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A WRKY transcription factor confers broad-spectrum resistance to biotic stresses and yield stability in rice

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Plants are subject to attack by diverse pests and pathogens. Few genes conferring broad-spectrum resistance to both insects and pathogens have been identified. Because of the growth–defense tradeoff, it is often challenging to balance biotic stress resistance and yield for crops. Here, we report that *OsWRKY36* suppresses the resistance to insects and pathogens via transcriptional repression of *Phenylalanine Ammonia Lyases* (*PALs*), a key enzyme in phenylpropanoid pathway in rice. Knocking out *OsWRKY36* causes elevated lignin biosynthesis and increased sclerenchyma thickness of leaf sheath, leading to enhanced resistance to multiple pests and pathogens. Additionally, loss of *OsWRKY36* also derepresses the transcription of *Ideal Plant Architecture 1* (*IPA1*) and *MONOCULM2* (*MOC2*), resulting in increased spikelet number per panicle and tiller number. These findings provide mechanistic insights into biotic stress tolerance in rice and offer a promising strategy to breed rice cultivars with broad-spectrum resistance to insects and pathogens while maintaining stable yield.

rice | broad-spectrum resistance | insect | disease | yield

Significance

Few genes have been identified that confer broad-spectrum resistance against both crop insects and diseases. Furthermore, balancing energy allocation between growth and defense to optimize crop yield remains a major challenge. Here, we found that knocking out *OsWRKY36* derepresses the transcription of *OsPALs*, leading to increased biosynthesis of lignin and thickening of the sclerenchyma in rice leaf sheaths, thereby conferring broad-spectrum resistance. Additionally, knocking out *OsWRKY36* also derepresses the transcription of *IPA1* and *MOC2*, resulting in increased spikelets per panicle and tiller number, contributing to stable yield maintenance. Our findings not only provide insights into balancing broad-spectrum resistance and development but also present a promising strategy for molecular breeding in rice to achieve a balance between resistance and yield.

Rice (*Oryza sativa*) is one of the most important staple crops globally. However, pests and diseases seriously threaten the yield and quality of rice annually (1, 2). Among them, rice planthopper, including brown planthopper (BPH), white-backed planthopper (WBPH), and the small brown planthopper (SBPH), is the most devastating insect pests. They not only inflict direct damage but also cause secondary harm through the transmission of various plant viruses (3–5). Besides insect pests, pathogens also pose substantial threats to rice production. Rice bacterial leaf blight and rice blast are common diseases of rice, which can reduce rice yield by up to 50 to 100% under severe conditions (6). Worldwide, chemical control is the most commonly used strategy to control insect pests and diseases. However, overuse or misuse of pesticides threatens the health of farmers, consumers, and the environment (7). There is an urgent need for the development of sustainable strategies to effectively control pests and pathogens in rice production.

To fend off diverse pathogens and insect pests, plants have evolved sophisticated defense mechanisms. Two phytohormones, jasmonic acid (JA) and salicylic acid (SA), play pivotal roles in plant defense against pathogens and herbivores. Generally, JAs-mediated defenses are typically activated in response to herbivores and necrotrophic pathogens, whereas SA is mainly associated with plant responses to biotrophic pathogens (8). However, JA and SA often act antagonistically in many plant species, leading to conflicting defense responses against insect pests and pathogens (9–11). For instance, overexpression of *OsNPR1*, the key regulatory factor of the SA pathway, enhances the resistance to rice bacterial leaf blight disease and rice blast disease, while reducing the resistance to WBPH (12). Consequently, it remains a significant challenge to breed cultivars with broad-spectrum resistance to both insects and pathogens.

In addition, strong defense responses triggered by pathogens or insect herbivores are often accompanied by reduced growth due to the reallocation of limited resources, a phenomenon known as the growth–defense trade-off (13–15). Previous studies demonstrate that hormones play a crucial role in regulating the conflict between the immunity and growth. Generally, auxins (AUX), gibberellins (GAs), cytokinins (CKs), and brassinosteroids (BRs) are considered plant growth hormones, typically suppressing immune responses to pathogens and herbivores. While the stress hormones SA and JA activate plant defense against pathogens and herbivores on one hand, they also impede plant growth and development on the other hand (9, 15). So far, few genetic factors that balance the growth–defense tradeoff have been identified in rice. Recent studies demonstrated that rice *IDEAL PLANT ARCHITECTURE1*

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(IPA1) promotes both yield and disease resistance by sustaining a balance between growth and immunity. In the absence of a pathogen, nonphosphorylated IPA1 activates the transcription of *DEP1* promoter, promoting plant growth and yield. Upon pathogen attack, phosphorylated IPA1 changes DNA binding specificity, leading to enhanced immunity to pathogen (16). Despite the progress, our understanding of the molecular mechanisms governing the trade-offs between growth and defense is still limited, hindering breeding of rice cultivars of broad-spectrum resistance without compromising crop yield.

In this study, we report that knocking out the transcription factor gene *OsWRKY36* in rice enhances the resistance to multiple rice planthopper species including BPH, WBPH, and SBPH, as well as to the rice blast fungus *Magnaporthe oryzae*. Mechanistically, we found that knocking out *OsWRKY36* leads to the derepression of *OsPAL6* and *OsPAL1* transcription, resulting in increased lignin biosynthesis and sclerenchyma thickness in leaf sheath. Additionally, knocking out *OsWRKY36* also derepresses the transcription of *IPA1* and *MONOCULM2 (MOC2)*, leading to increased number of spikelets per panicle and tiller. Our findings add insights into the balance between broad-spectrum resistance and yield, and genetic resources for molecular breeding of rice cultivars with balanced resistance and yield potentials.

Results

***OsWRKY36* Negatively Regulates the Resistance Against BPH.** To explore the mechanism underlying BPH resistance, we screened a collection of activation-tagged T-DNA insertion rice mutant library consisting of 446 mutant lines carrying insertions in genes encoding transcription factors, and identified a mutant with increased susceptibility to BPH based on seedling mortality measurements. This mutant was named *BPH susceptible 1-Dominant* (*Bphs1-D*) (Fig. 1 *A* and *B*). According to the flanking sequence tag from the T-DNA Insertion Mutant Database (http://orygenesdb.cirad.fr/tools.html), the insertion location of T-DNA was identified in the 5′-UTR of the WRKY transcription factor gene *OsWRKY36*, and the insertion was verified by PCR. qRT-PCR showed that the T-DNA inserted enhanced the expression of *OsWRKY36* in *Bphs1-D* (Fig. 1*C* and *SI Appendix*, Fig. S1). To confirm the role of *OsWRKY36* in BPH resistance, we generated two independent *OsWRKY36*-overexpressing transgenic lines (*OsWRKY36*-OE) and two independent homozygous knockout lines (*OsWRKY36*-KO) of *OsWRKY36* in the background of Ningjing1 (an elite *japonica* cultivar) using the CRISPR/Cas9 method (Fig. 1). Compared with wild type (WT), both overexpressing lines of *OsWRKY36* exhibited increased susceptibility to BPH (Fig. 1 *D–F* and *SI Appendix*, Fig. S2*A*); while both knockout lines

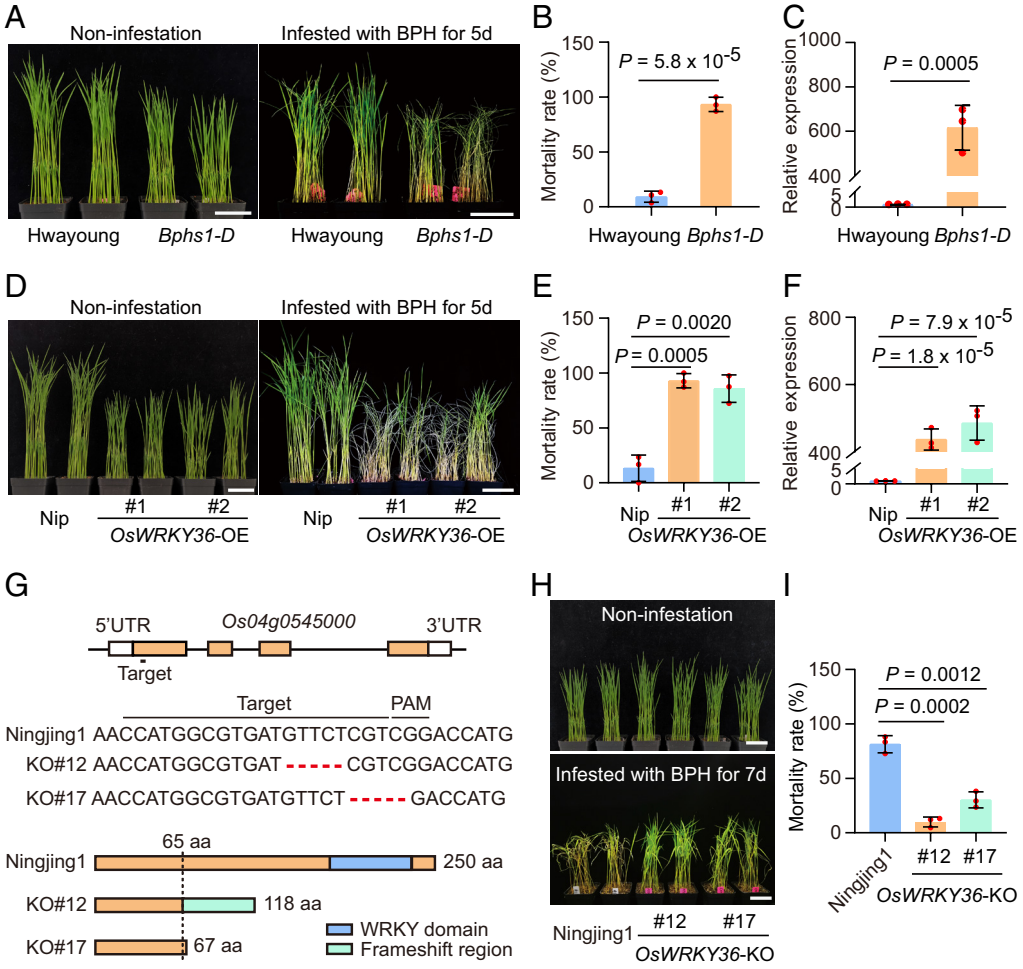

Fig. 1. *OsWRKY36* negatively regulates the resistance to BPH in rice. (*A*) Image of Hwayoung (WT) and *Bphs1-D* plants before and at 5 d postinfestation (dpi) with BPH. (Scale bar, 5 cm.) (*B*) Seedling mortality rates of Hwayoung (WT) and *Bphs1-D* plants at 5-dpi with BPH. (*C*) Relative transcript levels of *OsWRKY36* in Hwayoung (WT) and *Bphs1-D*, with the number in Hwayoung (WT) set as 1. *Ubiquitin* (*Os03g0234350*) was used as the internal reference. (*D*) Image of Nipponbare (WT) and *OsWRKY36*-overexpressing lines (*OsWRKY36*-OE) before and at 5-dpi with BPH. (Scale bar, 5 cm.) (*E*), Seedling mortality rates of Nipponbare (WT) and *OsWRKY36*-OE at 5-dpi with BPH. (*F*) Relative transcript levels of *OsWRKY36* in Nipponbare (WT) and *OsWRKY36*-OE, with the number in Nipponbare (WT) set as 1. (*G*) The sequence analysis of *OsWRKY36* between Ningjing1 (WT) and two distinct *OsWRKY36* knockout lines. (*H*) Image of Ningjing1 (WT) and the *OsWRKY36* knockout lines (*OsWRKY36*-KO) before and at 7 dpi with BPH. (Scale bar, 5 cm.) (*I*) Seedling mortality rates of Ningjing1 (WT) and the *OsWRKY36*-KO lines at 7-dpi with BPH. Values are means ± SD of at least three biological replicates. *P* values were derived by Student's *t* test.

displayed enhanced resistance to BPH at both the seedling stage and the mature stage (Fig. 1 *G–I* and *SI Appendix*, Fig. S2*B*). Notably, *Bphs1-D* mutant and *OsWRKY36*-OE lines exhibited obvious weaker growth than the WT. (Fig. 1 *A* and *D*). To exclude the possible effect of plant size on BPH resistance, *Bphs1*-D mutants and *OsWRKY36*-OE lines were sown earlier than the WT to ensure similar plant sizes prior to BPH infestation. Similarly, the results showed that the seedling mortality rates of *Bphs1*-D and *OsWRKY36*-OE were still significantly higher than their background parents (*SI Appendix*, Fig. S3). Together, these results demonstrate that *OsWRKY36* negatively regulates BPH resistance in rice.

qRT-PCR analysis showed that *OsWRKY36* is constitutively expressed in root, stem, leaf blade, leaf sheath, and panicle (*SI Appendix*, Fig. S4*A*). The expression of *OsWRKY36* was initially downregulated upon BPH infestation and then gradually recovered (*SI Appendix*, Fig. S4*B*). RNA in situ hybridization analysis showed that *OsWRKY36* was highly expressed in vascular sheath and sclerenchyma cells (*SI Appendix*, Fig. S4*C*). The OsWRKY36-GFP fusion protein was colocalized with the nuclear marker D53-mCherry, consistent with the predicted nuclear localization of OsWRKY36 (*SI Appendix*, Fig. S4*D*). A transactivation assay using yeast cells showed that the full-length OsWRKY36 protein exhibited low transactivation activity, while truncated OsWRKY36 protein lacking the C-terminal WRKY domain (ΔWRKY) strongly activated transcription (*SI Appendix*, Fig. S5*A*). Consistently, dual-luciferase assay also suggests that OsWRKY36 acts as a transcriptional repressor (*SI Appendix*, Fig. S5*B*).

***OsWRKY36* Participates in the Phenylpropanoid Pathway and Negatively Regulates Lignin Accumulation in Leaf Sheath.** To gain additional insights into the action mechanisms of *OsWRKY36* in regulating BPH resistance, we performed a high-throughput transcriptome sequencing assay using *OsWRKY36*-KO and WT seedlings infested with BPH for 12 h. Compared with the WT, a total of 3, 809 genes were differentially up-regulated in *OsWRKY36*-KO (*SI Appendix*, Fig. S6*A*). The top two enriched KEGG pathways for the differentially expressed genes belong to carbon metabolism and phenylpropanoid biosynthesis (*SI Appendix*, Fig. S6*B*). Previous studies reported that lignin, derived from the phenylpropanoid pathway, is the major component of the secondary cell wall in the sclerenchyma and it plays a crucial role in plant defense against pathogens and insect pests (17–19). Phenylalanine ammonia lyase (PAL) is a key enzyme in the phenylpropanoid pathway, which is essential to lignin biosynthesis (20, 21). We found that forty-eight genes involved in the phenylpropanoid and lignin biosynthesis, including *OsPAL1* and *OsPAL6*, were significantly up-regulated in the *OsWRKY36*-KO plants (*SI Appendix*, Fig. S6 *C* and *D*). The differential upregulation of these genes in the *OsWRKY36*-KO plants was also confirmed via qRT-PCR analysis (*SI Appendix*, Fig. S7 *A–C*). Moreover, the transcription of these genes was consistently down-regulated in *Bphs1-D* and *OsWRKY36*-OE (*SI Appendix*, Fig. S7 *D* and *E*). These results suggest that *OsWRKY36* participates in the phenylpropanoid pathway and lignin biosynthesis.

To verify whether *OsWRKY36* influences the biosynthesis of lignin, we measured the lignin content in fresh leaf sheath by histochemical stain with phloroglucinol (Fig. 2). We observed that the staining intensity of lignin in the vascular bundle of leaf sheath was significantly reduced in both the *Bphs1-D* and *OsWRKY36*-OE plants compared with WT, but was enhanced in the *OsWRKY36*-KO plants (Fig. 2 *B–D*). Moreover, the number of cell layers and the thickness of the sclerenchyma in the leaf sheath were significantly decreased in both the *Bphs1-D* and *OsWRKY36*-OE plants and even completely absent at the preferred probing sites by the insects (Fig. 2 *E* and *F*). By contrast, the number of cell layers and the thickness of the sclerenchyma were significantly increased in the *OsWRKY36*-KO plants (Fig. 2*G*). Consistent with the results of histochemical staining, lignin content was significantly reduced in the *Bphs1-D* and *OsWRKY36*-OE plants compared with WT, but increased in *OsWRKY36*-KO (*SI Appendix*, Fig. S7*F*). These results indicate that *OsWRKY36* plays a negative role in the accumulation of lignin and the thickness of sclerenchyma in the leaf sheath.

OsWRKY36 Represses the Transcription of *OsPAL6* and *OsPAL1*. In light of the positive role of *OsPAL6* in BPH resistance (21) and the up-regulated expression of *OsPAL6* and *OsPAL1* in the *OsWRKY36*-KO plants (*SI Appendix*, Figs. S6 and S7), we hypothesized that OsWRKY36 may regulate the transcription of *OsPAL6* and *OsPAL1*. This speculation was supported by the findings that OsWRKY36 directly bound to the promoter of *OsPAL6* and *OsPAL1* in a yeast one-hybrid assay (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S8 *A* and *B*) and that OsWRKY36 suppresses the expression of *OsPAL6* and *OsPAL1* in luciferase assays (Fig. 3 *C* and *D* and *SI Appendix*, Fig. S8 *C* and *D*).

The WRKY family transcription factors usually recognize the W-box [TTGAC(C/T)] elements in promoters (22). Three annotated W-box motifs were found in the promoter of *OsPAL6* (Fig. 3*A*). To test whether OsWRKY36 binds to these W-box motifs, we performed a yeast one-hybrid assay using three fragments (a, b, c) of the *OsPAL6* promoter. As shown in Fig. 3*B*, OsWRKY36 only bound to fragments b and c (which harbor the W-box motifs), but not a (which lacks the W-box motif). The binding of OsWRKY36 to the W-box motifs in the promoter of *OsPAL6* was further confirmed using an electrophoretic mobility shift assay (EMSA) (Fig. 3*E*) and ChIP-qPCR experiments (Fig. 3*F*). Similarly, we demonstrated that OsWRKY36 also bound to the W-box in the promoter of *OsPAL1* (*SI Appendix*, Fig. S8 *A-F*). These results suggest that OsWRKY36 negatively regulates the expression of *OsPAL6* and *OsPAL1* by directly binding to the W-box motifs in their promoters. Consistent with the role of OsWRKY36 in repressing the transcription of *OsPAL6* and *OsPAL1*, the transcript levels of *OsPAL6* and *OsPAL1* were reduced in the *OsWRKY36*-OE plants (*SI Appendix*, Fig. S7 *D* and *E*), but increased in the *OsWRKY36* knockout lines (*SI Appendix*, Fig. S7 *A–C*).

To test the role of *OsPAL1* and *OsPAL6* in the resistance against BPH, we evaluated BPH resistance level in two *OsPAL1*-overexpressing lines (23) and found that both lines displayed higher resistance compared with the controls (*SI Appendix*, Fig. S8 *G-I*). Similarly, overexpressing *OsPAL6* also significantly enhances the rice resistance against BPH (Fig. 3 *G–I*). Histological examination showed that overexpressing *OsPAL6* (*OsPAL6/GFP*-OE) significantly enhanced the lignin accumulation, and slightly increased the sclerenchyma thickness (*SI Appendix*, Fig. S9). To further verify whether the decreased expression of *OsPAL6* contributed to the susceptibility of *Bphs1-D* to BPH, we overexpressed *OsPAL6* in the *Bphs1-D* mutant background and found that the resistance to BPH was restored (Fig. 3 *J-L*). Histological examination staining showed that the intensity of lignin in the vascular bundle of leaf sheath and lignin accumulation was clearly enhanced in the *OsPAL6*-OE/*Bphs1-D* plants, compared with the *Bphs1-D* plants (Fig. 2*B* and *SI Appendix*, Fig. S7*F*). Moreover, the number of cell layers and the thickness of the sclerenchyma were also significantly increased in the *OsPAL6*-OE/*Bphs1-D* plants (Fig. 2*E*). Collectively, these results support that OsWRKY36 negatively regulates the resistance to BPH by repressing the transcription of *OsPAL6* and *OsPAL1*.

Knocking out *OsWRKY36* Confers Broad-Spectrum Resistance Against Multiple Insects and Pathogen. Given the function of *OsWRKY36* in the formation of sclerenchyma and the accumulation of lignin, we next assessed whether *OsWRKY36*

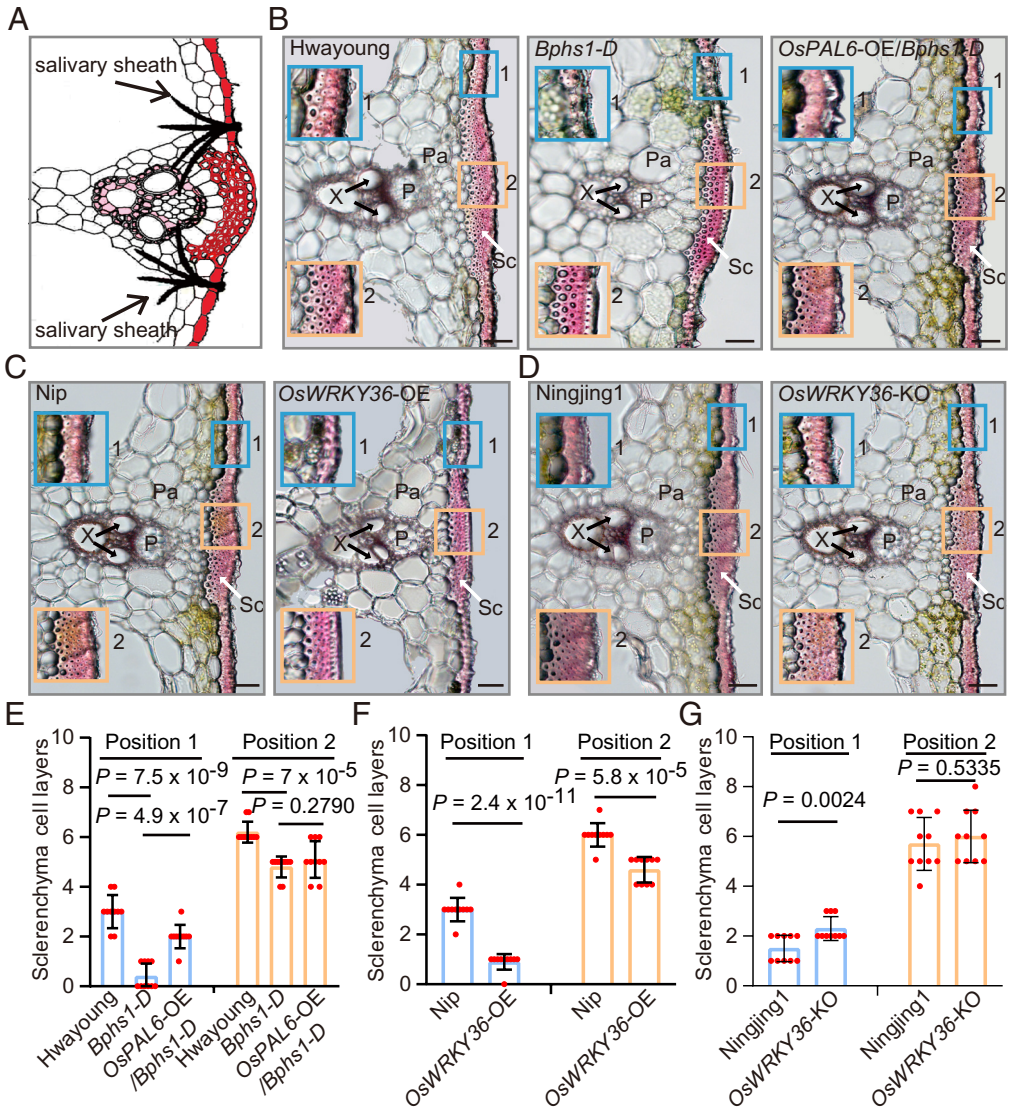

Fig. 2. *OsWRKY36* negatively regulates the thickness of sclerenchyma and the accumulation of lignin. (*A*) Structural model of the salivary sheaths of BPH inside a rice leaf sheath. (*B–D*) Histochemical staining showing the sclerenchyma and the accumulation of lignin in fresh leaf sheaths in *Bphs1-D*, *OsWRKY36*-OE, *OsWRKY36*-OE/*Bphs1-D*, *OsWRKY36*-KO, and the corresponding genetic background (WTs). Sc, sclerenchyma cells; Pa, parenchyma cells; P, phloem; X, xylem. (Scale bar, 50 μm.) (*E–G*) the sclerenchyma layers in fresh leaf sheaths in *Bphs1-D*, *OsWRKY36*-OE/*Bphs1-D*, *OsWRKY36*-OE, *OsWRKY36*-KO, and the corresponding WTs at positions 1 and 2 as shown in *B*, *C*, and *D*, respectively. Values are means ± SD of 10 biological replicates and *P* values were derived by Student's *t* test.

confers broad-spectrum insect resistance. Besides BPH, WBPH and SBPH are also major pests in rice production. While BPH and WBPH feed specifically on rice plants, SBPH has a broader host range, including some of the major staple crops such as wheat, maize, and barley (24–26). We evaluated the resistance level of *Bphs1-D*, *OsWRKY36*-OE, *OsWRKY36*-KO, *OsPAL6/GFP*-OE, and *OsPAL6*-OE/*Bphs1-D* to WBPH and SBPH. Strikingly, the *Bphs1-D* and *OsWRKY36*-OE plants exhibited increased susceptibility to both WBPH (*SI Appendix*, Fig. S10 *A*, *B*, *G*, and *H*) and SBPH (*SI Appendix*, Fig. S11 *A–D*), while the *OsWRKY36*-KO and *OsPAL6*-OE plants displayed higher resistance to these insects compared with the WT (*SI Appendix*, Figs. S10 *C–F* and S11 *E–H*). In addition, the *OsPAL6*-OE/*Bphs1-D* plants also showed higher resistance to both WBPH (*SI Appendix*, Fig. S10 *G* and *H*) and SBPH (*SI Appendix*, Fig. S11 *I* and *J*) than *Bphs1-D*. These results demonstrate that OsWRKY36 also negatively regulates the resistance to WBPH and SBPH, and thus plays a key role in modulating resistance to a broad range of insect pests.

OsPALs are known to contribute to broad-spectrum resistance to various pathogens, including the rice bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and the rice blast fungus *Magnaporthe oryzae* (23). We thus tested the resistance of *OsWRKY36* and *OsPAL6* to avirulent and virulent *Xoo* isolates. Similar to their genetic background parents, *OsWRKY36*-OE and *OsPAL6/GFP*-OE exhibited high resistance to the two avirulent *Xoo* isolates, PXO79 and GZ10 (*SI Appendix*, Fig. S12 *A* and *B*). However, infestation experiments with the virulent *Xoo* strain PXO99 showed that both the *OsWRKY36*-OE and *Bphs1-D* plants were more susceptible to the pathogen compared with their genetic backgrounds (*SI Appendix*, Fig. S12 *C*, *D*, *G*, and *H*). In contrast, overexpressing *OsPAL6* (*OsPAL6/GFP*-OE) significantly increased the resistance to PXO99 (*SI Appendix*, Fig. S12 *E* and *F*). Moreover, overexpression of *OsPAL6* restored the resistance of *Bphs1-D* to PXO99 (*SI Appendix*, Fig. S12 *G* and *H*). These observations hint that *OsWRKY36* negatively regulates the resistance to *Xoo*. However, as *Xoo* PXO99 is not virulent to Ningjing1, the background parent of knocking out lines (*SI Appendix*, Fig. S12*I*), we tested additional *Xoo* strains including PXO79, CR7, JS49-6, and Zhe173 on Ningjing1. The results showed that Ningjing1 was highly resistant to all the tested strains (*SI Appendix*, Fig. S12*J*).

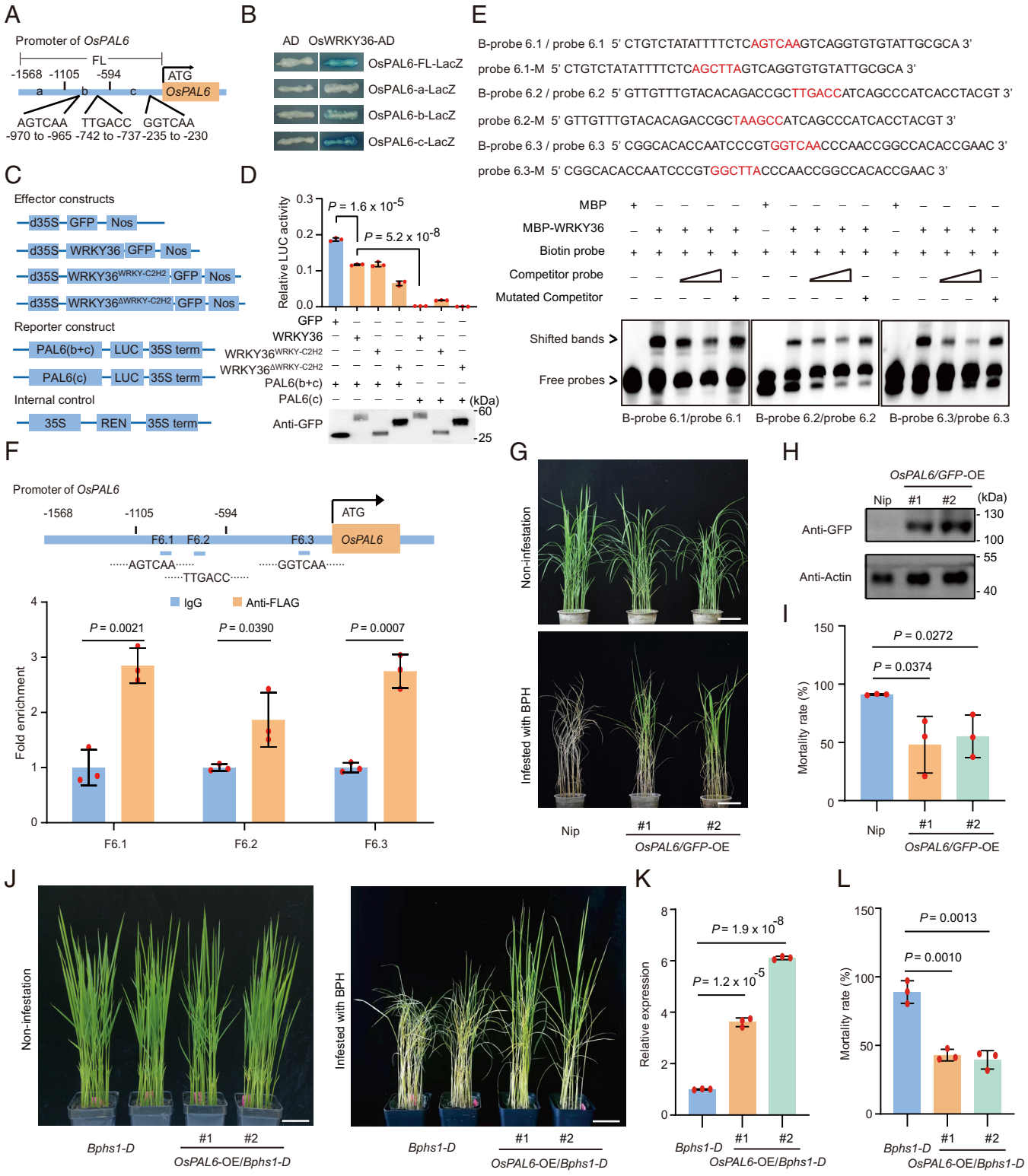

Fig. 3. OsWRKY36 reduces the resistance to BPH in rice by repressing the expression of *OsPAL6*. (*A*) Diagram of the *OsPAL6* promoter indicating the boundaries of the three sections and the locations of the predicted W-box motifs. (*B*) Yeast one-hybrid assay of the interaction between OsWRKY36 and the full-length or the three separate sections of the *OsPAL6* promoter as indicated in *A*. (*C*) Diagram of the vectors used in the transient expression assay. (*D*) Luciferase activity measured by coexpressing *pOsPAL6::LUC* and *p35S::OsWRKY36* or a control vector in rice protoplasts. (*E*) Electrophoretic mobility shift assay (EMSA) of the interaction between OsWRKY36 and the *OsPAL6* promoter. Sequences of the labeled probe (B-probe) and specific competitor (probe) or mutated competitor (probe-M) used for EMSA are indicated. The W-box motifs and mutated motifs are marked in red. All EMSA probes were double-stranded DNAs. For simplicity, the sequence of only one strand (5′ to 3′) is displayed. EMSA gels are representative of the experiment repeated at least three times. (*F*) Chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) analysis of the DNA binding activity of OsWRKY36 to *OsPAL6* promoter. (*G*) Images of WT and *OsPAL6/GFP*-OE plants without or with BPH for 7 d. (Scale bar, 5 cm.) (*H*) Express analysis of OsPAL6/GFP protein by Western blotting in WT and *OsPAL6/GFP*-OE. (*I*) Seedling mortality rates of WT and *OsPAL6/GFP*-OE plants infested with BPH. (*J*) Images of *Bphs1-D* and *OsPAL6*-OE/*Bphs1-D* (Overexpressing *OsPAL6* in *Bphs1-D*) plants without or with BPH for 5 d. (Scale bar, 5 cm.) (*K*) qRT-PCR analysis of *OsPAL6* in *Bphs1-D* and *OsPAL6*-OE/*Bphs1-D* plants, with the number in *Bphs1-D* set as 1. (*L*) Seedling mortality rates of *Bphs1-D* and *OsPAL6*-OE/*Bphs1-D* plants infested with BPH at 5 dpi. Values are means ± SD of three biological replicates. *P* values were derived by Student's *t* test.

Thus, the effect of knocking out *OsWRKY36* on the resistance to *Xoo* awaits confirmation in additional genetic backgrounds.

We next evaluated the resistance of *OsWRKY36*-KO and WT to the rice blast fungus *M. oryzae*. Compared with WT, the *OsWRKY36*-KO plants showed enhanced resistance when infected by mixed spores of *M. oryzae* races 522, 924, 863, 587, and 16-3, all of which are highly virulent races commonly found in rice-growing areas in the Jiangsu province. We also inoculated WT and *OsWRKY36*-KO plants with the above-mentioned five races of *M. oryzae* individually. Among the five races tested, races 522, 924, and 863 were virulent on Ningjing1. The *OsWRKY36*-KO plants were more resistant to race 522, and exhibited slightly enhanced resistance to race 924 and 863 compared with WT (*SI Appendix*, Fig. S13 *A* and *B*). Consistently, the *OsWRKY36*-OE plants exhibited increased susceptibility to virulent strains compared to the WT (*SI Appendix*, Fig. S13 *C* and *D*). In contrast, *OsPAL6* overexpression conferred enhanced resistance (*SI Appendix*, Fig. S13 *E* and *F*). Furthermore, overexpressing *OsPAL6* in the *Bphs1-D* mutant background successfully restored the resistance of *Bphs1-D* to virulent strains of *M. oryzae* (*SI Appendix*, Fig. S13 *G* and *H*). Together, these results demonstrate that knocking out *OsWRKY36* in rice also confers enhanced resistance to pathogens in rice.

Knocking out *OsWRKY36* Elevates the Number of Spikelets per Panicle and Tillers. To determine whether the broad-spectrum resistance conferred by the loss of *OsWRKY36* is accompanied by any altered agronomic traits, we investigated the effect of *OsWRKY36* on rice growth and yield under field conditions. To thoroughly examine the agronomic traits, we measured a wide range of parameters including plant height, panicle length, tiller number, number of spikelets per panicle, seed-setting rate, 1000-grain weight, and grain yield per plant. Compared with WT, both the *Bphs1-D* and *OsWRKY36*-OE plants exhibited a reduction in most of the parameters examined (*SI Appendix*, Fig. S14 *A*–*C*). Notably, the grain yield per plant of *Bphs1-D* and *OsWRKY36*-OE were sharply reduced by 42.2 to 56% (*SI Appendix*, Fig. S14*D*), while the grain yield per plot of *Bphs1-D* and the *OsWRKY36*-OE lines were reduced by over 60% (*SI Appendix*, Fig. S14 *E*–*G*). The above results demonstrate that overexpressing *OsWRKY36* in rice not only impairs host resistance, but also reduces yield.

In contrast, the tiller number, spikelet number per panicle, and grain yield per plant of the *OsWRKY36*-KO lines were significantly increased compared with WT under field conditions in three consecutive years (2019, 2020, and 2021) (Fig. 4 *A*–*C*). To further test the influence of knocking out *OsWRKY36* on the yield of rice, we measured the yield per plot according to the standard procedures for variety registration test in China. In 2021, when yield per plot was measured in Nanjing, the grain yield of *OsWRKY36*-KO per plot was significantly increased, which is consistent with the increased grain yield per plant (Fig. 4*D*). In 2023, we remeasured the grain yield at both Nanjing and Suzhou. Our results showed that the two knockout lines had similar grain yield compared with WT in Suzhou, but the grain yield of the two knockout lines was decreased in Nanjing (*SI Appendix*, Fig. S15). Further, we found that the tiller number and spikelet number per panicle were still increased in 2023, but the seed setting rate and 1,000-grain weight were significantly decreased, which resulted in the yield reduction in the knockout lines (*SI Appendix*, Fig. S15). We speculated that the reduced seed setting rate and 1000-grain weight in both Suzhou and Nanjing in 2023 is likely caused by continuous rainy weather and low temperature (the minimum temperature is just 18 °C) during the rice grain filling period in Jiangsu Province (https://lishi.tianqi.com/nanjing/202309.html). The more severe reduction in the seed setting rate and 1,000-grain weight in the *OsWRKY36*-KO plants (compared with the WT plants) suggests that *OsWRKY36*-KO is likely prone to be influenced by low temperature at the grain filling stage. Overall, the above results suggest that knocking out *OsWRKY36* in rice confers broad-spectrum resistance to insect pests and pathogen while maintaining crop yield by increasing spikelet number per panicle and tiller number.

***OsWRKY36* Suppresses the Transcript Level of *IPA1* and *MOC2* to Regulate the Number of Spikelets per Panicle and Tillers.** To explore the molecular mechanism underlying the increase of spikelet number per panicle and tiller number in the *OsWRKY36*-KO plants, we examined the high-throughput transcriptomic data between WT and *OsWRKY36*-KO, and found that *IPA1* and *MONOCULM 2* (*MOC2*) were significantly up-regulated in the *OsWRKY36*-KO seedlings (Fig. 4*E* and *SI Appendix*, Fig. S6*D*). Previous studies have reported that *IPA1* and *MOC2* plays a positive role in determining spikelet number and tiller number, respectively (27–29). Therefore, we analyzed the promoters of *IPA1* and *MOC2* and identified 6 and 15 W-box motifs upstream of their transcription start sites (TSS), respectively (Fig. 4*F* and *SI Appendix*, Fig. S16*C*). A yeast one-hybrid confirmed the binding of OsWRKY36 to the promoters of *IPA1* and *MOC2* (Fig. 4*F* and *SI Appendix*, Fig. S16*C*). Furthermore, EMSA, ChIP-qPCR, and bimolecular luciferase assays demonstrated that OsWRKY36 suppresses the transcription of *IPA1* by binding to the W-box motifs in *IPA1* promoter (Fig. 4 *G*–*I*). Consistently, qRT-PCR analysis showed that the expression level of *IPA1* and *MOC2* was significantly increased in the *OsWRKY36*-KO plants, but reduced in the *OsWRKY36*-OE plants, compared with WT (Fig. 4*E* and *SI Appendix*, Fig. S16 *A* and *B*). As a result, we concluded that OsWRKY36 influences tiller formation and number of grains per panicle by negatively regulating the expression of *IPA1* and *MOC2*.

Discussion

Breeding of rice cultivars of broad resistance to insect and pathogens, while maintaining stable yield is a priority goal for breeders. This objective is currently hindered by the lack of genes conferring broad-spectrum resistance against both insects and diseases and genes that can balance the trade-offs of growth and defense in response various biotic and abiotic stresses. In this study, we report that *OsWRKY36* defines a knob gene that not only confers broad-spectrum resistance to both insects and pathogen, but also balances the trade-off between resistance and yield in rice. We found that overexpressing *OsWRKY36* not only negatively regulates rice resistance against multiple insects and diseases, but also impairs rice yield. On the contrary, knocking out *OsWRKY36* not only displays broad-spectrum resistance against insects and pathogens, but also increases the number of spikelets per panicle and tillers. Mechanistically, our findings reveal that *OsWRKY36* acts as a transcriptional repressor, suppressing the transcription of *OsPALs*. Knocking out *OsWRKY36* results in increased lignin accumulation and sclerenchyma formation in the leaf sheath, resulting in broad-spectrum resistance against both herbivores and pathogens. Additionally, we found that *OsWRKY36* also suppresses the expression of *IPA1* and *MOC2*. Knocking out *OsWRKY36* results in increased tiller number and grains per panicle, thus contributing to maintaining stable yield (Fig. 4*J*). Thus, *OsWRKY36* represents a valuable genetic target for simultaneously improving grain yield and broad biotic resistance in rice.

Previous studies have documented ample evidence that lignin and other polyphenolic compounds derived from the phenylpropanoid pathway play crucial roles in plant defense responses to various pathogens and insect pests (19). For example, overexpressing *OsPAL8* in rice leads to increased lignin content and enhanced resistance to

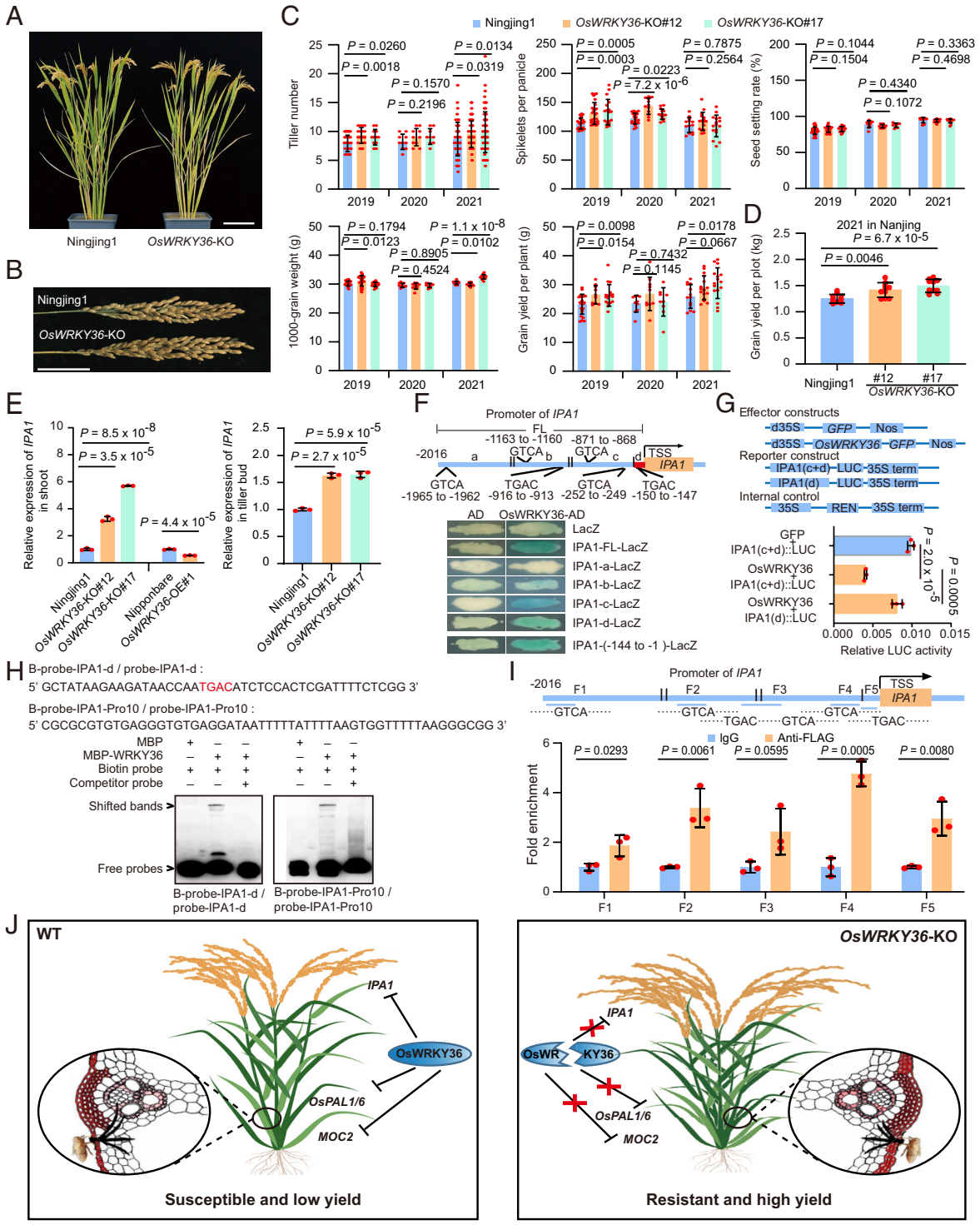

Fig. 4. OsWRKY36 represses the expression of *IPA1* and regulates the yield characteristics of rice. (*A* and *B*) Images of mature stage plants and panicles of *OsWRKY36*-KO and Ningjing1 (WT). (Scale bar, 10 cm in *A*, 5 cm in *B*.) (*C*) Tiller number, spikelets per panicle, seed setting rate, 1,000-grain weight, and grain yield per plant in WT, *OsWRKY36*-KO#12, and *OsWRKY36*-KO#17 measured in 2019, 2020, and 2021, respectively. Each point represents a single biological replicate and experimental groups in *C* consist of more than 10 biological replicates. *P* values were derived by Student's *t* test in comparison with WT. (*D*) Grain yield per plot for WT and *OsWRKY36*-KO (KO#12, KO#17). Data are presented as means ± SD (n = 3 plots, 50 plants within a plot) and analyzed by Student's *t* test. (*E*) Relative transcript levels of *IPA1* in the shoot and tiller buds of *OsWRKY36*-KO, *OsWRKY36*-OE, and background parent Ningjing1. *Ubiquitin* (*Os03g0234350*) was used as the internal reference. (*F*) *Top*: Diagram of the *IPA1* promoter. *Bottom*: Yeast one-hybrid assay of OsWRKY36 and the W-box motifs in the promoter region of *IPA1*. (*G*) Diagram of the vectors used in the transient expression assay. Luciferase activity was measured in rice protoplasts coexpressing *pIPA1::LUC* and *p35S::OsWRKY36* or a control vector. Error bars, mean ± SD of three biological replicates. *P* values were derived by Student's *t* test. (*H*) The gel electrophoresis mobility shift assay (EMSA) is used to detect the binding of WRKY36 to W-box motif in the *IPA1* promoter. Sequences of the labeled probe (B-probe) and specific competitor (probe) used for EMSA are shown. The W-box motif is marked in red. (*I*) Chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) analysis of the DNA binding activity of OsWRKY36 to *IPA1* promoter. (*J*) A model of OsWRKY36 simultaneously regulating rice resistance and yield. In the WT, OsWRKY36 represses the transcription of *OsPAL6* and *OsPAL1* to reduce the lignin accumulation and sclerenchyma thickness, consequently diminishing resistance to insect pests and pathogens. Additionally, suppressed expression of *IPA1* and *MOC2* negatively impacts spikelets per panicle and tiller number. Conversely, in the OsWRKY36 knockout mutant, elevated biosynthesis of lignin leads to thickened sclerenchyma, thereby enhancing resistance to insect pests and pathogens. Moreover, the up-regulated expression of *IPA1* and *MOC2* results in increased spikelets per panicle and tiller number.

BPH (30). *OsPAL1* and *OsPAL4* have been reported to confer broad-spectrum resistance to rice bacterial leaf blight and rice blast, likely by reinforcing the cell wall through the deposition of lignin (23, 31). In this study, we find that knocking out *OsWRKY36* confers broad-spectrum resistance against both herbivores and pathogens, resulting from increased lignin accumulation and sclerenchyma formation in the leaf sheath. Together, these findings reinforce the notion that manipulating the expression of specific *PAL* family member genes might represent a feasible approach to breed rice cultivars with broad-spectrum resistance to pathogens and insect pests. Additionally, the finding that *OsWRKY36* is a direct upstream regulator of the phenylpropanoid pathway and lignin biosynthesis adds insights into lignin biosynthesis in rice and probably in other crops. Earlier studies reported several classes of transcriptional regulators of *PALs*, including OsMYB30, BSR-K1, and PIBP1 (23, 30, 32). Elucidating their regulatory relationship with *OsWRKY36* will deepen our understanding of the regulatory mechanisms of lignin biosynthesis in rice.

Trade-offs between plant growth and immunity have long been a limiting factor for breeding rice cultivars with balanced resistance and yield (13). Here, we found that OsWRKY36 negatively coregulates the expression of *OsPAL*, *IPA1,* and *MOC2* by directly binding to the W-box motifs within their promoters. Knocking out OsWRKY36 results in increased transcript levels of *OsPAL*, *IPA1,* and *MOC2*, leading to enhanced resistance against pathogens and insects, and increased number of spikelets per panicle and tillers. Our finding expands the understanding of the coregulatory networks of growth and defense, and provides valuable gene targets for boosting resistance while maintaining yield stability.

Knocking out *OsWRKY36* confers broad-spectrum resistance against both herbivores and pathogens by regulating lignin biosynthesis. Previous studies have also shown that lignin plays a crucial role in the formation of the cell wall and vascular bundles, providing structural support and facilitating long-distance water and nutrient transport in plants. Therefore, in addition to its role in plant defense, lignin is also essential for plant growth (33, 34). These findings suggest that fine-tuning lignin content could be an effective strategy for simultaneously regulating both plant growth and defense.

Overall, the findings of the present study not only broaden our understanding of the mechanisms involved in plant resistance to biotic stress but also propose a strategy for simultaneously enhancing crop broad-spectrum resistance to multiple biotic stresses without compromising yield.

Methods

Plant Materials and Growth Conditions. *Bphs1*-D was an activation-tagged T-DNA insertion rice mutant in the background of *japonica* rice cultivar Hwayoung, and screened from a T-DNA insertion mutant library kindly provided by Professor Gynheung An (Department of Plant Systems Biotech, Kyung Hee University, Korea). *OsWRKY36* overexpressing and knock-out transgenic plants are in the background of *japonica* rice cultivars Nipponbare or Ningjing1, respectively. *OsPAL6*-overexpressing transgenic plants were achieved in the background of the *Bphs1-D*. *OsPAL1* overexpressing transgenic plants in *japonica* rice cultivar TP309 were kindly provided by Professor Xuewei Chen (State Key Laboratory of Crop Gene Exploration and Utilization in Southwest China, Sichuan Agricultural University, China). Rice plants were grown in the paddy fields at the Nanjing Agricultural University under natural conditions with conventional management. Field experiments were carried out as a randomized block design with 300 kg/ha N fertilizer, and P and K fertilizers were applied at 100 kg/ha each. The transplanting distances are 20 cm between rows and 17 cm between individuals within the same row. Rice seeds were sown on around 10 May and transplanted. The grain yield per plant were measured on a single-plant per hole, then the grain yield per plot were measured on 2 or 3-plants per hole according to the regional variety trial. All field experiments were carried out without planthopper infestation.

BPH Maintenance. A colony of the BPH was collected from rice fields in Nanjing, and maintained on the susceptible cultivar Taichung Native1 (TN1) under greenhouse conditions at the Nanjing Agricultural University.

BPH Resistance Evaluation. BPH resistance of rice seedling was scored according to the standard evaluation systems of IRRI (35). A seedling bulk test was conducted to phenotype plant reaction to BPH feeding. To ensure all seedlings were at the same growth stage for BPH infestation, seeds were pregerminated in petri dishes in a 30 °C incubator for 2 to 3 d. About 30 seeds from a single plant were sown in a 10 cm–diameter plastic pot with a hole at the bottom. Seven days after sowing, seedlings were thinned to 25 plants per pot. At the two–leaf stage, the seedlings were infested with 2nd to 3rd instar BPH nymphs at 10 insects per seedling. For the knock-out plants, the seedling mortality was recorded when nearly all the Ningjing1 plants were dead. For *Bphs1-D* and *OsPAL6* overexpressing lines, the seedling mortality rates were recorded when over 80% of WT were dead. Three replicates were used for each cultivar or line.

To evaluate the BPH resistance of rice varieties at the mature stage, two seedlings of each variety were planted in a pot and infested with approximately 20 adult female BPHs per plant during the peak tillering stage. After infestation, the tested variety and the background parental plants were transferred into a cage sealed with nylon. Data were collected when the susceptible individuals died within the same group. Three replicates at least were used for each variety or line.

Blast Resistance Test. Growth conditions of the blast fungus and methods for the punch infection of the leaf blade with the blast fungus were performed as described (23). The five individual strains of *M. oryzae* were isolated from the field by the Jiangsu Academy of Agricultural Sciences, China. The mixed spores or spores from individual strains of *M. oryzae* were used for infestation and the phenotypes were investigated at 7 d postinoculation. 5×10^4 spores/ml of each conidial suspension were used for spraying. Disease lesion area (DLA) was assessed by Image J. Lesions were photographed and measured or scored at 7 d postinoculation.

***Xoo* Inoculation and the Measurement of Lesion Length.** 6-week-old rice seedlings were inoculated with the *Xoo* strain (OD_{600} of 0.5) by the scissors-dip method (23). After inoculation, rice plants are transferred to a growth chamber with a 16 h-photoperiod at 25 °C, 80% relative humidity. Lesion length was measured using a ruler 2 wk after inoculation to evaluate the resistance of the different families against pathogens. Three biological replicates were conducted and 10 to 20 representatives were used for measurement.

Vector Construction and Plant Transformation. To overexpress *OsWRKY36* and *OsPAL6*, the coding sequences (CDS) of *OsWRKY36* and *OsPAL6* were amplified using gene-specific primers (*SI Appendix*, Table S1) from Hwayoung, and cloned into pCAMBIA1390. All constructs were verified by DNA sequencing. Subsequently, the two recombinant constructs were introduced into Nipponbare and *Bphs1-D* by *Agrobacterium*-mediated transformation as described previously, respectively (36).

To knock out *OsWRKY36*, 20-bp gene-specific spacer sequences were cloned into the sgRNA-Cas9 vector (37) and subsequently introduced into Ningjing1 via *Agrobacterium*-mediated transformation. Approximately 30 positive T_0 transgenic individuals were identified by sequencing; two different gene-edited lines were utilized for subsequent experiments.

RT-qPCR Analyses. Total RNA was extracted from various tissues using the RNA prep Pure Plant Kit (Tiangen) according to the manufacturer's instructions. Real time-qPCR was performed on Bio-Rad CFX96 Real-Time System using a SYBR Premix Ex Taq™ RT–PCR Kit (Takara) with *Ubiquitin* as the endogenous control. Relative expression levels of genes were quantitated based on three biological replicates via the $2^{-\triangle\triangle Ct}$ method (38). All primers used for Real time-qPCR are listed in *SI Appendix*, Table S1.

Subcellular Localization. CDS of *OsWRKY36* without the stop codon was amplified and inserted between the *Xba* I and *BamH* I sites of the pAN580 vector to generate the *OsWRKY36-GFP* construct (primers listed in *SI Appendix*, Table S1). The *OsWRKY36-GFP* plasmid was introduced into rice protoplasts according to the protocols described by Zhang et al (39). Fluorescence of GFP was observed using a confocal laser scanning microscope (Leica SP8).

RNA In Situ Hybridizations. Leaf sheaths from Nipponbare was fixed in a FAA (RNase free) fixative solution at 4 °C. Through dehydration in a series of ethanol and xylene, samples were embedded in paraffin (Paraplast Plus, Sigma).

A probe was prepared by amplifying a fragment from *OsWRKY36* cDNA and cloned into the pGEMT Easy vector (Promega). The probe was then transcribed in vitro using a DIG Northern Starker Kit (Cat. no. 2039672, Roche) following the manufacturer's instructions. RNA in situ hybridization with the probes was performed on transverse sections of leaf sheath. After blotting with Antidigoxigenin AP-conjugated (Roche, 11093274910) and incubation with the NBT solution (Roche, 11383213001), the slides were observed and photographed using a light microscope (Leica DM5000 B). Primers used are listed in *SI Appendix*, Table S1.

Transactivation Activity Assay. *OsWRKY36*, *OsWRKY36*$^{\triangle WRKY\text{-}C2H2}$ and *OsWRKY36*$^{WRKY\text{-}C2H2}$, were cloned into pGBKT7. Each of the three fusion constructs or the empty pGADT7 was transformed into the *Saccharomyces cerevisiae* strain *AH109*, respectively. All procedures were performed according to the manufacturer's recommendations (Clontech Laboratories Inc., Mountain View, CA).

To analyze the transactivation activity of *OsWRKY36*, the assay in rice protoplast system was performed as previously described (40). The reporter vector was constructed by inserting four copies of *GAL4* binding element and a minimal TATA region of 35S promoter of *CaMV* into the upstream of the firefly LUC in pPCV814 vector. The control effector vector *35S-Gal4DB* was constructed by inserting the GAL4DB into pRTL2-*GUS* vector. The *OsWRKY36* was cloned into control effector vector to generate 35S-*Gal4DB-OsWRKY36* as the effector. The pPTRL was used as the internal control. The effectors, reporters, and the internal control were cotransformed into rice protoplasts. After culturing for 16 h, the luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and quantitated by the GloMax 20/20 luminometer (Promega, Madison, WI). The experiment was performed with three biological replicates.

RNA-seq Assay. Twenty-day-old rice seedlings of Ningjing1 and *OsWRKY36*-KO were infested with BPH for 12 h. Three biological replicates of each pedigree were collected and flash frozen in liquid nitrogen. The library construction, sequencing, and bioinformatic analyses were performed at the Novogene Bioinformatics Institute (Beijing, China). Differential gene expression analysis was performed at Novogene (https://magic.novogene.com).

Histochemical Staining and Lignin Analysis. Histochemical staining and observation of the leaf sheath cross-sections of rice for lignin detection was performed as previously described (41). The cross-sections of leaf sheaths were dissected transversely in advance with razor blades. In Phloroglucinol–HCl staining, after treatment with 37% HCl for 30 s, the sections were stained with 1.0% (w/v) phloroglucinol for 30 s. The leaf sheath cross-sections were then observed under a Zeiss Axio microscope (Zeiss, Oberkochen, Germany). Lignin content (total lignin) was defined as a sum of Klason lignin residue and acid soluble lignin, and measured following the method described by Jurak, et al. (42).

Yeast One-Hybrid Assays. The full-length coding region of *OsWRKY36* was cloned into the pB42AD vector at the *EcoR* I restriction site to generate the pB42AD-*OsWRKY36* construct. The full-length and various truncated promoter regions of *OsPAL6*, *OsPAL1*, *IPA1,* or *MOC2* were cloned into the *Xho* I restriction site of pLacZi reporter vector, respectively. Primers used are listed in *SI Appendix*, Table S1. Constructs were then cotransformed into the yeast strain *EGY48* according to the manufacturer's manual (Clontech Laboratories Inc., Mountain View, CA). Transformants were grown on SD-Trp/-Ura plates for 3 d at 30 °C and then transferred onto 5-bromo-4-chloro-3-indolyl-b-d-galactopyranoside plates for blue color development. Yeast strains containing the empty pB42AD in combination with the LacZ reporter were used as negative controls.

Transient Expression Assay in Protoplasts and LUC Activity Determination. Full-length CDS of *OsWRKY36* was amplified (primers listed in *SI Appendix*, Table S1) and inserted into the *Xba* I/*Pst* I restriction sites of the pAN580 vector to generate the *35S:OsWRKY36* effector construct. 1.5 to 2.0 kb of promoter regions of *OsPAL6*, *OsPAL1*, and *IPA1* were amplified using the primers of *OsPAL6-LUC*, *OsPAL1-LUC,* and *IPA1-LUC* as listed in *SI Appendix*, Table S1, and then cloned into the upstream of *Luciferase* (*LUC*) reporter gene from *Renilla reniformis* (Ren) to generate the reporter construct. *LUC* under the control of *CaMV35S* promoter was used as the internal control. The combined reporter and effector plasmids were cotransformed into the rice protoplasts according to a protocol described by Zhang et al. (39). The LUC activity was quantified with a Dual-Luciferase Assay Kit (Promega, Madison, WI) following the manufacturer's recommendations and the relative LUC activity is calculated as the ratio of LUC/Ren.

EMSA. MBP-WRKY36 fusion protein was purified using the pMAL™ Protein Fusion and Purification System following the manufacturer's instructions (New England Biolabs, Ipswich, MA). For the EMSA, a modified 5′-biotin-binding oligonucleotide probe containing the W-box element was synthesized prior to the binding experiment. EMSA was manipulated following the Light Shift™ EMSA Optimization and Control Kit instructions (Thermo Fisher Scientific, Waltham, MA). Briefly, equal amounts of complementary oligonucleotides were incubated at 95 °C for 10 min, cooled down slowly to 15 °C (0.1 °C/s), and diluted to 50 fmol/mL final concentrations. The DNA binding reaction was performed with 100 fmol probe, 2 mg poly (dI-dC), and 100 ng purified MBP or MBP-WRKY36 proteins and incubated at room temperature for 30 min. Then the samples were immediately applied to the prerun native polyacrylamide gel containing 6.5% acrylamide in Tris-borate-EDTA buffer. After electroblotting onto a nylon membrane (Millipore, Billerica, MA), the oligonucleotides were crosslinked using UV-light. Detection of biotin on the probe by streptavidin was carried out using a Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific, Waltham, MA).

ChIP Assay. ChIP was performed by standard protocols for ChIP-qPCR and ChIP. Briefly, the appropriate pairs of plasmids were transfected into rice protoplasts. Then, cells were fixed by 1% formaldehyde for 8 min at room temperature (RT) with rotation, quenched by 0.125 M glycine. The cells were digested with MNase (NEB, M0247S), and chromatin was sonicated to an average fragment size of 200 to 500 bp. Then the chromatin was incubated with indicated primary antibodies with rotation overnight at 4 °C. The antibody–chromatin complex was immunoprecipitated with magic beads with rotation at 4 °C for 4 h. Then the immunoprecipitated DNA was extracted followed by qPCR. ChIP-qPCR results of indicated primary antibodies were calculated by normalization to ChIP-INPUT. ChIP-qPCR assays were performed in triplicates and the data are presented as mean ± SD. The ChIP-qPCR primers are listed in *SI Appendix*, Table S1.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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