uninfected plants (Figure S1A). Furthermore, several members of the bHLH family have previously been implicated in regulating JA signaling (Goossens et al. 2017) and are likely involved in the disease response. Four of the bHLH TF genes were downregulated in the *M. oryzae*-infected plants while seven were upregulated, including *OsbHLH6* (LOC_Os04g23550), the expression of which was up to 16-fold higher in the presence of the pathogen (Figure S1B). Previously, *OsbHLH6* was reported to retard plant growth when overexpressed in rice and was named *RERJ1* (Kiribuchi et al. 2004); however, the roles of this gene in plant disease resistance are not known.

To verify the transcriptome data, we examined *OsbHLH6* expression in rice leaves during *M. oryzae* infection. *OsbHLH6* expression was induced approximately 7.8 folds after inoculation with *M. oryzae* and peaked at 24 hpi, after which it decreased (Figure 1A). Moreover, we also treated a suspension of rice cells with the typical PAMPs, chitin and bacterial flagella-derived 22 (flg22) peptide, to examine *OsbHLH6* expression during PAMP-triggered immunity (PTI). The transcription of *OsbHLH6* was induced by chitin (2.9 folds higher) but not flg22 (Figure 1B), indicating that *OsbHLH6* is responsive to fungal pathogens. We then investigated the role of OsbHLH6 during rice blast infection. High levels of *ObHLH6* expression is known to cause a dwarf phenotype (Kiribuchi et al. 2004); therefore, we expressed *OsbHLH6* driven by a constitutive promoter and a dexamethasone (Dex)-inducible promoter (Figure S2A, B). We selected the constitutive-overexpression lines that were phenotypically normal but with higher *OsbHLH6* expression levels than the wild-type (WT) for use in the disease resistance analysis (Figure S2A). Overexpressing *OsbHLH6* in rice leads to enhanced disease susceptibility; the two representative lines, *OsbHLH6*-OE-2 and *OsbHLH6*-OE-11, contained much more fungal biomass than the WT plants at 5 dpi (Figure 1C, D). Similarly, the Dex-induced overexpression lines were more susceptible to *M. oryzae* than the WT (Figure 1E, F).

We generated *OsbHLH6*-RNAi transgenic plants, which were confirmed to have reduced expression of *OsbHLH6* (Figure S2C). We found that two representative RNAi lines, *OsbHLH6*-RNAi-1 and *OsbHLH6*-RNAi-3, were more resistant than the WT to *M. oryzae*, and contained much less fungal biomass (Figure 1G, H). We also generated *OsbHLH6* mutant lines using the CRISPR/Cas9 system (Figure S2D). The two independent mutant lines contained a nucleotide insertion at the same site (G67), which resulted in the early termination of translation. Consistently, the two mutant lines were also more resistant to *M. oryzae* than the WT (Figure 1I, J). These data indicate that OsbHLH6 is a negative regulator of blast disease resistance in rice.

Jasmonic acid activates *OsbHLH6* expression

A previous study reported that OsbHLH6 is a JA-inducible transcription activator in rice (Kiribuchi et al. 2004). A sequence analysis shows that OsbHLH6 contains a bHLH domain in its N-terminal region (Figure S3A), indicating that it is a bHLH family protein. We therefore followed the standard nomenclature and used the name OsbHLH6 instead of RERJ1. A phylogenetic analysis revealed that OsbHLH6 is more closely related to the orthologs of wheat (*Triticum aestivum*), *Brachypodium distachyon*, or maize (*Zea mays*) than to bHLH proteins in the dicot soybean (*Glycine max*) and *Arabidopsis* (Figure S3B). As there are no OsbHLH6 orthologs in dicots, it suggests that

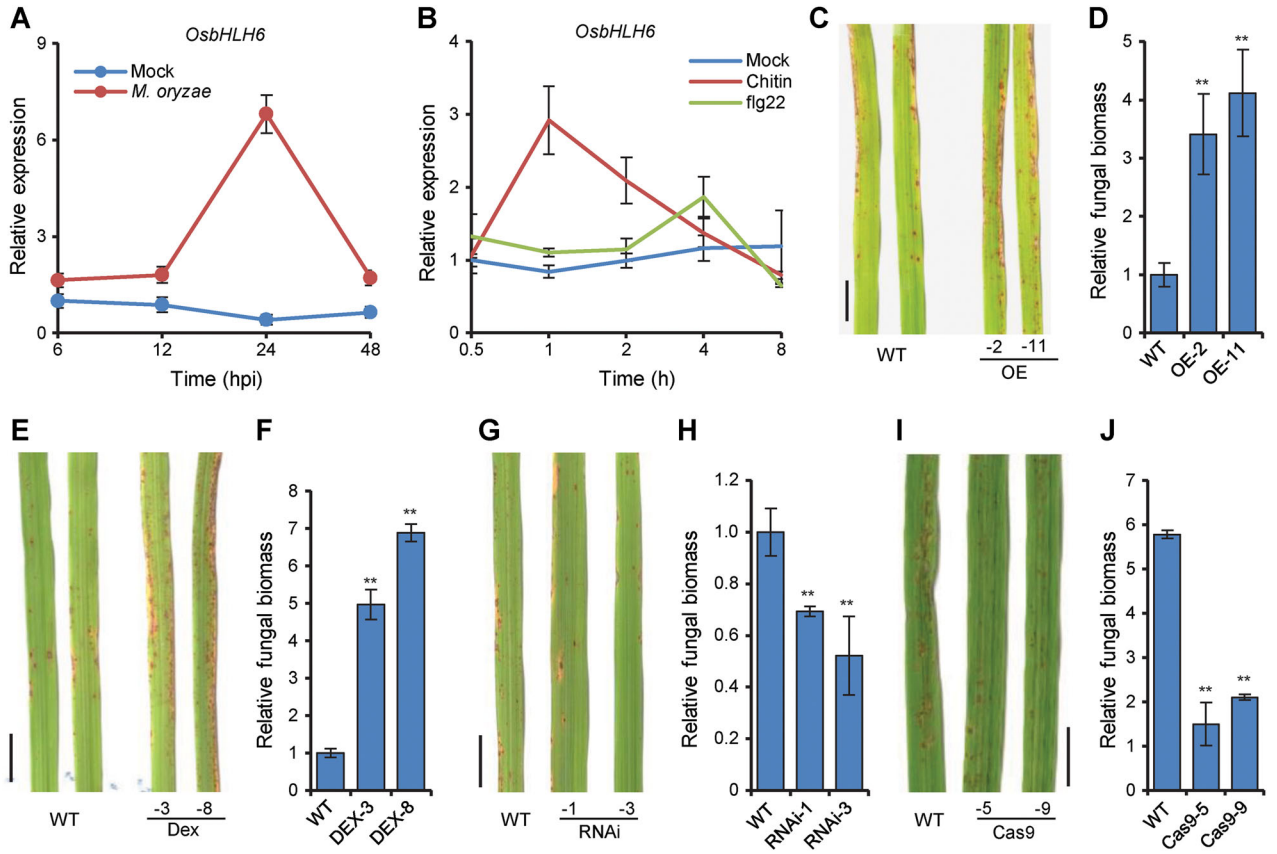

Figure 1. OsbHLH6 is a negative regulator of blast disease resistance in rice

(**A**) Transcription analysis of *OsbHLH6* over time after inoculation with blast fungus in rice. Conidial suspensions (1×10^5 conidia per mL in 0.02% Tween-20) were sprayed onto the leaf surface of 2-week-old rice seedlings. The infected leaves were sampled at indicated time points for quantitative reverse transcription polymerase chain reaction (RT-qPCR) assays. 0.02% Tween-20 serves as mock. Values are means ± *SD* (n = 3 biological replicates). (**B**) Transcription analysis of *OsbHLH6* in rice suspension cells treated by 100 μM flg22 or 10 μM chitin. RT-qPCR were used to evaluate the gene expression. Values are means ± *SD* (n = 3 biological replicates). (**C**) and (**D**) Disease symptoms and relative fungal biomass of WT and *OsbHLH6*-OE lines. Two independent lines (OE-2 and OE-11) were used for inoculation assays. Images were taken at 5 dpi. (**E**) and (**F**) Disease symptoms and relative fungal biomass of WT and *Dex:OsbHLH6* lines after *M. oryzae* infection. Two independent *Dex:OsbHLH6* lines were pretreated with 10 μM Dexamethasone (Dex). 24 h later, the plants were sprayed with fungal spores. (**G**) and (**H**) Disease symptoms and relative fungal biomass of WT and *OsbHLH6*-RNAi lines. Two independent RNAi lines (RNAi-1 and RNAi-3) were used. (**I**) and (**J**) Disease symptoms and relative fungal biomass of WT and *OsbHLH6*-Cas9 lines. Two independent knock-out lines generated by CRISPR/Cas9 technique were used for the assays. In (**C**)–(**J**), conidial suspensions (1×10^5 conidia per mL in 0.02% Tween-20) were sprayed onto the leaf surface. The fugal biomass was determined by RT-qPCR of *M. oryzae Pot2* gene against rice *OsUbi1* gene. Values are means ± *SD* (n = 3 biological replicates). ** Indicates significant differences from WT by student's *t*-test ($P < 0.01$).

OsbHLH6 and its orthologs may be unique to the monocots.

Consistent with a previous report (Kiribuchi et al. 2004), we found that the expression of *OsbHLH6* was significantly induced by 100 μM methyl jasmonate (MeJA) in rice leaves (Figure 2A). We also found that the levels of JA increased following *M. oryzae* infection (Figure 2B). OsMYC2 is known to be a major regulator of JA signaling; therefore, we examined whether OsMYC2 could directly activate *OsbHLH6* transcription. The bHLH domains of MYC-type TFs bind to G-box (CACGTG and CACATG) or G-box-like (CANNTG) elements in the promoters of their target genes (Cai et al. 2014). We analyzed the promoter region of *OsbHLH6* and found three putative bHLH-binding motifs (G1, G2, and G3; Figure 2C). To test the binding specificity, we then performed an electrophoresis mobility shift assay (EMSA) using recombinant OsMYC2 DNA -binding domains (500–751aa) and the fragment

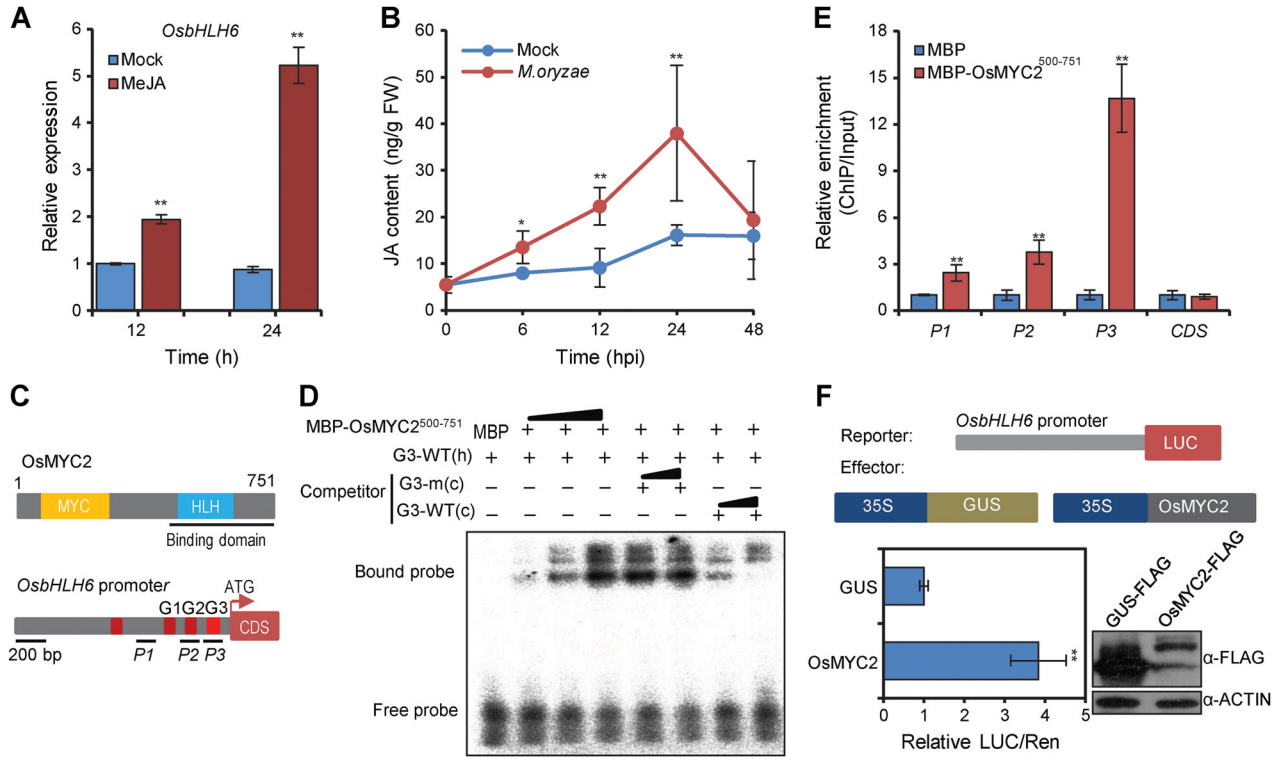

Figure 2. *OsbHLH6* expression is regulated by jasmonic acid (JA)

(**A**) Transcription analysis of *OsbHLH6* in two-week-old rice leaves treated by 100 μM MeJA. The mRNA was extracted at 12 and 24 h post MeJA treatments. RT-qPCR was used to evaluate the gene transcription levels. Values are means ± *SD* (n = 3 biological replicates). (**B**) *M. oryzae* infection induces JA accumulation. Two-week-old rice seedlings were treated with *M. oryzae* spores. Conidial suspensions (1×10^5 conidia per mL in 0.02% Tween-20) were sprayed onto the leaf surface. The JA content was measured at indicated time points. The experiment was repeated three times with similar results. Values are means ± *SD* (n = 3 biological repeats). FW, fresh weight. (**C**) Schematic diagrams of the OsMYC2 binding domain and the putative OsMYC2 binding sites (G1, G2, and G3) in the promoter of *OsbHLH6*. (**D**) OsMYC2 protein binds to G3 region of the *OsbHLH6* promoter. MBP-tagged OsMYC2$^{500\text{-}751}$ protein was incubated with γ^{32} P-labeled DNA fragments. The fragments containing G3 motif (G3-WT) and G3 motif mutant (G3-m) were examined for the binding specificity. Competition for the γ^{32}P-labeled promoter region was performed by adding an excess of unlabeled G3 containing fragment (Competitor). "h" represents isotope-labeled hot probe, and "c" represents unlabeled fragment. Two biological replicates were performed with similar results. (**E**) Chromosome immunoprecipitation (ChIP) assays showing OsMYC2 binding with the *OsbHLH6* promoter. DNA fragments co-incubated with MBP was used as a negative control. Relative enrichment was represented as the normalized ratio of the ChIP DNA to the input genomic DNA at the site. P1, P2, and P3 are the fragments of the promoter, amplified using the primers in Table S1. Values are means ± *SD* (n = 3). (**F**) OsMYC2 activates the promoter activity of *OsbHLH6* in rice protoplasts. The *OsbHLH6* promoter was fused with LUC reporter. GUS serves as a negative control. Luciferase activities were measured with a dual-luciferase reporter assay system. Values are means ± *SD* (n = 5 biological repeats). Right panel indicates the protein abundance. * and ** indicate significant differences from the control by student's *t*-test at $P < 0.05$ and $P < 0.01$, respectively.

containing G3 motif, which demonstrated that OsMYC2 can bind to the G3 region of the *OsbHLH6* promoter *in vitro* (Figures 2D, S4). Non-labeled DNA fragment G3-WT was able to effectively compete with the binding of OsMYC2 to the promoter of *OsbHLH6*, but G3-m (G3 motif was mutated to AAAAAA) could not (Figures 2D, S4), indicating the binding specificity of OsMYC2 with G3 motif.

To verify the EMSA result, we then performed a chromatin immunoprecipitation-quantitative polymerase chain reaction (ChIP-qPCR) assay using purified recombinant MBP-OsMYC2$^{500\text{-}751}$ protein (containing DNA binding domain) and rice genomic DNA fragments. The result showed that MBP-OsMYC2$^{500\text{-}751}$ efficiently bound P1, P2, and P3 fragments, where P3 is the major fragment that was bound with MBP-OsMYC2$^{500\text{-}751}$ (Figure 2E). By

contrast, MBP did not bind any of the above fragments. Furthermore, we were able to demonstrate that OsMYC2 activated *OsbHLH6* expression in rice protoplast using a dual transactivation luciferase system (LUC/REN). As shown in Figure 2F, OsMYC2 induced the expression of *LUC* driven by the *OsbHLH6* promoter, but GUS did not. These results indicate that OsMYC2 binds directly and specifically to the *OsbHLH6* promoter and activates its transcription.

OsbHLH6 regulates JA and SA signaling

In order to further assess the roles of OsbHLH6 in plant immune response, we sequenced the transcriptomes of WT, *OsbHLH6*-OE, and *OsbHLH6*-Cas9-9 rice seedlings. We found that 2,212 DEGs in *OsbHLH6*-OE, and 711 DEGs in *Cas9-9* line, which generates 509 and 202 terms in *OsbHLH6*-OE and *Cas9-9* lines, respectively (Figure 3A, B). *OsbHLH6*-OE and cas9-9 lines share 175 common terms, and importantly, by focusing on the signaling and biological terms, it clearly shows that JA, SA, and ABA signaling are enriched in *OsbHLH6-OE* and *Cas9-9* lines (Figure 3B, C). Jiang et al. (2010) showed ABA suppressed SA signaling to impair the blast disease resistance in rice. By rice plasma membrane proteomic analysis, we previously showed that SA and ethylene signaling was suppressed in *M. oryzae*-infected rice leaves at early infection stage (Cao et al. 2016). These data indicate that the hormone signaling pathway is significantly influenced by OsbHLH6. Indeed, OsbHLH6 appears to play an essential role in JA signaling, as the JA-responsive gene *OsJAR1* transcription was significantly impaired in the mutant *Cas9-9* after treatment with MeJA (Figure S5A).

Because SA/JA either positively or negatively interacts in rice (De Vleesschauwer et al. 2014) and OsbHLH6 acts in JA signaling (Figure S5A), we then compared the expression of the SA/JA-responsive genes in these plants, revealing that they were highly influenced in the *OsbHLH6-OE* or Cas9-9 plants (Figure 3D, E). The annotated SA or JA signaling genes in the common DEGs were pulled out to make the heatmaps. Strikingly, the known JA positive responsive genes, *OsJAR1*, *OsOPR1*, *OsMYC2*, *OsLOX1*, and *OsCOI1*, expressed at higher levels in *OsbHLH6*-OE line (Figure 3D), confirming that JA signaling was activated in these plants.

The JA- and SA-mediated signaling pathways often antagonize each other in higher plants; therefore, we analyzed the DEGs for SA-signaling genes. Consistently, the major SA-responsive genes, *OsNPR1* and *OsWRKY45*, expressed at lower levels in the *OsbHLH6*-OE lines but at higher levels in the mutant lines (Figure 3E). Due to the lack of clear SA signaling annotation in rice, we were unable to identify more SA responsive genes in our assays. Nevertheless, the above data indicate that SA signaling may be repressed in *OsbHLH6*-OE lines but activated in *OsbHLH6* mutant lines.

To verify the transcriptome data, we examined the expression of the key responsive genes of SA- and JA-signaling pathways, *OsWRKY45* and *OsJAR1* (JASMONATE RESISTANT 1) (Riemann et al. 2008; Shimono et al. 2012), respectively. *OsJAR1* expression was significantly upregulated in the two *OsbHLH6*-overexpressing lines, but has a reduced expression in the mutant lines *Cas9-5* and *Cas9-9* (Figure 3F, G). By contrast, the SA responsive gene *OsWRKY45* and *OsPR1b* expressed at a lower level in the overexpression lines, whereas it expressed at much higher levels in mutant lines when compared to the WT (Figure 3H–K).

The OsbHLH6-activated JA signaling is likely due to the elevated levels of JA biosynthesis. The *OsbHLH6*-OE lines contained over two folds more JA than the WT plants (Figure S5B). In addition, we also observed that JA-Ile was significantly accumulated in the overexpression line (Figure S5C). Many of the JA-related genes are known to be involved in the progress of senescence, including *RCCR1*, *Osh36*, and *SGR* (Liang et al. 2014). An analysis of these genes in the *OsbHLH6*-OE lines revealed a strong upregulation in their expression relative to the WT (Figure S6). The above-mentioned results indicate that OsbHLH6 plays a critical role in regulating JA and SA signaling in rice.

Cytosolic OsbHLH6 cannot activate JA signaling

Many TFs are exclusively located in the nucleus; however, while our subcellular localization analysis revealed that GFP-OsbHLH6 is predominantly located in the nucleus, a certain amount of GFP-OsbHLH6 was also observed in the cytoplasm, as revealed using rice protoplast expression system (Figure 4A). This notion was further verified by subcellular fraction assays in rice. We generated an antibody that could specifically recognize OsbHLH6 in rice (Figure S7). Using this antibody, we showed that OsbHLH6 localized in nucleus

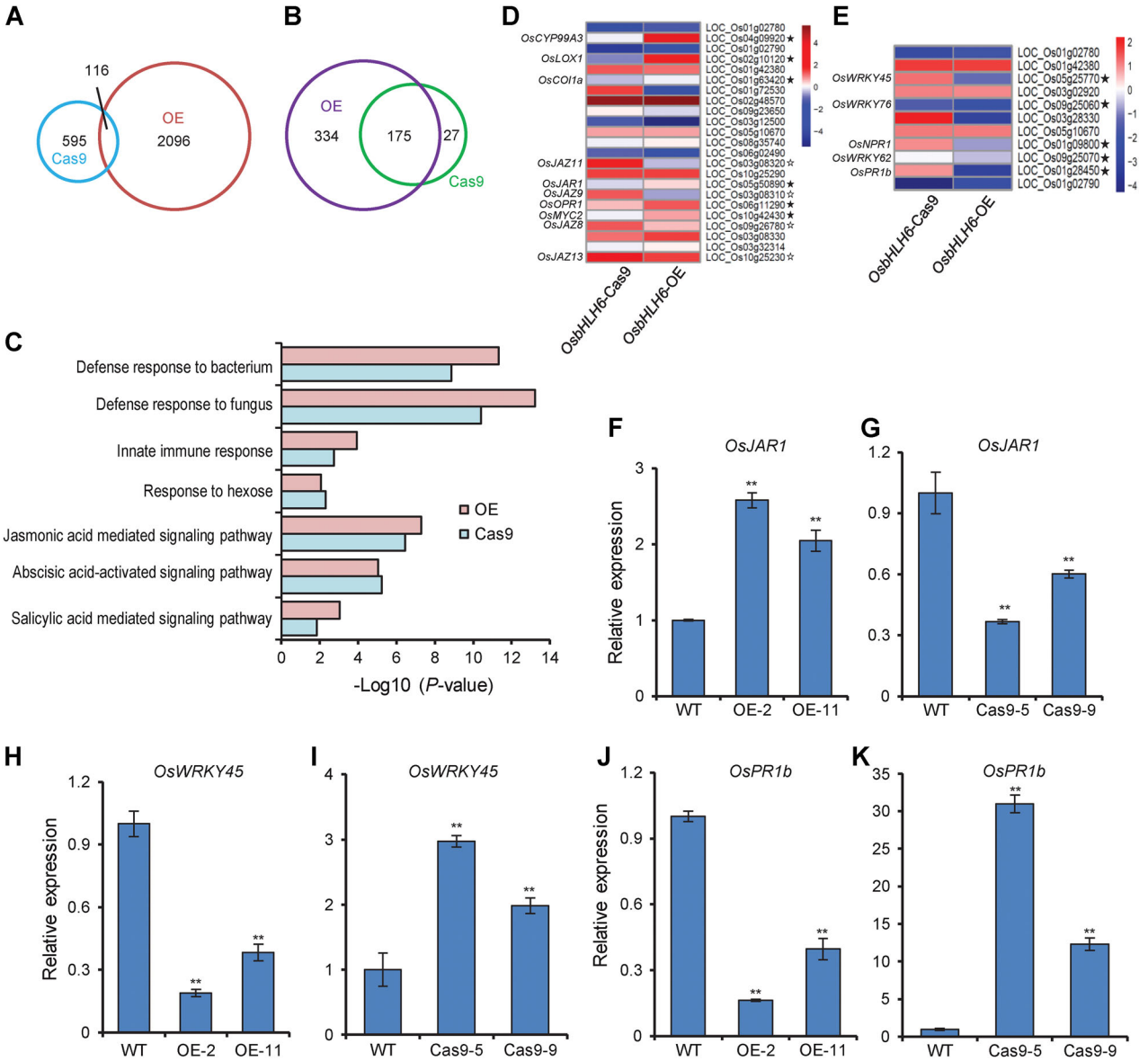

Figure 3. OsbHLH6 regulates jasmonic acid (JA) and salicylic acid (SA) signaling

(**A**) Differential expressed genes (DEGs) in *OsbHLH6* overexpression and mutant lines. Numbers indicate the DEGs in *Ubi:OsbHLH6* and Cas9-9 lines by transcriptome assays when compared to WT plants. The genes with fold change >1.5 and *P* value <0.05 were analyzed. (**B**) Gene terms enriched in the differentially expressed genes in *OsbHLH6*-OE and *OsbHLH6*-Cas9 plants. The indicated terms were identified differentially expressed in *OsbHLH6* overexpression and knockout plants, respectively. $P < 0.05$ and the number of DEGs in each term over 20 were analyzed. (**C**) Gene ontology terms for response and signaling pathways. The common terms (175) in *Ubi:OsbHLH6* and Cas9-9 lines were analyzed. Note the prevalence of terms associated with the JA, SA, and abscisic acid (ABA) pathway. (**D**) and (**E**) JA- and SA-responsive genes were differentially expressed in *OsbHLH6*-OE and *OsbHLH6*-Cas9 plants. Shown is hierarchical clustering of JA and SA-responsive genes as differentially expressed in the pairwise comparison between WT and *OsbHLH6*-OE and *OsbHLH6*-Cas9 in their common terms, respectively. Solid stars indicate the positive regulators, and hollow stars indicate the negative regulators. (**F**) and (**G**) The JA responsive gene *OsJAR1* expression in the *OsbHLH6*-OE and mutant lines. The *Ubi:OsbHLH6* (OE-2 and OE-11) and two independent Cas9 lines (5 and 9) were sampled for *OsJAR1* expression assay by RT-qPCR. The experiment was repeated three times. Values are means ± *SD* (n = 3 biological replicates). (**H**–**K**) The *OsWRKY45* and *OsPR1b* expression levels in *Ubi:OsbHLH6* and Cas9 lines. Others are as in (**F**). ** indicates significant differences from WT by student's *t*-test at $P < 0.01$.

as well as the cytoplasm fractions in the *Dex:OsbHLH6* plants after treatment with Dex (Figure 4B). Notably, the nuclear OsbHLH6 protein migrated slightly slower than those in total or cytoplasm, which may be post-translationally modified.

Therefore, we next explored the effect of cytosolic OsbHLH6 on plant defense. We generated *OsbHLH6*-NES transgenic rice plants, in which OsbHLH6 was fused with a nuclear export signal (NES) to force OsbHLH6 to be localized in the cytosol (Figure S8A). Unlike the *OsbHLH6*-OE plants whose over 75% of which displayed a dwarf and yellowish phenotype, the *OsbHLH6*-NES transgenic plants had a similar phenotype to the WT at the seedling stage (Figure 4C).

Because overexpression of OsbHLH6 significantly reduced the blast disease resistance in rice (Figure 1),

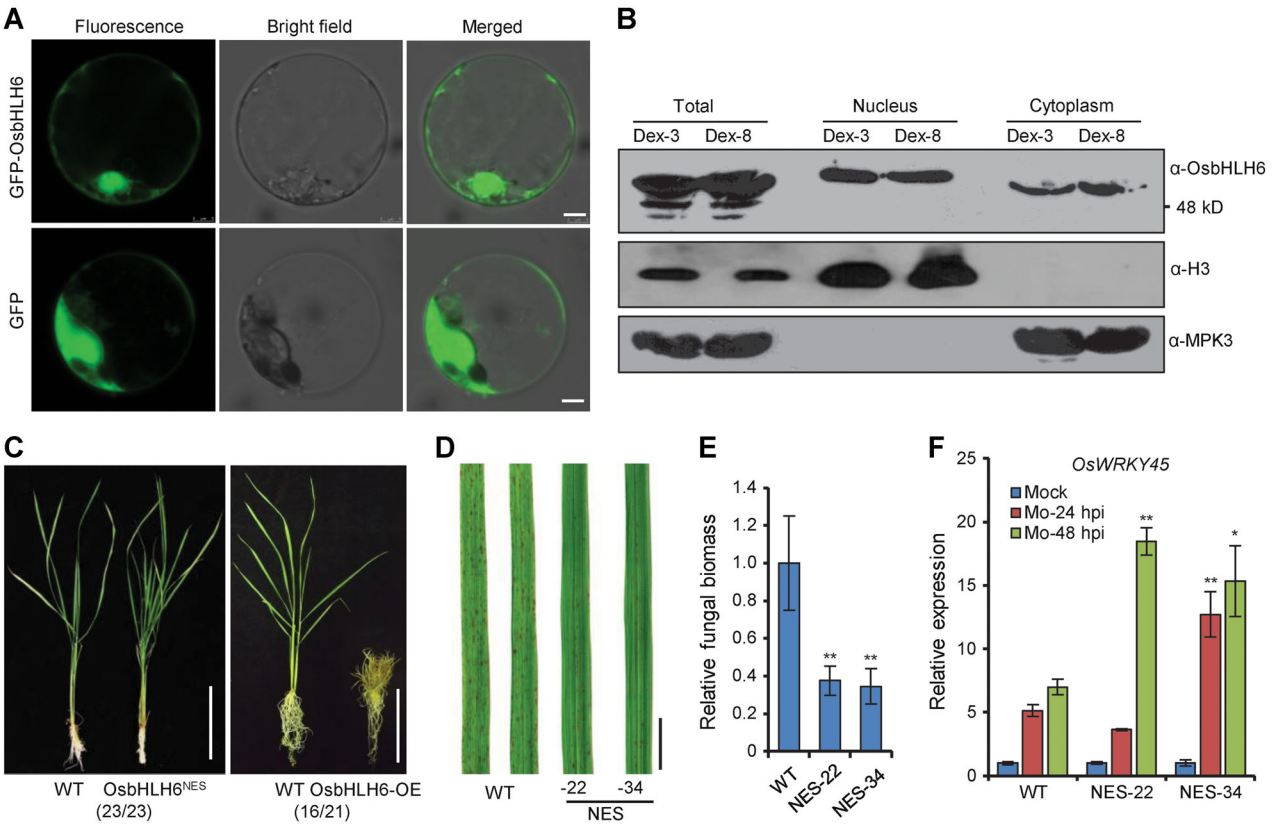

Figure 4. OsbHLH6 functions in nucleus

(**A**) The subcellular localization of OsbHLH6. OsbHLH6 fused with GFP fluorescence protein was transiently expressed in rice protoplasts. The images were captured by confocal microscope at 16 h after transformation. GFP alone serves as a negative control. Bar = 5 μm. (**B**) Immunoblot analysis showed the subcellular localization of OsbHLH6 in rice. Two independent Dex inducible *OsbHLH6* expression lines, Dex-3 and Dex-8, were used. 10 μM Dexamethasone were used to induce the gene expression. Total proteins were extracted from 2-week-old rice seedlings; nuclear and cytoplasm proteins were separated using CelLytic PN Isolation/Extraction kit. Proteins were detected by western blot with an α-OsbHLH6 antibody. α-MPK3 and α-H3 antibodies were used as cytosolic and nuclear markers, respectively. The experiment was repeated twice with similar results. (**C**) The phenotype of *OsbHLH6*-OE and *OsbHLH6*-NES transgenic plants. *OsbHLH6*-OE and *OsbHLH6*-NES constructs driven by ubiquitin promoter were transformed to the Nipponbare (WT) plants, respectively. T_1 transgenic plants were photographed. (**D**) Disease symptoms of WT and *OsbHLH6*-NES plants. The pathogen inoculation is same as in Figure 1B. Images were taken at 4 dpi. (**E**) Relative fungal biomass in (**D**). ** Indicates significant differences from WT by student's *t*-test ($P < 0.01$). Values are means ± *SD* (n = 3 biological repeats). (**F**) *OsWRKY45* expression levels in the *OsbHLH6*-NES plants after *M. oryzae* infection. RT-qPCR was used to examine the gene expression. The 2-week-old plants were spray-inoculated with *M. oryzae* conidial suspensions at a concentration of 5×10^5 conidia per mL in 0.02% Tween-20. The infected leaves were sampled at 24 and 48 hpi. Two independent lines were used for the gene expression assays.). * and ** indicate significant differences from WT by student's *t*-test at $P < 0.05$ and $P < 0.01$, respectively. Values are means ± *SD* (n = 3 biological replicates).

we then investigated the effect of cytosol OsbHLH6 on the disease resistance. The pathogen inoculation assays revealed that the *OsbHLH6*-NES plants were more resistant than the WT to *M. oryzae* infection, as revealed by the much smaller disease lesions and reduced fungal biomass in the *OsbHLH6*-NES leaves (Figure 4D, E). These results demonstrate that restricting OsbHLH6 to the cytosol leads to enhanced blast disease resistance. Because OsWRKY45 is known to be a positive regulator in rice blast disease resistance and SA signaling (Shimono et al. 2012), we then examined its expression. The result showed that *M. oryzae* infection activated *OsWKRY45* expression, but its expression levels were much higher in the *OsbHLH6*-NES plants (Figure 4F), suggesting that SA signaling was further activated in the transgenic plants. It is worth noting that expression of OsbHLH6^{NES} led to the reduction of nuclear OsbHLH6, as they interact with wild type OsbHLH6 in the cytosol (Figure S8B–D). The above-mentioned data show that containing OsbHLH6 in the cytosol largely abolishes its function.

OsbHLH6 interacts with OsMYC2

Previous studies have shown that proteins containing bHLH domains can form homodimers or heterodimers (Toledo-Ortiz et al. 2003). We therefore speculated that, in addition to being transcriptionally regulated by the JA-signaling mediator OsMYC2, OsbHLH6 might interact with this protein, which also contains a bHLH domain. Split luciferase (LUC) assays showed that OsbHLH6 indeed interacted with OsMYC2 in the plant leaves by *Agrobacterium*-mediated transient expression in *Nicotiana benthamiana,* and the interaction strength was comparable to the interaction of OsJAZ1 and OsMYC2 (Figure 5A). Further, the bimolecular fluorescence complementation (BiFC) assays revealed that the combination of nYFP-OsMYC2 and cYFP-OsbHLH6 reconstituted fluorescence in the nucleus, whereas the close homologous cYFP-OsbHLH3 and nYFP-OsMYC2 did not produce any fluorescence (Figure 5B), indicating that OsMYC2 specifically interacts with OsbHLH6 in plant nucleus.

OsbHLH6 competes with OsJAZ1 to bind OsMYC2

JAZ proteins act as transcriptional repressors of the JA response by interacting with OsMYC2 to repress its activation of downstream JA-signaling genes (Gimenez-Ibanez et al. 2014). OsbHLH6 interacts with OsMYC2 (Figure 5A, B); therefore, we considered whether OsbHLH6 competes with OsJAZs to bind OsMYC2. By split LUC assays in *N. benthamiana*, we showed that OsMYC2 interacted with OsJAZ1 (Figure 5A, C); however, their interaction was attenuated by OsbHLH6. Following the addition of OsbHLH6, the interaction of OsMYC2 and OsJAZ1 was greatly diminished in a dose-dependent manner; by contrast, GFP did not interfere with their interactions (Figure 5C).

Next, we investigated whether OsbHLH6 could affect OsMYC2-mediated gene transcription. MYC2 has been reported to bind to the promoter of *SAG29* (*SENESCENCE-ASSOCIATED GENE29*) and activate its expression in *Arabidopsis* (Qi et al. 2015). We therefore used the *SAG29* promoter as the binding target of OsMYC2. If OsbHLH6 can interfere with the association of OsMYC2 and OsJAZ1, it should enhance the transcription of OsMYC2-targeted genes. Our LUC activation assays showed that the expression level of *LUC* driven by the *SAG29* promoter was transiently upregulated by OsMYC2; however, this activation was dramatically repressed by the co-expression of *OsJAZ1* (Figure 5D). The activation of *SAG29:LUC* expression by OsMYC2 in the presence of JAZ1 was partially restored by co-expressing *OsbHLH6* (Figure 5D). The above results indicate that OsbHLH6 activates OsMYC2-targeted gene transcription by interfering with the association of OsJAZ1 with OsMYC2.

OsbHLH6 interacts with OsNPR1 in the cytoplasm

Our results indicate that overexpression of *OsbHLH6* suppresses SA signaling in rice (Figure 3H–K); therefore, we next investigated how this suppression might occur. We examined the interaction of OsbHLH6 with several key SA regulators using BiFC assays, and the result revealed that OsbHLH6 but not the close homologous OsbHLH3 interacts with the SA master regulator OsNPR1 in rice protoplast (Figure 6A). It has to be pointed out that YFP fluorescence was found to be aggregated in the cytosol of the rice protoplasts, demonstrating that OsNPR1 and OsbHLH6 interact in the cytoplasm, as indicated by previous cytosolic aggregations of proteins in BiFC assays (Huh et al. 2017). Similarly, BiFC assays were performed in *N. benthamiana*, which also showed that the interaction occurred in the cytoplasm (Figure S9A). The interaction was further confirmed by immunoprecipitation (IP) assays using rice protoplast that were co-expressed with OsNPR1 and OsbHLH6

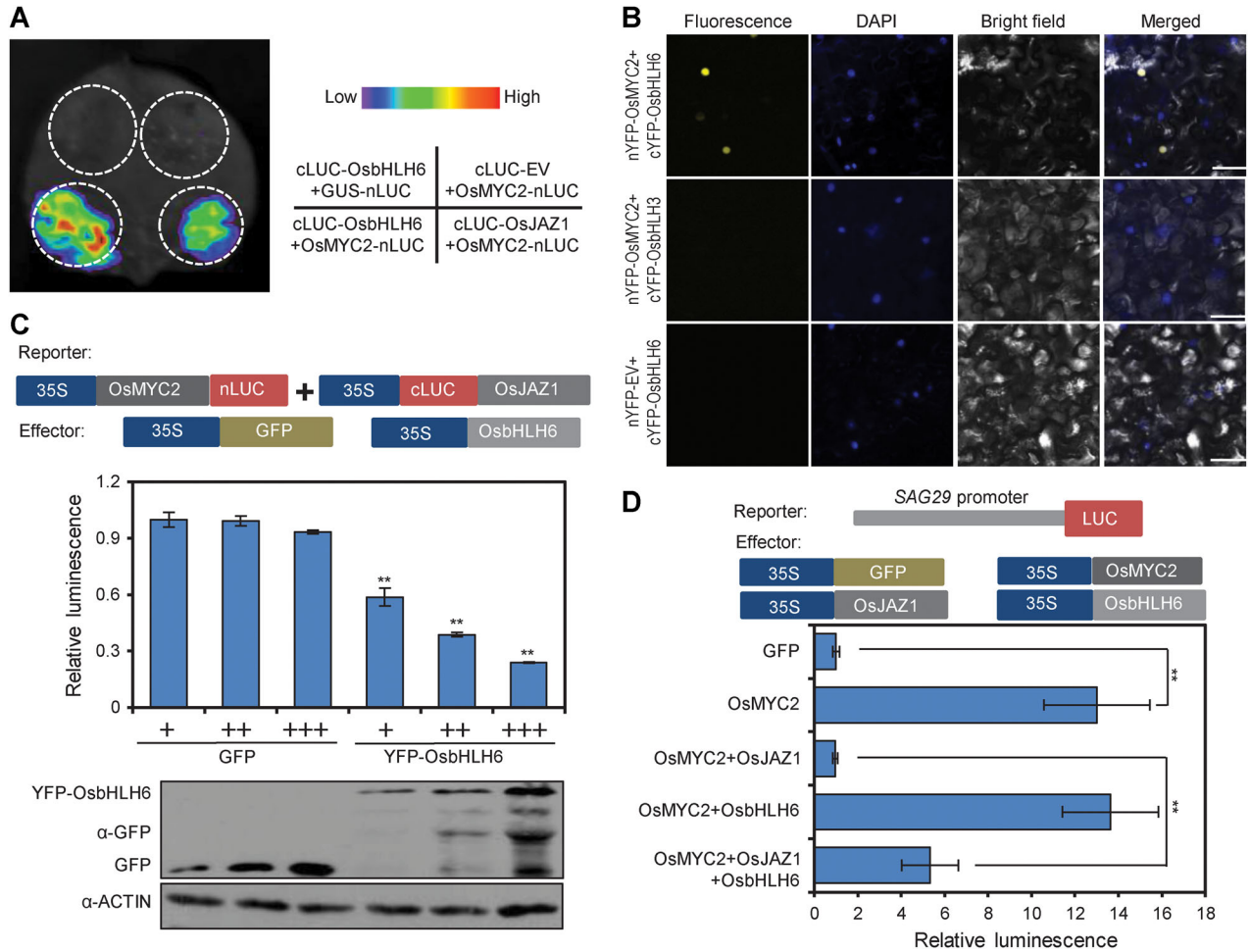

Figure 5. OsbHLH6 activates jasmonic acid (JA) signaling by reducing the suppression of OsJAZs on OsMYC2

(**A**) OsMYC2 interacts with OsbHLH6 by split-luciferase complementation assays. The OsMYC2-nLUC and cLUC-OsbHLH6 were transiently expressed in *N. benthamiana* leaves by *Agrobacteria*-mediated expression system. Co-expression of OsMYC2-nLUC and cLUC-OsJAZ1 served as a positive control. (**B**) Bimolecular fluorescence complementation (BiFC) analysis shows the interaction between OsbHLH6 and OsMYC2. The proteins were transiently expressed in *N. benthamiana* leaves. nYFP, N-terminal fragment of YFP; cYFP, C-terminal fragment of YFP. Nucleus of leaf epidermal cells were stained with DAPI. OsbHLH3 served as a negative control. The experiment was repeated at least three times with similar results. Bar = 50 μm. (**C**) OsbHLH6 competes with OsJAZ1 to bind OsMYC2 in planta. OsMYC2-nLUC and cLUC-OsJAZ1 were transiently expressed in *N. benthamiana* leaves. Incremental expression of OsbHLH6 or GFP was used to examine the effect on the association of OsMYC2 and OsJAZ1. GFP serves as a negative control. Lower panel indicated the protein abundance. (**D**) OsbHLH6 undermines OsJAZ1's suppression on the OsMYC2-targeted gene expression. Transient luciferase activities in *N. benthamiana* were analyzed by co-transforming with the LUC reporter and different combinations of effectors. *LUC* gene is driven by the *SAG29* promoter. GFP serves as the negative control. ** indicates significant differences from the control by student's *t*-test ($P < 0.01$). Values are means ± *SD* (n = 5 biological repeats).

(Figure 6B). These data indicate that OsNPR1 and OsbHLH6 are physically associated in rice cells. OsNPR1 contains a BTB domain in its N-terminal region and an ankyrin-repeat domain in the C-terminus (Figure 6C). MBP pull-down assays revealed that MBP-OsbHLH6 directly associated with the ankyrin-repeat domain in OsNPR1, but not with its BTB domain (Figure 6D).

Previous studies have shown that treatment with SA/DTT leads to the translocation of NPR1 from the cytoplasm to the nucleus in both *Arabidopsis* and rice (Mou et al. 2003; Yuan et al. 2007; Spoel et al. 2009). We therefore investigated whether the association of OsbHLH6 with OsNPR1 is affected by SA. OsNPR1-T7 and OsbHLH6-FLAG were co-expressed in *N.*

benthamiana, and IP assays further indicated an interaction between OsNPR1 and OsbHLH6; however, the interaction was reduced by a treatment of 100 μM SA (Figure S9B, C). This observation was further confirmed by BiFC assays in both rice protoplast and *N. benthamiana*, where SA treatment remarkably reduced the interaction (Figure S9D, E). These results indicate that the interaction of OsbHLH6 and OsNPR1 is regulated by SA. However, OsNPR1^{2CA}, the mutation in C76A/C216A sites which causes that OsNPR1 cannot form oligomer and is localized in the nucleus (Yuan et al. 2007), interacts much weaker than OsNPR1 (Figure S9F), suggesting that the OsNPR1 oligomer is most likely the major form to interact with OsbHLH6.

OsbHLH6 is primarily localized in the nucleus (Figure 4A, B), yet we have shown that its interaction with OsNPR1 likely occurs in the cytoplasm (Figure 6A). We therefore investigated whether the nucleo-cytosolic trafficking of OsbHLH6 occurs when the level of OsNPR1 in the cytosol is increased. We found that the fluorescence intensity of the nuclear localization of YFP-OsbHLH6 was significantly weakened when

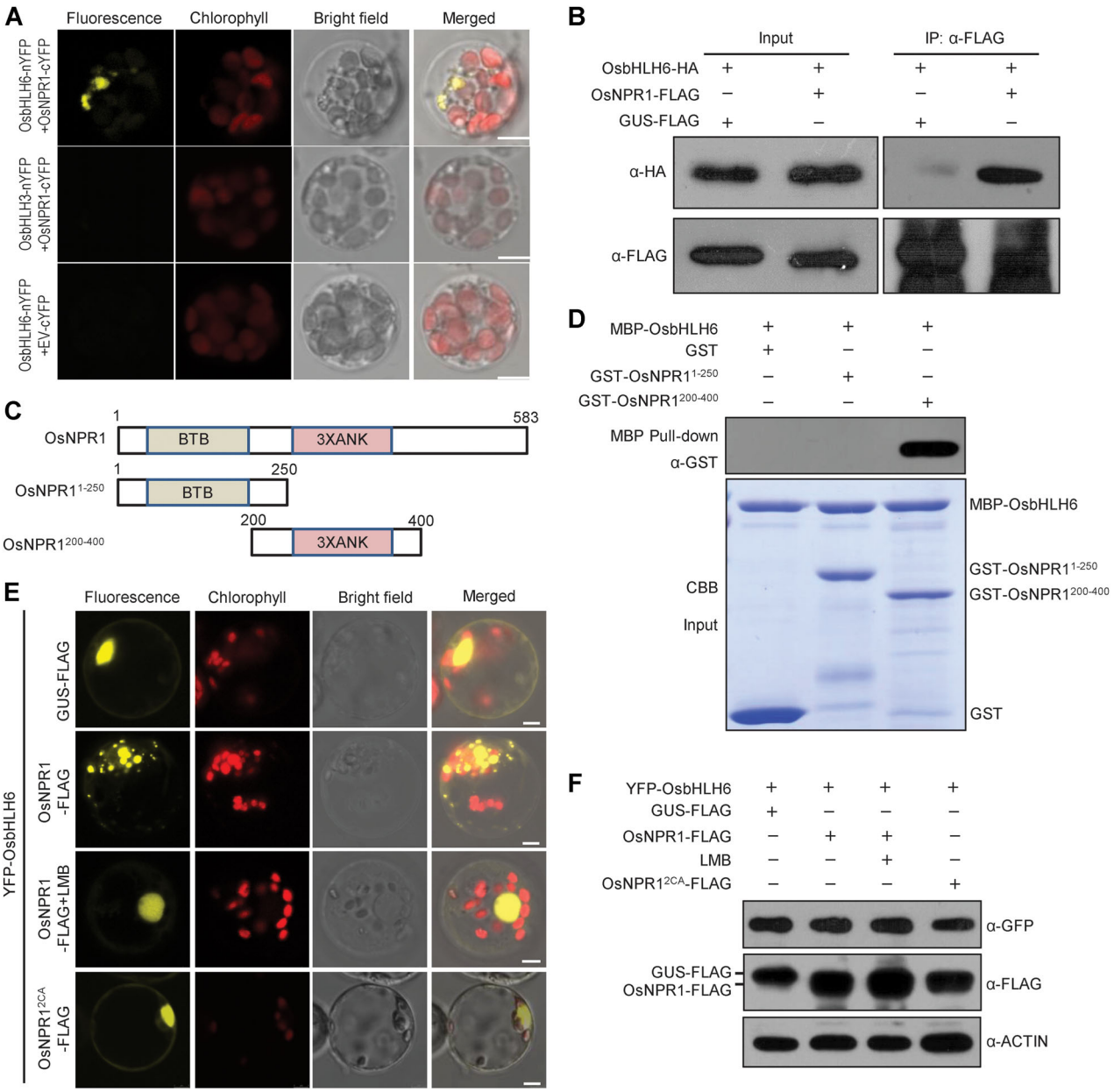

Figure 6. Continued

co-expressed with OsNPR1 (Figure 6E); however, the OsNPR1-mediated relocation of YFP-OsbHLH6 could be restored by a treatment with 200 nM LMB (cytotoxin leptomycin B, a nuclear export inhibitor) (Haasen et al. 1999) (Figure 6E). Interestingly, OsNPR1^{2CA} was unable to induce the OsbHLH6 export from nucleus. Immunoblots using an anti-GFP antibody revealed that the levels of YFP-OsbHLH6 were similar when YFP-OsbHLH6 was co-expressed with GUS or OsNPR1, or with OsNPR1 followed by the LMB treatment (Figure 6F). Furthermore, we found that the OsNPR1-induced export of OsbHLH6 from the nucleus could be reconstituted in *N. benthamiana* (Figure S10A–C). OsNPR1-induced OsbHLH6 export from nucleus appears to be specific, as it does not induce relocation of OsEIL1, a transcription factor of ethylene signaling that is known to be localized in rice nucleus (Figure S10D, E).

OsbHLH6 interferes with the association of OsNPR1 with rTGA2.1 and suppresses SA signaling

It is known that NPR1 interacts with TGAs to activate SA responsive gene expression in *Arabidopsis* (Kesarwani et al. 2007). Because OsbHLH6 interacts with the OsNPR1 ANK domains (Figure 6D) and ANK domains are known to facilitate protein interactions (Zhang et al. 1999), leading us to speculate that OsbHLH6 may interfere with the interaction of OsNPR1 and the TGAs in rice. We choose rice rTGA2.1 to test this hypothesis, as it interacts with OsNPR1 in rice and can bind to the *as-1*-like element of the *PR-1* gene promoter (Chern et al. 2005). Our GST-OsNPR1 pull-down assays revealed that MBP-OsbHLH6 effectively caused the dissociation of OsNPR1 and rTGA2.1, but MBP alone did not (Figure 7A). We confirmed this result using split LUC assays in *N. benthamiana*, which demonstrated that OsbHLH6 significantly represses the interaction between OsNPR1 and rTGA2.1 in a dose-dependent manner (Figure 7B), while the negative control GFP did not affect this interaction.

We then determined whether OsbHLH6 could suppress the transcription of the SA-responsive genes *in vivo*. As expected, we found that OsNPR1 could coordinate with rTGA2.1 to activate *PR1b*-promoter-driven *LUC* expression; however, OsbHLH6 remarkably repressed this *LUC* expression, but the negative control (GFP) did not (Figure 7C). This result indicates that OsbHLH6 represses rTGA2.1-mediated *PR1b* transcription by interfering with the association between OsNPR1 and rTGA2.1. As a result, overexpression of OsbHLH6 may lead to the reduced expression of *OsPR1b* in the OE-2 and OE-11 lines (Figure 3J).

OsNPR1^{NES} suppresses JA signaling and releases SA signaling

Because OsNPR1 expression is induced by benzothiadiazole (Yuan et al. 2007) and OsNPR1 could sequester OsbHLH6 in the cytosol (Figure 6E), we hypothesize that SA signaling might dynamically regulate JA signaling in rice. To explore the biological significance of OsNPR1 sequestering OsbHLH6 in cytosol, we generated *OsNPR1^{NES}* (NES, nuclear export signal) transgenic plants in *OsbHLH6*-OE background. As we showed earlier, the *OsbHLH6*-OE plants displayed elevated JA signaling but

Figure 6. OsNPR1 interacts with OsbHLH6 and sequesters OsbHLH6 in cytoplasm

(**A**) Bimolecular fluorescence complementation (BiFC) analysis shows the interaction between OsbHLH6 and OsNPR1 in rice protoplasts. The plasmids carrying OsbHLH6-nYFP and OsNPR1-cYFP constructs were co-transformed into rice protoplasts. The fluorescence was observed using a confocal microscope at 16 h after transformation. OsbHLH3 served as a negative control. The experiment was repeated at least three times with similar results. Bar = 10 μm. (**B**) OsNPR1 interacts with OsbHLH6 in rice. The OsbHLH6-HA and OsNPR1-FLAG were transiently expressed in rice protoplasts. After 16 h transformation, the proteins were subjected to immunoprecipitation (IP) assays. GUS-FLAG served as a negative control. The experiment was repeated three times with similar results. (**C**) Schematic diagram of OsNPR1 constructs. (**D**) MBP pull-down assays show the interaction between OsbHLH6 and OsNPR1$^{200\text{-}400}$ *in vitro*. The recombinant OsNPR1 fragments GST-OsNPR1$^{1\text{-}250}$ and GST-OsNPR1$^{200\text{-}400}$ were incubated with MBP-OsbHLH6, and the interacting proteins were visualized by probing with anti-GST antibodies. The experiment was repeated three times with similar results. (**E**) OsNPR1 induces OsbHLH6 export from nucleus to the cytoplasm. GUS-FLAG, OsNPR1-FLAG, and YFP-OsbHLH6 were transiently expressed in rice protoplasts. GUS-FLAG was used as a negative control. 200 nM nucleus export inhibitor LMB was added in the protoplast at 12 h before the fluorescence observation. Confocal microscope was used to observe the YFP-OsbHLH6 subcellular localization. Bar = 10 μm. (**F**) The immunoblots show the protein levels of YFP-OsbHLH6, GUS-FLAG, OsNPR1-FLAG, and OsNPR1^{2CA}-FLAG in (**E**). The expressed proteins were probed with respective antibodies to show the similar levels in rice protoplasts. ACTIN served as the internal reference.

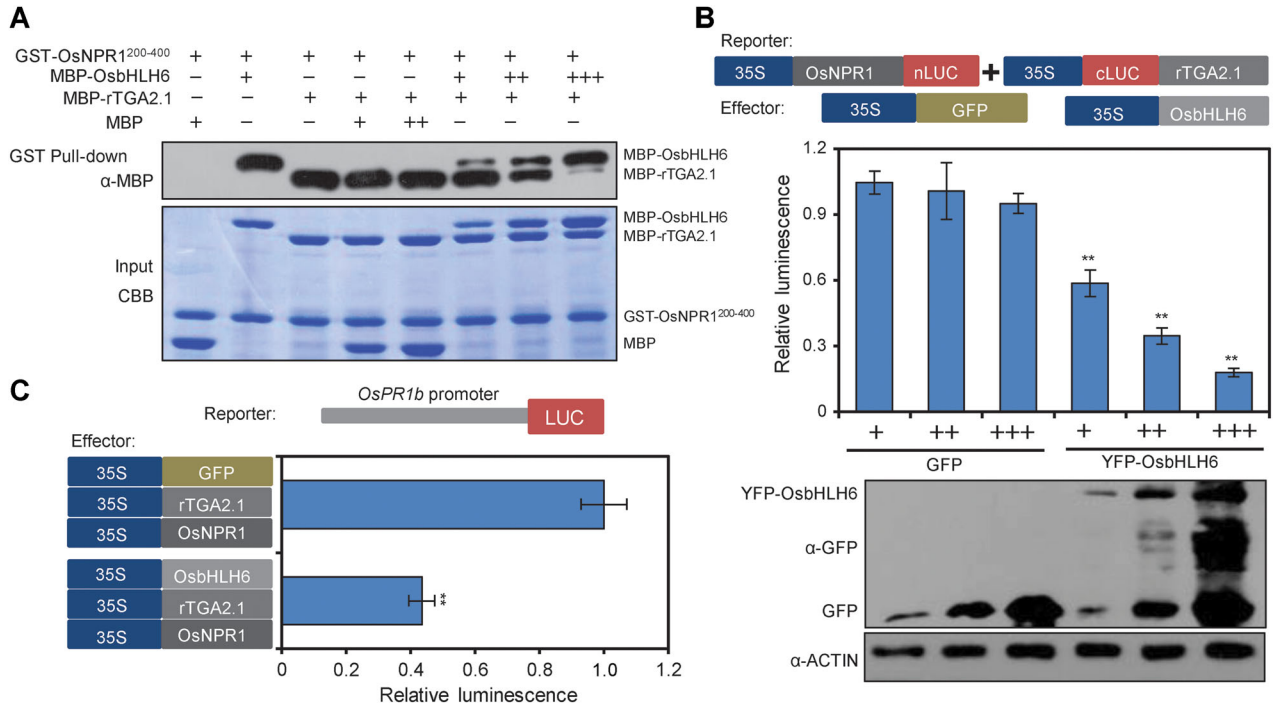

Figure 7. OsbHLH6 represses OsNPR1-mediated *OsPR1b* transcription

(**A**) OsbHLH6 reduces the interaction of rTGA2.1 and OsNPR1. GST pull-down assays were used to examine the effect of OsbHLH6 on the interaction of OsNPR1 and rTGA2.1. The proteins were expressed in *E. coli* and the purified recombinant proteins were used in the assays. Incremental MBP and MBP-OsbHLH6 were used for the competition assays. The interacting proteins were visualized by probing with anti-MBP antibodies. The experiment was repeated three times with similar results. (**B**) The split luciferase assays show that OsbHLH6 reduces the interaction of rTGA2.1 and OsNPR1. OsNPR1-nLUC and cLUC- rTGA2.1 were transiently expressed in *N. benthamiana* leaves. Incremental OsbHLH6 were co-transformed to examine the effect on the association of OsNPR1 and rTGA2.1. GFP serves as a negative control. Lower panel showing the protein abundance. (**C**) OsbHLH6 attenuates the promoter activity that is activated by OsNPR1 and rTGA2.1. The *OsPR1b* promoter was fused with LUC reporter. The transcription activity was analyzed in the combination of OsbHLH6, rTGA2.1, and OsNPR1. GFP serves as a negative control. ** indicates significant differences from the control by student's *t*-test ($P < 0.01$). The experiment was repeated three times with similar results.

decreased SA signaling (Figure 3); however, with expression of *OsNPR1*^{NES}, the JA signaling was significantly impaired as indicated by reduced expression of *OsJAR1* in the *OsNPR1*^{NES}/*OsbHLH6*-OE lines (Figure 8A). By contrast, the expression of SA responsive gene *OsWRKY45* was significantly activated (Figure 8B). These results indicate that OsNPR1-induced OsbHLH6 export from rice nucleus substantially suppresses OsbHLH6-mediated activation of JA signaling.

To further investigate the biological significance for OsbHLH6 shuttling, we examined the signaling of SA and JA dynamic changes during blast infection. Following *M. oryzae* infection, JA signaling is continuously activated; however, SA signaling shows limited activity until 24 hpi, as indicated by the upregulated expression of two SA master regulator genes, *OsNPR1* and *OsWRKY45* (Figure S11). This event suggests that SA signaling facilitates the export of OsbHLH6 from the nucleus at 24 hpi via elevated amounts of OsNPR1.

DISCUSSION

Plants must fine tune and time their immune responses to pathogen infection to maximize their defensive outputs (Caarls et al. 2015). Plant hormones are known to play essential roles in regulating the immune response; however, many pathogens can suppress the plant immune system by rewiring the complex hormonal networks (Zheng et al. 2012; Cao et al. 2016; Gimenez-Ibanez et al. 2016). In particular,

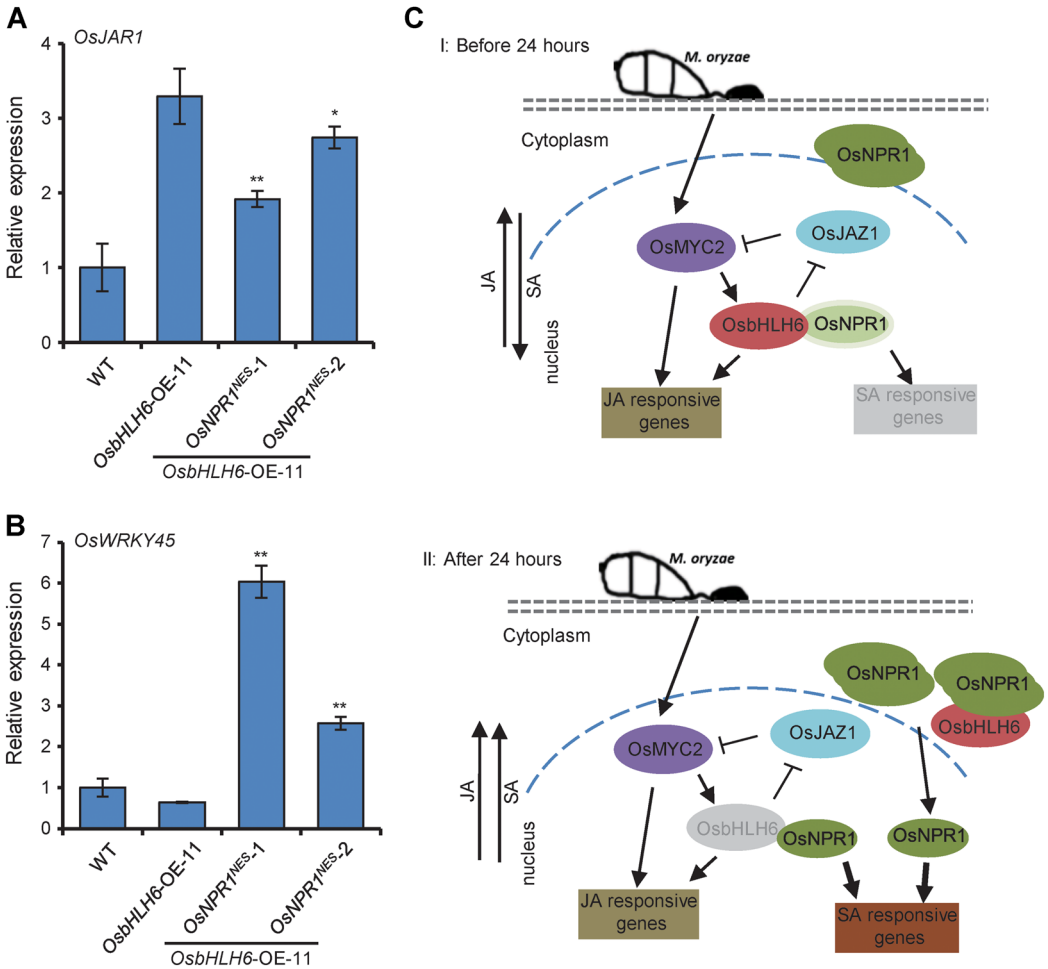

Figure 8. OsNPR1-NES attenuated OsbHLH6-activated jasmonic acid (JA) signaling
(**A**) and (**B**) *OsJAR1* and *OsWRKY45* expression levels in the *OsNPR1*^{NES}/*OsbHLH6*-OE lines. The 2-week-old *OsNPR1*^{NES} overexpression lines (T_2 lines) in *OsbHLH6*-OE-11 background were sampled for gene expression assays by RT-qPCR. * and ** indicate the differences from *OsbHLH6*-OE-11 lines at $P < 0.05$ and 0.01 by student's *t*-test, respectively. Values are means ± *SD* (n = 3 biological replicates). (**C**) A model shows the OsbHLH6 regulatory module. At early infection stage (before 24 h), *M. oryzae* infection activates *OsbHLH6* expression, which subsequently activates the JA signaling pathway and suppresses SA signaling pathway. At later infection stage (after 24 h), *OsNPR1* expression leads to the sequestering of OsbHLH6 in the plant cytosol. As the result, SA signaling pathways is activated.

infections by hemibiotrophic pathogens, such as *M. oryzae*, involve concurrent biotrophic and necrotrophic phases in different cells (Khang et al. 2010), causing an even more complex alteration of hormone signaling in the host. Indeed, the transcriptomes of rice plants during blast infection revealed that JA signaling is activated at the early stages of the disease, while SA signaling is activated at later stages (Figure S11). Although the exact role of SA is controversial in rice immune response, overexpression of the key SA regulators *OsNPR1* or *OsWRKY45* significantly enhances the resistance of the host plant to *M. oryzae* (Chern et al. 2005; Shimono et al. 2012), suggesting that restriction of SA signaling activation at the early stages of infection (biotrophic stage) contributes to pathogen infection.

Here, we demonstrated that OsbHLH6 regulates both SA and JA signaling (Figure 3). By competing with the OsJAZs and binding to OsNPR1, the activities of OsbHLH6 enable OsMYC2 to activate the transcription of the JA-responsive genes while preventing the TGAs from activating SA-responsive gene expression (Figures 5, 7). Although it remains to be investigated for why the SA signaling is repressed at the early infection

stage, *OsNPR1* transcription is only induced at later *M. oryzae* infection stages (Figure S11). We therefore speculated that, at later blast infection stages, OsNPR1 sequesters OsbHLH6 in the cytosol to prevent its activation of JA signaling and remove its inhibition on SA signaling. Notably, it has been hypothesized that NPR1 may sequester a JA-responsive TF in the cytosol to repress JA signaling in plants (Caarls et al. 2015). Several reports have suggested that cytoplasm-localized NPR1 is required for SA/JA antagonism (Spoel et al. 2003; Yuan et al. 2007). Indeed, the nucleus-localized OsNPR1^{2CA} is unable to sequester OsbHLH6 in cytosol (Figure 6E); whereas, introducing *OsNPR1*^{NES} to *OsbHLH6*-OE plants led to the suppression of JA signaling and activation of SA signaling (Figure 8A, B), supporting the notion that OsNPR1 sequesters OsbHLH6 in the cytosol to suppress the JA signaling. It has been known that SA treatment induces NPR1 oligomer to monomer and, monomeric NPR1 enters nucleus (Mou et al. 2003; Spoel et al. 2009). OsNPR1^{2CA} cannot form oligomer and is mainly located in nucleus, mimicking the SA treatment (Yuan et al. 2007). However, we also observed a weak interaction of OsNPR1^{2CA} and OsbHLH6 (Figure S9F), suggesting that the oligomeric OsNPR1 is the major form to interact with OsbHLH6. Nevertheless, it is plausible to conclude that a certain amount of monomeric OsNPR1 could hold OsbHLH6 in the cytosol before it enters the nucleus. Therefore, OsNPR1-sequestered OsbHLH6 cytosolic localization should occur early in SA signaling. Unfortunately, we were unable to show the nucelocytoplasm shuttling of OsbHLH6 by exogenous SA treatments due to incredibly high levels of SA in rice plants. In fact, rice plants appear to be insensitive to SA treatment and manipulation of endogenous SA does not alter the *PR* gene expression (Yang et al. 2004). One of the major reasons is that rice plants carry high basal levels of SA (8-37 μg/g fresh weight) (Silverman et al. 1995). However, either overexpressing *OsNPR1* or *OsWRKY45* in rice enhances its disease resistance, suggesting that SA signaling, rather than SA levels, is important for mediating defense in rice (Chern et al. 2005; Shimono et al. 2012). Nevertheless, our finding supports the observation that cytosolic NPR1 is required for suppression of JA signaling in rice and Arabidopsis (Spoel et al. 2003; Yuan et al. 2007). Based on the results, we propose that OsbHLH6 plays an essential role in regulating SA and JA signaling (Figure 8C).

OsbHLH6-mediated SA/JA signaling regulation may be unique to monocot plants, as we showed that OsbHLH6 is phylogenetically distant from the bHLH genes in dicots (Figure S3B). Although it remains unclear how SA antagonizes JA signaling in dicot plants, we speculate that NPR1 may induce the relocation of some JA-responsive TFs to the cytosol to suppress JA signaling. In fact, the regulatory mechanism of sequestering TFs in the cytosol has also been observed previously in the interaction between the SA and ABA signaling pathways (Shang et al. 2010). In this example, SA induces the TF WRKY40 to inhibit the ABA-responsive genes, such as *ABI4* and *ABI5*, by binding to their promoters. The ABA receptor ABAR can interact with WRKY40 however, and recruits WRKY40 to the cytosol following an ABA treatment, removing the transcriptional restriction on the ABA-responsive genes in the nucleus.

The induction of JA-responsive genes and suppression of SA-responsive genes is widely adopted by the biotrophic and hemibiotrophic pathogens; for example, the hemibiotrophic *Pseudomonas* pathogens secrete coronatine, a JA mimic that can significantly induce JA signaling and suppress SA signaling (Zheng et al. 2012). Likewise, the *Pseudomonas* pathogens secrete the effectors HopZ1a and HopX1, which can bind and degrade the JAZ repressor proteins and activate JA signaling (Jiang et al. 2013; Gimenez-Ibanez et al. 2014). In addition, SA signaling can be directly manipulated by the biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis*, as reported by Caillaud et al. (2013) that the *H. arabidopsidis* effector HaRxL44 targets Mediator subunit 19 (MED19) for degradation and rewires SA-responsive and JA-responsive gene expression. We previously used a proteomic approach to show that *M. oryzae* can suppress the rice immune response at the early stages of infection (before 24 hpi, biotrophic stage), and found that the immune response was activated at later infection stages (after 24 hpi) (Cao et al. 2016). Given the rice variety "Nipponbare" used in this study is susceptible to the fungi, the upregulated OsbHLH6 expression at early infection should contribute to the suppression of SA signaling, which consequently suppresses the immunity and promotes the infection.

Manipulating the plant hormone pathway has been proven to be effective in controlling pathogen infections (De Vleesschauwer et al. 2014; Berens et al. 2017). Our current work identifies an OsbHLH TF that

is involved in the regulation of SA and JA signaling pathways in rice. It would be interesting to explore the roles of OsbHLH6 in defending other pathogens. Future studies should also focus on the bHLH families in other plants in terms of understanding the SA/JA regulation.

MATERIALS AND METHODS

Plant and fungi growth conditions

Rice (*Oryza sativa subsp. Japonica* cv Nipponbare) was used as the wild type (WT) in this study. The *OsbHLH6* coding sequence was PCR amplified and cloned into the pCAMBIA1390 binary vector with an Ubi promoter or pTA7001 with a Dex-inducible promoter. For the OsbHLH6-RNAi vectors, two copies of the 484 bp specific coding sequence were assembled in opposite orientation into pTCK303 vector to form a hairpin. The CRISPR/Cas9 target in the *OsbHLH6* genomic locus was designed through the website http://www.e-crisp.org/E-CRISP/. The gRNA expression cassette was inserted into the pYLCRISPR/Cas9-MTmono binary plasmid. All the vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 through electroporation, and the resulting strains were used to transform the WT. All the plants were grown at 28°C with a 16-h/8-h light/dark photoperiod and 75% relative humidity.

M. oryzae strain ZHONG-10-8-14 was grown on oatmeal agar (50 g/L oatmeal, 30 g/L agar) for about 2 weeks at 28 °C. Conidial formation was then induced under light for 2–3 d and then the spores were collected in sterile water and used for inoculation.

Plant infection assay

Plant inoculation assays were performed as described previously (Yang et al. 2017). Briefly, 2-week-old seedlings were used for the plant infection assays. Conidial suspensions ($1 \times 10^5 \sim 5 \times 10^5$ conidia per mL in 0.02% Tween-20) were sprayed onto the surface of rice plants. The rice plants sprayed with 0.02% Tween-20 were used as mock control. Fungal biomass in infected rice leaves was determined at 5 dpi by qPCR using specific primers for the *Pot2* gene of *M. oryzae* and normalized to *OsUbi1* gene. All the experiments were performed with four replicates.

Measurement of JA, JA-Ile, and SA by HPLC-MS/MS

The rice leaves were harvested at the indicated time. For each sample, 100 mg of fresh tissue was homogenized in liquid nitrogen and extracted following the method described previously (Yang et al. 2017). 6 ng d_5-JA and 6 ng d_6-SA was used as the internal standards, respectively. JA-Ile was determined using external standard method. Endogenous JA, JA-Ile, and SA were purified and measured by Agilent 1200 HPLC coupled with Agilent 6410 triple quadrupole mass spectrometer (MS) equipped with an electrospray interface (ESI). The MS was operated in negative mode and Multiple Reaction Monitoring (MRM) of ion pairs was used for analysis of d_5-JA (214 > 61.8), endogenous JA (209.2 > 59), JA-Ile (322.2 > 130.1), d_6-SA (142.1 > 97.6), and endogenous SA (136.9 > 93). Experiments were performed with three independent biological replicates.

RNA extraction and RT-qPCR assay

Gene expression was determined from a pool of at least four leaves at the same developmental stage of soil-grown plants. Total RNA was extracted using TRIZOL reagent (Invitrogen, USA). The complementary DNAs were synthesized using PrimeScript Reagent kit with gDNA Eraser (Takara) and quantified with CFX96TM Real-time System (Bio-RAD) with the SYBR Green kit (Takara) according to the manufacturer's instructions. *OsActin1* was used as the internal control. The primers are listed in Table S1.

Library preparation and bioinformatics analysis

Two-week-old rice seedlings of WT, *OsbHLH6-Cas9*, and *OsbHLH6-OE-11* plants that were inoculated with ZHONG-10-8-14 were used for RNA-seq library preparation. The leaves were harvested at indicated time and the RNA was extracted. The messenger RNA (mRNA) was purified from 2 μg total RNA using Dynabeads mRNA purification kit (Invitrogen, USA), and then submitted to RNA-seq library construction for the transcriptome experiments using the ultra RNA library prep kit (NEB, Singapore). Multiplex paired-end adapters were used to multiplex libraries. The RNA-seq libraries were quantified using bioanalyzer (Agilent, USA), then sequenced (paired-end, 100 bp each) in the Illumina genome analyzer (Hiseq 2000). After removing adaptor and low-quality reads, clean reads were mapped to rice genome MSU7.0 using TopHat, and analyzed using Cufflinks according

to Trapnell et al. (2012) with slight modification. Poisson-dispersion model of fragment was used to conduct statistical analysis (FDR <0.05) and responsive genes were identified by fragments per kilobase per million reads (FPKM) requiring more than two-fold change between two samples. Each experiment performed with two biological replicates. Two biological replicates were used and their repeatability and correlation were evaluated by the Pearson's Correlation Coefficient. Gene Ontology enrichment analyses were performed with the Cytoscape plugin ClueGO with the *P*-value below 0.05 and the default parameters (Bindea et al. 2009). JA or SA responsive genes are referred to the articles of Ogawa et al. (2017) and Sugano et al. (2010), respectively.

Rice protoplast transformation and BiFC assay

Rice protoplast preparation and plasmid transformation were performed according to the methods of Ma et al. (2018). The pSAT1-nEYFP and pSAT1-cEYFP plasmids were used for the BiFC assay. The CDS of *OsbHLH6* was cloned into the pSAT1-nEYFP and pSAT1-cEYFP vectors. The CDS of *OsMYC2* and *OsNPR1* was cloned into the pSAT1-nEYFP and pSAT1-cEYFP vectors, respectively. The fluorescence was observed at 16 h after co-transformation. For agrobacteria-mediated protein transient expression, *A. tumefaciens* strains (C58C1) carrying the BiFC constructs were infiltrated in 5-week-old *N. benthamiana* leaves as described in Luo et al. (2017). Infiltrated leaves were observed 36–48 h later using a confocal laser scanning microscope (Leica SP8).

GST and MBP pull-down assays

The *OsbHLH6* coding sequence was cloned into pMAL-C4X and pGEX-4T-1, and the coding sequence of *OsMYC2* fragments and *rTGA2.1* were cloned into pMAL-C4X. *OsNPR1* fraction was cloned into pGEX-4T-1. A one-step cloning kit (Vazyme Biotech) was used for subcloning and the recombinant proteins were expressed in *Escherichia coli* strain BL21. Protein purification and pull-down assays were performed according to the method described by Liu et al. (2011). MBP and GST fused recombinant proteins were detected by immunoblotting using anti-MBP antibody (TransGen Biotech, HT701) and anti-GST antibody (TransGen Biotech, HT601), respectively.

Chromatin immunoprecipitation–quantitative polymerase chain reaction

Total DNA of Nipponbare (WT) and purified MBP-OsMYC2$^{500\text{-}751}$ were used for ChIP assays. The total DNA was sheared into 100–500 bp fragments using ultrasonic crusher. MBP-OsMYC2$^{500\text{-}751}$ and DNA fragments were co-incubated for 4 h in the incubation buffer (50 mM Tris, 1 mM EDTA, 100 mM KCl, adjust pH to 7.0 by HCl, 5% Glycerol, 0.1% Triton X-100, and freshly 1 mM DTT). After co-incubation, MBP beads were washed five times using incubation buffer. Reverse protein DNA cross-linking was performed by incubating the immunoprecipitated complexes in NaCl at 65°C for 4 h. DNA was recovered with a QIAquick PCR purification kit (Qiagen) and analyzed by qPCR using primers as described (Table S1). DNA fragments co-incubated with MBP was used as a negative control, while the fragments isolated before precipitation were used as an input control.

Co-immunoprecipitation assays

The CDS of *OsbHLH6* and *OsNPR1* was cloned to the pMD1-Flag and pMD1-T7, respectively. For transient protein expression, the C58C1 carrying the *pMD1-OsbHLH6-Flag* and *pMD1-OsNPR1-T7* were used for infiltration of *N. benthamiana* leaves. The C58C1 carrying the *pMD1-GFP-Flag* and *pMD1-OsNPR1-T7* served as the negative control. For SA treatment, 100 mM SA was sprayed onto the leaves 6 h after infiltration for 1 d. The samples were harvested at 48 hpi. The plant leaves were ground in liquid nitrogen and extracted in extraction/wash buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% TritonX-100, 0.2% Nonidet P-40, 1 mM DTT, and 1× complete protease inhibitor (Roche). The homogenate was centrifuged at 15,000 *g* for 20 min. Anti-FLAG antibody-conjugated agarose beads (Sigma) were added to the supernatant. After incubation at 4°C on an end-over-end shaker for 1.5 h, the beads were spun down at 1,500 *g* for 2 min and washed with wash buffer for at least 6 times. The bound proteins were eluted by 1.5× Laemmli loading buffer and resolved by 12% SDS-PAGE and then subjected to immunoblot analysis using anti-GFP antibody (TransGen Biotech, HT801), anti-FLAG antibody (Sigma, F3165), or anti-T7 antibody (Abcam, ab9115).

Electrophoresis mobility shift assay

The EMSA assays were performed as described by Yang et al. (2017). The fusion protein contained the C-terminal of OsMYC2 (500-751 amino acids, containing binding domain) was purified from *E. coli*. Single-stranded complementary oligonucleotide fragments corresponding to region of *OsbHLH6* promoter harboring the bHLH binding motif were synthesized (Generay). Oligonucleotide pairs were annealed to make double-stranded probes and competitors by mixing together at equal amounts, boiling for 5 min, and cooling down 30 min at room temperature. The DNA probes were labeled with [γ-^{32}P]-ATP by T4 polynucleotide kinase (Thermo Scientific). The DNA probes and proteins were co-incubated in the reaction buffer (10 mM Tris, 50 mM KCl, 1 mM DTT, pH 7.5) for 30 min at room temperature. DNA loading buffer was added to stop the reaction. The protein–probes mixture was separated by 5% polyacrylamide native gel and transferred to a zip bag. The isotope-labeled DNA shift was exposed to a phosphor screen for 2–4 h and scanned using a PhosphorImage system (GE Healthcare, Chicago, IL USA). Non-isotope-labeled (cold) probes were used for the competitive binding experiments.

OsbHLH6 polyclonal antibody preparation

The full length CDS of *OsbHLH6* was cloned into the pGEX-4T-1 vector, which was expressed in *E. coli* DE3 (BL21) and purified using Affinity Resin to produce rabbit polyclonal antibodies (prepared by Abclonal of China). The antibody (1:1,000) was tested by immunoblot analysis using total proteins and nuclear proteins extracted from Dex-inducible promoter-driven lines and Cas9 mutant line, which detected a single band size at 50 kD.

Transactivation assay in *N. benthamiana* leaves

The transactivation expression assays were performed in *N. benthamiana* leaves as previously described (Yang et al. 2017). The 2.0 kb sequences upstream from the ATG codons of *OsbHLH6*, *SAG29*, *PR1*, and *OsWRKY45* were cloned into binary vector pGWB435 to generate promoter::LUC reporter constructs using Gateway® technology (Invitrogen). All the effectors were driven by 35S promoter. The reporter and effector plasmids were transformed into C58C1. Equal amounts of bacterial suspensions were infiltrated into the leaves of 5-week-old *N. benthamiana* plants using a needleless syringe. The inoculated leaves were sprayed with 100 μM luciferin (Promega) at 48 hpi and kept in dark for 5 min. Luminescence images were taken with a low-light cooled charge-coupled device imaging apparatus (iXon; Andor Technology). Experiments were performed with three independent biological replicates.

For the transactivation expression assays in rice protoplasts, the 2.0 kb sequences upstream from the ATG codons of *OsbHLH6* were cloned into pGreenII-LUC vector to generate the LUC/REN reporter. The OsMYC2 effector or GUS control was co-transfected with reporter into protoplasts prepared from 2-week-old rice seedlings. Luciferase activities were measured with a dual-luciferase reporter assay system (Promega).

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AUTHOR CONTRIBUTIONS

J.L., F.M., and C.Y. conceived the original screening and research plants; J.C. and Q.Z. performed the bioinformatics experiments; Z.W. and J.P. provided technical assistance; F.M. and C.Y. performed most of the experiments and data analysis; J.L., F.M., C.Y., H.C, and Z.Q.F. wrote the article. All authors read and approved the manuscript.

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SUPPORTING INFORMATION

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Figure S1. bHLH transcription factors were differentially expressed upon *M. oryzae* infection in rice

(**A**) The differentially expressed transcription factors in response to *M. oryzae* invasion at 24 hpi in rice. The DEGs were acquired by applying $q < 0.05$ and two-fold as the cutoff. (**B**) Differentially expressed *bHLH* family genes in 2-week-old rice plants after infection with *M. oryzae*. Shown is hierarchical clustering of the 21 genes identified as differentially expressed in the pairwise comparison between Mock and 12, 24, and 48 hpi samples.

Figure S2. The *OsbHLH6* expression level in *OsbHLH6* overexpression, silencing, and mutant plants

(**A**–**C**) The *OsbHLH6* expression levels in transgenic plants. Two-week-old rice seedling leaves were sampled for RT-qPCR assays. Values are means ± *SD* (n = 3 biological repeats). Two independent lines were used in each genotype. ** Indicates significant differences from WT by student's *t*-test ($P < 0.01$). (**D**) Verification

of the knockout lines by sequencing. *OsbHLH6*-Cas9-5 and -9 generated by CRISPR/Cas9 system showed single-nucleotide insertion in the *OsbHLH6* target sequence, leading to the early *OsbHLH6* translation termination.

Figure S3. Phylogenetic analysis and sequence alignment of OsbHLH6 and its homologs

(**A**) Sequence alignment of OsbHLH6, OsbHLH7, At4g29930, and At5g57150. Sequence alignments were performed by ClustalW (www.clustal.org). (**B**) Phylogenetic analysis of OsbHLH6 and its homologs from different plant species. The genes are from *Oryza sativa* (LOC), *Zea mays* (Zm), *Brachypodium distachyon* (BRADI), *Triticum urartu* (TRIUR), *Glycine max* (GLYMA), *Solanum lycopersicum* (Solyc), and *Arabidopsis thaliana* (At). The phylogenetic tree was generated using MEGA 7.0 (Kumar et al. 2016).

Figure S4. Sequence analysis of the *OsbHLH6* promoter

The *OsbHLH6* promoter was used to search the OsMYC2 binding motifs. G1, G2, and G3 indicate the three putative OsMYC2 binding sites. The black underlined sequence was used for the binding specificity assays. G3-m is fragment containing the sequence of CCACGCG(G3)G that is mutated to AAAAAAAA in the EMSA assay.

Figure S5. OsbHLH6 overexpression activates JA signaling and biosynthesis in rice

(**A**) OsbHLH6 acts in JA signaling. Two-week-old rice leaves were treated with 100 μM MeJA, and the leaves were sampled 8 hrs later. RT-qPCR was used to examine the OsJAR1 gene expression. ** Indicates significant differences from WT by student's *t*-test ($P < 0.01$). (**B**) and (**C**) The JA and JA-Ile content in *OsbHLH6* overexpression and mutant lines. The two-week-old transgenic plant leaves were used to measure the JA and JA-Ile content. The experiment was repeated twice. Values are means ± *SD* (n = 5 biological repeats). * and ** indicate the significant differences from WT by student's *t*-test at $P < 0.05$ and $P < 0.01$, respectively.

Figure S6. Overexpression of *OsbHLH6* leads to senescence-related gene expression

(**A**–**C**) The senescence-related gene expression in WT and *OsbHLH6*-OE plants. Two-week-old rice seedlings were used to evaluate the gene expression levels. *RCCR1* (Os10g0389200); *Osh36* (Os05g0475400); *SGR* (Os09g0532000). The experiments were repeated three times with similar results. D-3 and D-4 are two independent T_1 *OsbHLH6* overexpression lines. ** indicates the differences from WT by student's *t*-test at $P < 0.01$. Values are means ± *SD* (n = 3 biological replicates).

Figure S7. α-OsbHLH6 antibody specifically recognizes OsbHLH6 in rice

(**A**) Crude OsbHLH6 protein extract was probed by anti-OsbHLH6 antibody. Two-week-old *Dex:OsbHLH6* and Cas9-9 mutant rice leaves were treated with or without 10 μM Dex for 6 h. The plant leaves were sampled and the crude protein extract was subjected to immunoblotting assay. Anti-OsbHLH6 antibody was used to probe OsbHLH6 protein. ACTIN was used to indicate the equal loading of the total proteins. (**B**) The OsbHLH6 was detected in rice nucleus. The rice leaves in (**A**) were used to purify nucleus. The OsbHLH6 protein in the nucleus was probed by the anti-OsbHLH6 antibody after separated by the SDS-PAGE gel. Anti-Histone3 (H3) antibody was used to probe the histone3 to indicate the equal loading for the immunoblotting.

Figure S8. $OsbHLH6^{NES}$ reduces OsbHLH6 in nucleus

(**A**) YFP-$OsbHLH6^{NES}$ showing the cytosolic localization *in N. benthamiana*. DAPI staining showing the nucleus. The confocal microscope was used to observe the fluorescence signals. The experiment was repeated at least three times. Bar = 50 μm. (**B**) BiFC analysis shows $OsbHLH6^{NES}$ and OsbHLH6 form dimers in *N. benthamiana* cytoplasm. Unfused cYFP was used as a negative control. Bar = 50 μm. (**C**) $OsbHLH6^{NES}$ reduces the nuclear localization of OsbHLH6. YFP-OsbHLH6 and cLUC-$OsbHLH6^{NES}$ were transiently co-expressed in *N. benthamiana* leaves. Co-expressed YFP-OsbHLH6 and cLUC-EV were used as the negative control. Confocal microscope was used to observe the YFP-OsbHLH6 localization. Right panel showing the fluorescence intensity of YFP-OsbHLH6 in nucleus crossing the red bars. Bar = 50 μm. (**D**) The YFP-OsbHLH6 proteins in (**C**). The protein expression was examined by immunoblotting assays, showing the similar expression levels. The anti-GFP antibody was used to probe the YFP-OsbHLH6.

Figure S9. SA attenuates the interaction of OsbHLH6 and OsNPR1

(**A**) BiFC analysis shows the interaction of OsbHLH6 and OsNPR1 in *N. benthamiana*. Nucleus of leaf epidermal cells were stained with DAPI. Merged image shows that the fluorescence is not in the nucleus. Unfused cYFP was used as a negative control. Bar = 50 μm. (**B**) SA attenuates the

interaction of OsbHLH6 and OsNPR1. The proteins were transiently expressed in *N. benthamiana* leaves. After 24 h of the transformation, 100 μM SA was sprayed onto the leaves every 6 h. Co-immunoprecipitation assays were used to assess the protein interaction. (**C**) YFP-OsbHLH6 intensity of indicated position in (B). ** indicates significant differences from mock at $P < 0.01$, by student's *t*-test. Values are means ± *SD* (n = 3 biological repeats). (**D**) BiFC analysis shows SA attenuates the interaction of OsbHLH6 and OsNPR1 in rice protoplasts. The plasmids carrying OsbHLH6-nYFP and OsNPR1-cYFP constructs were co-transformed into rice protoplast. 16 h after transformation, rice protoplasts were treated with 200 μM SA for 6 h. Lower panel shows the protein levels in the samples. The experiment was repeated at least three times. Bar = 5 μm. (**E**) BiFC analysis shows SA attenuates the interaction of OsbHLH6 and OsNPR1 in *N. benthamiana*. 36-40 h after transformation, the leaves were treated with 200 μM SA for 6 h. The experiment was repeated at least three times. Bar = 50 μm. Others are same as in (**D**). (**F**) $OsNPR1^{2CA}$ interacts weakly with OsbHLH6 by Split luciferase assays. The OsbHLH6 was fused with cLUC fragments, and OsNPR1 and $OsNPR1^{2CA}$ were fused to the nLUC fragments. cLUC-EV and GUS-nLUC serve as controls. The proteins were co-expressed in *N. benthamiana* leaves.

Figure S10. OsNPR1 sequesters OsbHLH6 in cytoplasm

(**A**) OsNPR1 induces OsbHLH6 export from nucleus to the cytoplasm. YFP-OsbHLH6 and OsNPR1-T7 were transiently expressed in *N. benthamiana* leaves. 200 nM LMB was injected to the leaves 12 h before the observation. Confocal microscope was used to observe the YFP-OsbHLH6 localization. Bar = 50 μm. (**B**) YFP-OsbHLH6 intensity of indicated position in (**A**) by red bar. (**C**) The immunoblotting shows the protein levels of YFP-OsbHLH6, GUS-T7, and OsNPR1-T7 in (**A**). The expressed proteins were probed with respective antibodies to show the equal levels in *N. benthamiana* leaves. ACTIN served as the internal reference. (**D**) OsNPR1 did not affect the subcellular localization of OsEIL1 in rice protoplasts. GUS-FLAG, OsNPR1-FLAG, and OsEIL1-GFP were transiently expressed in rice protoplasts. GUS-FLAG was used as a negative control for OsNPR1-FLAG. Bar = 5 μm. (**E**) The immunoblotting shows the protein levels of GUS-FLAG, OsNPR1-FLAG, and OsEIL1-GFP in (**D**). The expressed proteins were probed with respective antibodies to show the equal levels. ACTIN served as the internal reference.

Figure S11. SA and JA signaling pathways are spatio-temporally regulated during *M. oryzae* infection

(**A**) Expression of JA and SA responsive genes in rice plants. Two-week-old WT seedling leaves were inoculated for transcriptome assays. The *z*-scores of RPKM expression values are shown. **** indicates significant differences from WT at $P < 0.0001$. Each experiment was performed with two biological replicates. (**B**) The expression of the SA responsive genes, *OsNPR1* and *OsWRKY45*, in rice seedlings after *M. oryzae* infection. RT-qPCR was used to evaluate the gene expression. Values are means ± *SD* (n = 3 biological replicates). * and ** indicate the significant differences from WT by student's *t*-test at $P < 0.05$ and $P < 0.01$, respectively.

Table S1. Primers used for this study

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