

Constitutive expression of pathogen-inducible *OsWRKY31* enhances disease resistance and affects root growth and auxin response in transgenic rice plants

Juan Zhang¹, Youliang Peng¹, Zejian Guo¹

¹State Key Laboratory of Agrobiotechnology, Department of Plant Pathology, China Agricultural University, Yuanmingyuan West Road 2, Beijing 100094, China

WRKY transcription factors have many regulatory roles in response to biotic and abiotic stresses. In this study, we isolated a rice *WRKY* gene (*OsWRKY31*) that is induced by the rice blast fungus *Magnaporthe grisea* and auxin. This gene encodes a polypeptide of 211 amino-acid residues and belongs to a subgroup of the rice *WRKY* gene family that probably originated after the divergence of monocot and dicot plants. *OsWRKY31* was found to be localized to the nucleus of onion epidermis cells to transiently express *OsWRKY31*-eGFP fusion protein. Analysis of *OsWRKY31* and its mutants fused with a Gal4 DNA-binding domain indicated that *OsWRKY31* has transactivation activity in yeast. Overexpression of the *OsWRKY31* gene was found to enhance resistance against infection with *M. grisea*, and the transgenic lines exhibited reduced lateral root formation and elongation compared with wild-type and RNAi plants. The lines with overexpression showed constitutive expression of many defense-related genes, such as *PBZ1* and *OsSci2*, as well as early auxin-response genes, such as *OsIAA4* and *OsCr11* genes. Furthermore, the plants with overexpression were less sensitive to exogenously supplied IBA, NAA and 2,4-D at high concentrations, suggesting that overexpression of the *OsWRKY31* gene might alter the auxin response or transport. These results also suggest that *OsWRKY31* might be a common component in the signal transduction pathways of the auxin response and the defense response in rice.

Keywords: WRKY transcription factor, *Oryza sativa*, defense response, lateral root
Cell Research (2008) 18:508-521. doi: 10.1038/cr.2007.104; published 11 December 2007

Introduction

Plants have evolved elaborate mechanisms to defend against pathogen invasion. Upon pathogen recognition, host cells undergo various biochemical and structural changes, such as burst of active oxygen species, hypersensitive response, accumulation of antimicrobial proteins and phytoalexins, deposition of callose papillae, and formation of cork and abscission layers [1]. The transcriptional activation of a large number of genes upon perception of external stimuli, including pathogenic infections, has often been demonstrated. Some of the ac-

tivated genes encode proteins that have regulatory functions, such as kinases and transcriptional factors, which may have important roles in signaling cascades [2].

Many reports indicate that plant mutants with enhanced disease resistance are usually small in size [3]. A possible explanation for the dwarf phenotype could lie in changes in the metabolic activities towards defense-related pathways, thus leading to the expression of defense genes and the accumulation of antimicrobial products against pathogens [4, 5]. However, detailed knowledge about the pathway interactions between plant disease resistance and growth or development is still limited. Recently, SGT1b, first identified in yeast, was found to be a factor involved in plant disease resistance signaling and a factor necessary for the SCF^{TIR1} (SKP1-CDC53/CUL1-F-box)-mediated degradation of auxin/indoleacetic acid (Aux/IAA) proteins [6-8]. The *Arabidopsis sgt1b-1* mutant exhibits a reduction in the lateral root

Correspondence: Zejian Guo

Tel: +86-10-62733849

E-mail: guozj@cau.edu.cn

Received 12 February 2007; revised 15 June 2007; accepted 18 October 2007; published online 11 December 2007

development in comparison with wild-type seedlings [8]. Loss of *SGT1* function disables the early plant defense mechanisms conferred by multiple *R* gene-mediated and non-host pathogen resistance, showing the positive effect of auxin on disease resistance at an early stage [7, 9]. However, some researches have found that in other conditions, auxin also has a role in disease susceptibility. For example, Navarro *et al.* [10] have demonstrated that a flagellin-derived peptide induces a plant microRNA that represses auxin signaling and leads to the restriction of *Pseudomonas syringae* growth in *Arabidopsis*. These results suggest that, for different pathogens or in different infection stages, crosstalk between the defense signaling and auxin response pathways is varied and complicated.

Auxin regulates the formation of lateral branches in roots and shoots, and regulates other aspects of plant growth and development [11]. *Aux/IAA* genes are rapidly upregulated in response to auxin [12]. *Aux/IAA* proteins, which are negative regulators of auxin signaling, regulate gene expression by interacting with auxin response factors (ARFs), which function as positive regulators by binding to AREs within the promoters of auxin-inducible genes [12, 13]. Mutants that are deficient in auxin transport or insensitive to auxin have been reported to have a reduced number of lateral roots in both *Arabidopsis* [14, 15] and rice [16–18]. For example, a rice mutation in *cr11* (*crown rootless 1*) decreases lateral root numbers and auxin insensitivity occurs during lateral root formation. *Cr11* encodes a positive regulator for crown and lateral root formation and its expression is directly regulated by an ARF in the auxin signaling pathway [16]. The loss-of-function mutants of *ARF3*, *ARF5* and *ARF7* also exhibit reduced sensitivity to auxin, which results in defects in specific auxin-dependent phenomena, such as the formation of the gynoecium, the development of the vascular system and the differential growth responses of hypocotyls [19–22]. Whereas overexpressing *ARF8* in *Arabidopsis* represses the growth of lateral roots, *ARF8* may control free IAA levels in a negative feedback fashion by regulating the expression of *GH3* genes, which may be involved in maintaining IAA homeostasis [22, 23]. These results indicate that *ARF* family genes may have a central role in auxin-triggered responses by regulating both auxin sensitivity and homeostasis in *Arabidopsis*. However, it is imperative to identify the mediators that link the control of *Aux/IAAs* by auxin to root formation and elongation.

Many WRKY proteins have been found to be involved in defense against pathogenic attack in *Arabidopsis*, tobacco and parsley [24–29]. In rice, *OsiWRKY* and *OsWRKY52* were induced by *Xanthomonas oryzae* pv. *oryzae* and *Magnaporthe grisea*, respectively [30, 31], and

OsWRKY03 was shown to be a transcriptional activator in defense signaling cascades [32]. Furthermore, WRKY proteins have been shown to have roles in developmental and physiological processes, such as in senescence [33], metabolism [34–36], trichome development [37] and root development [38]. In this study, we report that *OsWRKY31* (named as *WRKY55* by Xie *et al.* [35]), a rice WRKY transcription factor gene, was strongly induced by the rice blast fungus *M. grisea* and auxin. Further analyses indicate that *OsWRKY31* may be a common component in the signal transduction pathways of auxin and defense responses in rice.

Results

Cloning of the OsWRKY31 gene and sequence analysis

From a rice (IR72) cDNA library generated with elicitor-treated rice suspension cells, the *OsWRKY31* gene was amplified with gene-specific primers designed according to the deposited genomic sequence and the expressed sequence tags (GenBank acc. no: AK101653). The open reading frame was 633 bp in length, encoding a protein of 211 amino-acid residues with a molecular weight of 24.0 kDa and a *pI* of 7.35. There was one nucleotide change from T in Nipponbare to C in IR72, leading to an amino-acid change of Ser to Pro at position 116 of the encoded protein. The putative protein harbors two regions that are rich in Ser (aa 120–137) and acidic amino acids (aa 181–197) in the C-terminal of *OsWRKY31*, which suggests that they may be involved in transcriptional activation (Figure 1). Besides a putative nuclear localization signal (NLS) sequence RKEKRMRK, *OsWRKY31* has only one WRKY domain that contains an extended finger motif of CX₇CX₂₇HX₁C, compared with the typical WRKY domain (CX₇CX₂₃HX₁C) from other species [39, 40]. A phylogenetic tree generated with *OsWRKY31*, and several closely related WRKY proteins from different plant species based on BLAST analyses, showed that *OsWRKY31* clusters only with rice WRKYs of the same subgroup (data not shown). This result agrees with those obtained previously and indicates that *OsWRKY31* belongs to the rice IIIb subgroup and was possibly generated after the divergence of monocot and dicot plants [40]. The extended zinc-finger motif in *OsWRKY31* may also reflect the different divergent force for this family of genes between rice and *Arabidopsis*. There is also a change of Q to E in *OsWRKY31* within the conserved septimal WRKYGQK residues of WRKY proteins.

Nuclear localization of OsWRKY31

Sequence analysis showed that *OsWRKY31* contains

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1      ATGTCTCTGTGCCAGTCCGCATCAATCACACCATCTAGCCATGGCTCAAGGAAAGAG
1      M S P V P S P H Q S H H L G H G S R K E
61     AAGCCGATGAGGAAGTGGATACCTTTGGCCGCAACAACGACGGCCACCACTGGAGGAAG
21     K R M R K V D T F A P H N D G H Q W R K
121    TACGCCGAGAAGATAAACAACCTGTAAATTCGCCAGATACTACTACAGATGCACCTAT
41     Y G E K K I N N C N F P R Y Y Y R C T Y
181    AAAGATAACATGAATTGCCAGCAACGAAGCAGATTACGACAGAAAGATTATAGTGATCCA
61     K D N M N C P A T K Q I Q Q K D Y S D P
241    CCATTGTACTCAGTCACTACTACAATGAGCATACATGTAATAGTGCTTTCTTCCTCTT
81     P L Y S V T Y Y N E H T C N S A F L P L
301    AGCCCTCAGAGTCCAGCTGCAAGCTGCACTCTGGGAAGGCACTCCCATCTGCTTTGAA
101    S P S E F Q L Q T A S G K A V P I C F E
361    TCATCTGGGGCTCAAGAACCAATGACCAATGCCAGCTCACCTTCTTCAAGCGCAGCACGG
121    S S G A Q E P M T N A S S P S S A A R
421    CGTAGCACACCTTCAGAGAACAAGAATCAGCCTCTTCCACGGCATTCCGAAGCCTATTCT
141    R S T P S E N K N Q P L P R H S E A Y S
481    TGGGGGTGGTGTGTAGAAACAAAGCCGCTGCACTGAGCTCCAATCTTCAGCAGCC
161    W G V G V E Q K P S C T E L Q S C S T
541    GAATGTCAAGGATGCAATTTTCAGCTGGTACGATTCCTGAAGACAGTAGATGACGGAAGA
181    E C Q D A F S A G T I P E E T V D A G R
601    TTTGGTCTATCAGATCTTCCATTTCTCGAGTAA
201    F G S I R F F H F L E *
    
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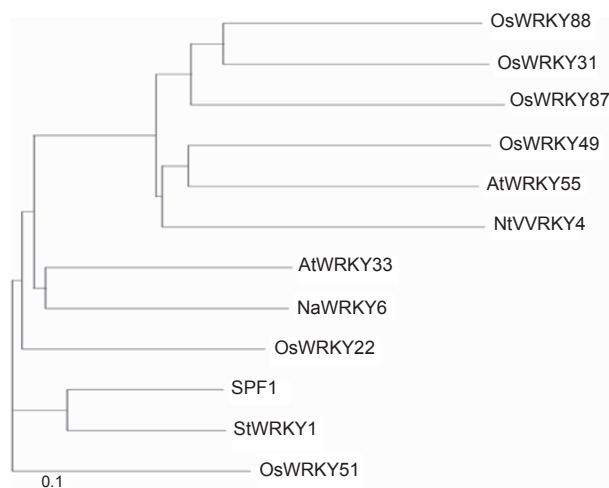


Figure 1 OsWRKY31 sequences and phylogenetic analysis. **(A)** The nucleotide sequence of *OsWRKY31* cDNA and its deduced amino-acid sequence. The 5'-untranslated region is italicized. The residues forming the WRKY domain are in bold and shaded, including the conserved amino-acid residues. Cysteine and histidine residues making up the potential zinc-finger motif are underlined. The putative nuclear localization signals are boxed. The asterisk indicates the stop codon. The acidic residues underlined suggest a potential transactivation region. **(B)** The phylogenetic tree of *OsWRKY31* with close homologs from a BLAST search. The deduced amino-acid sequence of *OsWRKY31* was aligned with some of the published members of WRKY using the CLUSTAL W program and the phylogenetic tree was drawn using the TREEVIEW program to produce a neighbor-joining tree of the full-length amino-acid sequences from WRKY proteins. The GenBank accession numbers are listed in brackets for each of the following WRKY proteins: *OsWRKY88* (AK073243), *OsWRKY87* (AC123514), *OsWRKY49* (AC136227), *AtWRKY55* (AAY78725), *NtWRKY4* (AAF61864), *AtWRKY33* (AAM34736), *NaWRKY6* (AAS13440), *OsWRKY22* (AK107199), *SPF1* (BAA06278), *StWRKY1* (BAE46417) and *OsWRKY51* (AK121190).

a putative NLS and may be a nuclear factor in rice cells. To examine this possibility, the *OsWRKY31* gene was fused in frame with a fluorescent reporter *gfp* gene. The chimeric gene was put under the control of a CaMV35S promoter (pCaMV35S::*OsWRKY31*-eGFP) and then bombarded into inner epidermal onion cells. After cultivation in the dark at 28 °C for 18 h, fluorescence was observed under a laser confocal microscope. As shown in Figure 2, the *OsWRKY31*-eGFP fusion protein was exclusively localized in the nucleus, whereas the control eGFP (pCaMV35S::eGFP) had spread into the cytoplasm as well as into the nucleus. This result reveals that *OsWRKY31* is a nuclear protein.

OsWRKY31 has transactivation activity in yeast

All transformants harboring *OsWRKY31* or its deletion mutants grew well on the synthetic SD-Trp medium (Figure 3A) and pBD-W31 grew normally on the SD-Trp-His-Ade medium. Deletion of the 61 C-terminal amino acids (pBD-C1) decreased β-galactosidase activity of pBD-W31 by half, and pBD-dC1 exhibited transactivation activity (Figure 3B). Further deletion of the putative acidic region (pBD-C2) abolished the transactivation activity.

However, pBD-N1 produced only 38.4% of the activity produced by pBD-W31. To clarify the possible effect of the 93 N-terminal regions, we generated several plasmids with different deletions of the N-terminal amino acids based on the pBD-C1 construct. The pBD-N3, -N4 and -N5 mutants almost completely lost β-galactosidase activity (Figure 3B), which indicates that the entire N-terminal region is required for transcription activity. Alternatively, the WRKY domain could suppress tran-

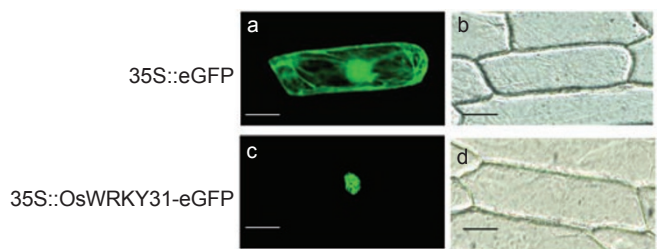


Figure 2 Localization of the *OsWRKY31*-eGFP fusion protein in the nucleus. The *OsWRKY31*-eGFP fusion protein was localized in the nucleus using a transient expression assay in onion. The control 35S::eGFP and 35S::*OsWRKY31*-eGFP plasmids were transformed into onion epidermal cells and examined after 18 h. GFP fluorescence (left panel) and differential contrast imagery (right panel) were compared to show the subcellular localization of eGFP (cytoplasmic and nuclear) and *OsWRKY31*-eGFP (nuclear). Scale bars indicate 100 μm.

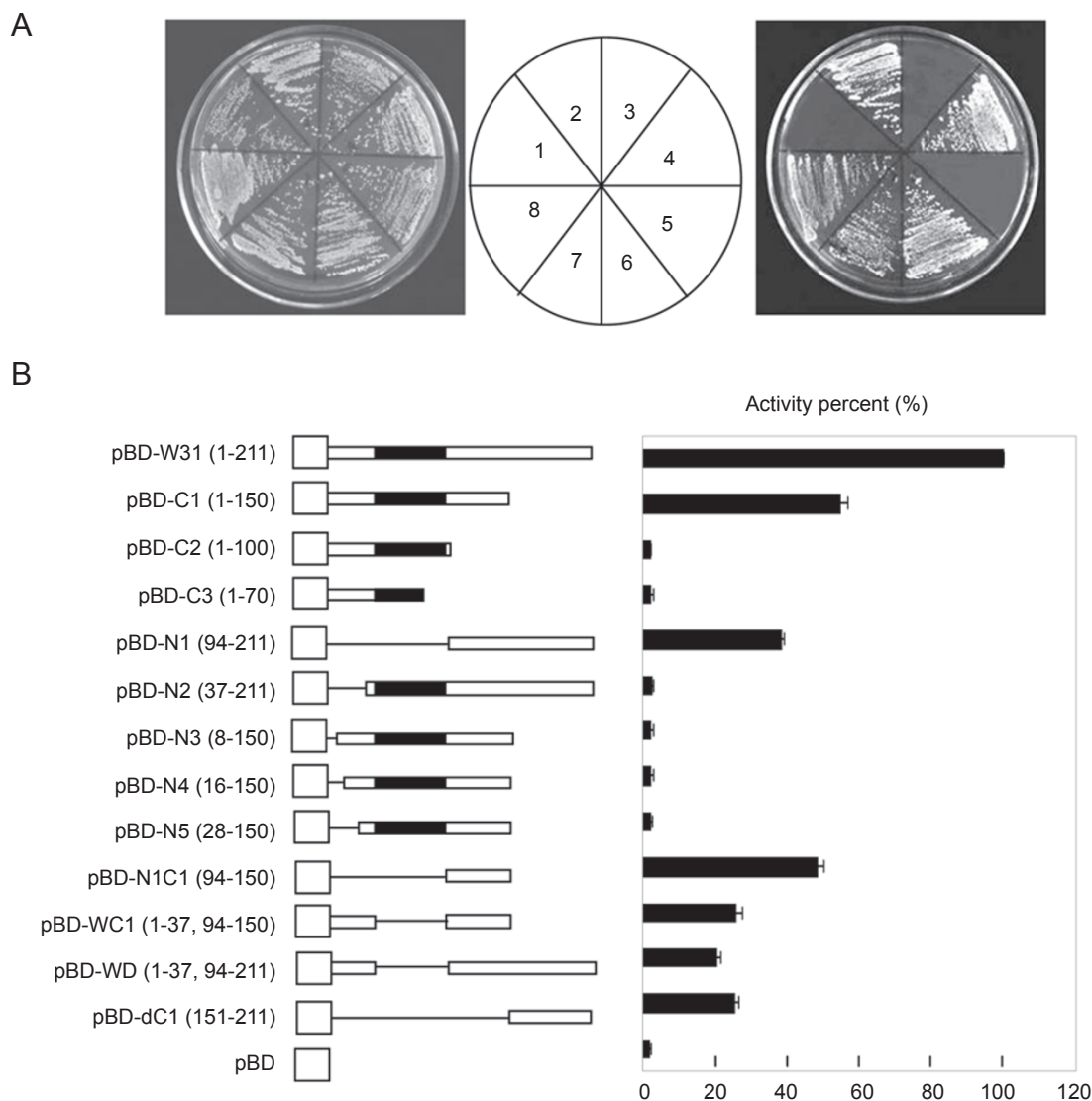


Figure 3 Analysis of the OsWRKY31 transactivation activity and activation domain in yeast. **(A)** The constructed vectors were transformed into the AH109 strain of yeast, and were grown on SD-Trp medium (left) and SD-Trp-Ade-His selective medium (right) at 30 °C for 3 days. 1, pBD; 2, pBD-W31; 3, pBD-C2; 4, pBD-C1; 5, pBD-N2; 6, pBD-N1; 7, pBD-N1C1; 8, pBD-WD. **(B)** β -galactosidase activity of transformants was assayed using *O*-nitrophenyl β -D-galactopyranoside as a substrate. The values from three independent experiments were shown as the mean \pm SE. The white square represents pBD in pGBKT7, the black rectangle represents the WRKY domain of OsWRKY31, the white rectangle represents the rest of OsWRKY31 and the line represents the deleted part. The numbers in brackets are the start and end positions of each translated product of OsWRKY31 in the construct.

scription activity when the N-terminus is incomplete, which is supported by evidence from the pBD-N1C1 and pBD-N2 transformants. But the deletion of the WRKY domain region (pBD-WD and pBD-WC1) did not increase transactivation activity in comparison with their parent plasmids pBD-W31 and pBD-C1. These findings suggest that OsWRKY31 is a transcriptional activator in yeast at least. The active regions were found to be the Ser-rich and the acidic amino-acid-rich C-terminal

regions. The region of the WRKY domain might have a repressive role, in which the effect could be counteracted by the presence of the N-terminal region.

OsWRKY31 expression was induced by pathogens and wounding

In a preliminary microarray study on the expression patterns of rice *WRKY* genes, we found that *OsWRKY31* expression was induced by the *M. grisea* pathogen (Guo

et al., unpublished results). To learn its more detailed expression profile, *OsWRKY31* expression was examined in the rice cultivar Zhonghua17 under different treatments. As shown in Figure 4, the mRNA accumulation of the *OsWRKY31* gene was induced as early as 4 h after inoculation with an avirulent strain (P131) of *M. grisea*, and lasted throughout the period studied. Induction of the *OsWRKY31* gene was also observed following infection with a compatible strain MS220, although the expression level was lower than that of P131 at the same time point (Figure 4). To examine the quality of pathogenic inoculation, the same blot was striped and re-hybridized with an *OsSci2* gene belonging to the subtilisin chymotrypsin inhibitor gene family, which is strongly induced by *M. grisea* (Zhao and Peng, personal communication). Induction of *OsSci2* by the P131 and MS220 strains in Zhonghua17 revealed that the expression of *OsWRKY31* is the consequence of pathogenic infection.

Analysis of this gene's expression was extended to mechanical wounding and methyl jasmonate (MeJA), an important signal molecule in plant defense responses. The accumulation of *OsWRKY31* mRNA occurred soon after wounding, with the mRNA levels increasing during the later time points (Figure 4). *OsWRKY31* gene expression was also stimulated by treatment with MeJA (Figure 4), although the induced response was relatively slow in comparison with the responses induced by pathogens or wounding.

OsWRKY31 overexpression enhances resistance to *M. grisea* and induces expression of defense-related genes

Transgenic rice plants were generated to explore the possible biological functions of *OsWRKY31*; 18 overexpression lines and 16 RNAi lines were obtained using an *Agrobacterium*-mediated methodology and were allowed to set seeds. Some of the T₁ progeny lines were then randomly selected to determine the expression of the transgene. As shown in Figure 5A, constitutive expression of the *OsWRKY31* gene was observed in the overexpression lines, whereas expression of the gene was not detected in the control or RNAi lines.

To examine whether *OsWRKY31* overexpression enhances disease resistance, transgenic and control plants were inoculated with a strong virulent strain of *M. grisea* (P140) by spraying. The development of disease was noticeably less in the *OsWRKY31* overexpressing progenies compared with control and RNAi lines (Figure 5B). Disease incidence was evaluated 1 week after inoculation by measuring the number and size of lesions. The results revealed a significant decrease in both occurrence and symptom development in the *OsWRKY31* overexpression lines compared with the control (Figure 5C and 5D). However, the level of disease found in the RNAi lines was similar to the control (data not shown). Analysis of mRNA accumulation by northern blotting revealed that the expression of *OsWRKY31* was suppressed in the RNAi lines even after inoculation with the pathogen

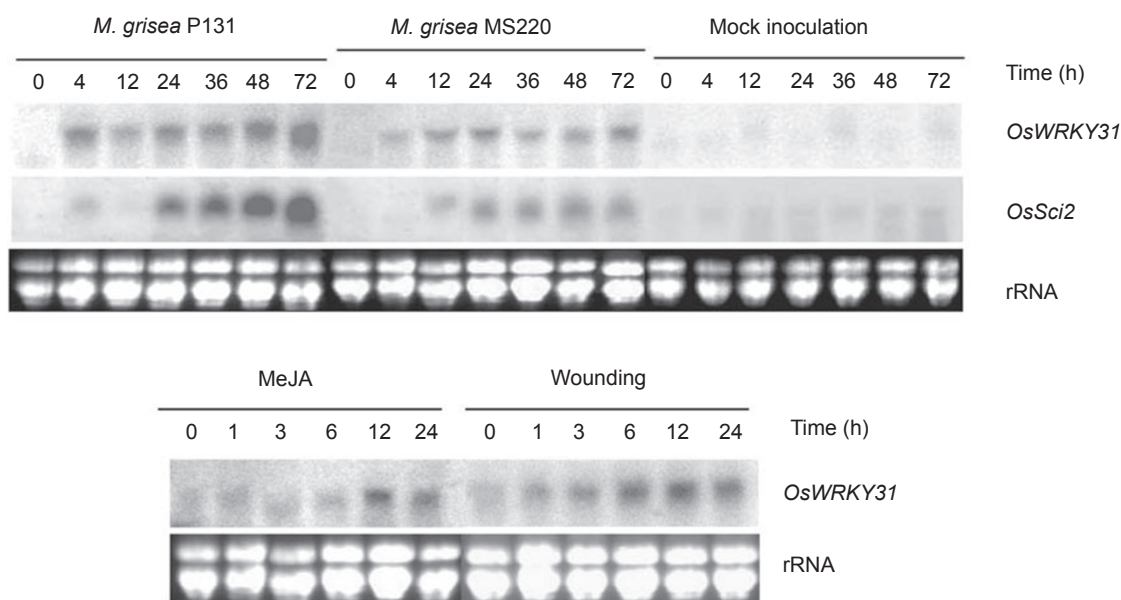


Figure 4 *OsWRKY31* expression is induced by biotic and abiotic stresses. Total RNA was isolated from the leaves of rice seedlings at different time points. An equal loading of 20 μg RNA to each lane was checked using ethidium bromide staining. ³²P-labeled *OsWRKY31* and *OsSci2* were used as probes for hybridization.

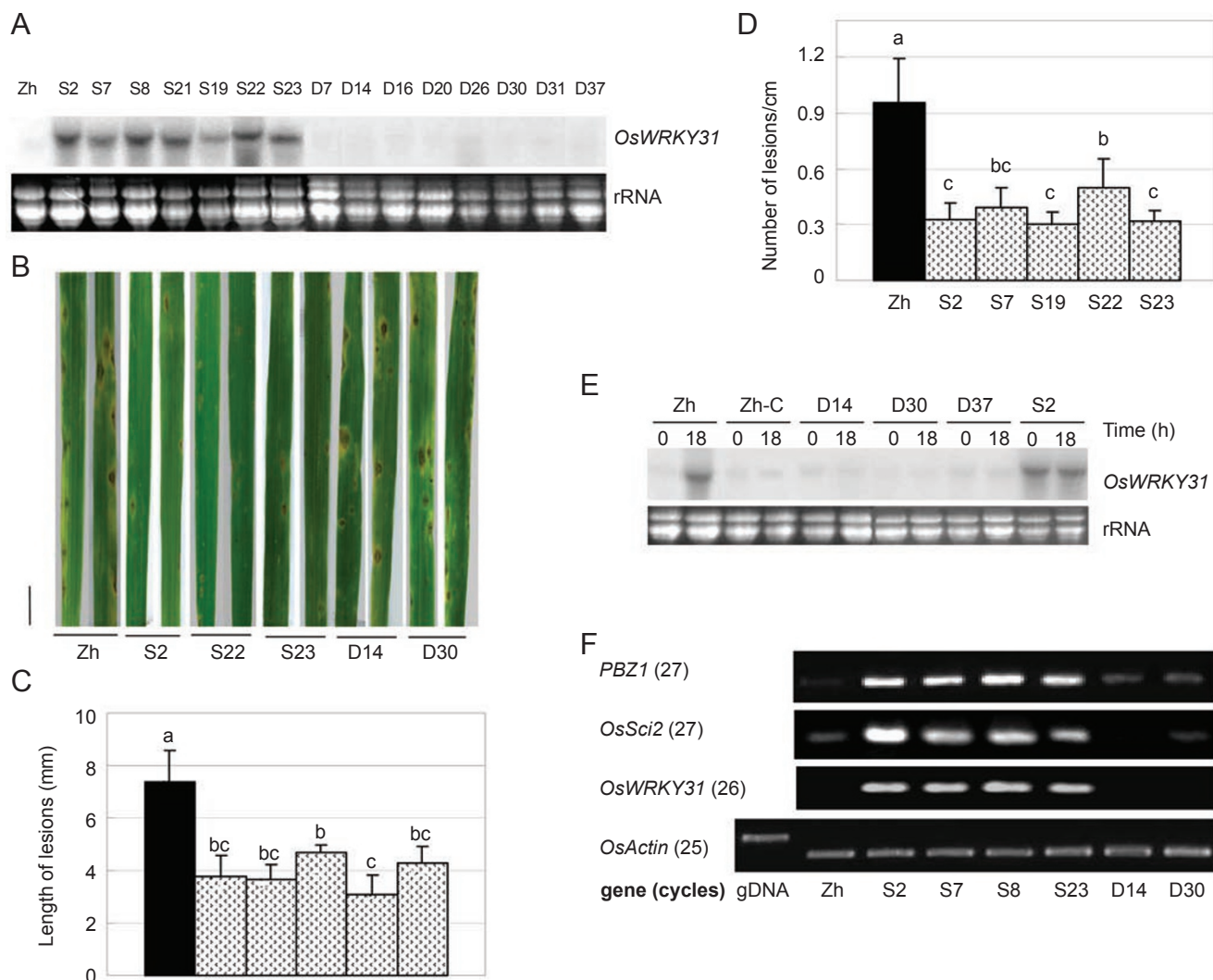


Figure 5 *OsWRKY31* overexpression enhances disease resistance and induces expression of defense-related genes. **(A)** *OsWRKY31* expression in the overexpression (S), RNAi (D) and control (Zh) plants. An equal loading of 15 μ g RNA to each lane was checked using ethidium bromide staining. 32 P-labeled *OsWRKY31* was used for hybridization. The numbers indicate the different transgenic lines. **(B)** Three-week-old transgenic rice seedlings were inoculated with a virulent strain of *M. grisea* P140. Leaves were detached from the inoculated plants 5 days after the infection and then scanned. **(C and D)** Histograms showing the lesion length **(C)** and number **(D)**. The disease incidence was evaluated 7 days post-inoculation. Each bar indicates the average and standard deviation of at least five leaves. Different letters (a, b and c) indicate statistically significant differences as analyzed by the SAS software (Duncan's multiple range test; $\alpha = 0.01$). **(E)** *OsWRKY31* transcription in RNAi lines (D14, D30 and D37), control plants (Zh and Zh-C), and overexpression line (S2) after inoculation or mock inoculation (Zh-C) with *M. grisea* P140 for 18 h. **(F)** Gene expression was determined by semiquantitative RT-PCR. The RNAs from the overexpression (S2, S7, S8, S23), RNAi (D14, D30) and control (Zh) plants were reverse-transcribed and used as templates for PCR. The actin gene was used as an internal standard and also amplified using genomic DNA (gDNA) as a template to check the gDNA contamination in the transcribed RNAs.

(Figure 5E).

To study whether the enhanced disease resistance was related to enhanced expression of defense-related genes, we selected four overexpression and two RNAi lines to detect mRNA levels using a semiquantitative RT-PCR method. Constitutive expression of *OsWRKY31* was

observed in the overexpression lines, whereas the accumulation of *OsWRKY31* mRNA was low in the control and RNAi plants (Figure 5F). Similarly, we observed the constitutive expression of *PR* genes, such as *OsSci2* and *PBZ1*, in the overexpressor transgenics, whereas these genes were expressed at much lower levels in the control

and RNAi progenies, suggesting that the increase of disease resistance might be due to the constitutive expression of the defense genes in the *OsWRKY31* overexpressing lines.

Ectopic expression of OsWRKY31 reduced the formation and elongation of lateral roots

When handling the rice seedlings, we noticed a difference in the formation of lateral roots between the *OsWRKY31* overexpression and control lines. Therefore, we selected some of the transgenic lines for more detailed examination. Seeds of the transgenic plants of the T₂ progeny were germinated and grown on a 1/2 MS

medium with 0.6% agar. Evaluation of 7-day-old rice seedlings revealed that the number of lateral roots was significantly less in the *OsWRKY31* overexpressing lines than in the control plants (Figure 6A-F). The length of the lateral roots was also shorter in the overexpressor progenies in comparison with the control plants. Furthermore, 10 out of 18 of the overexpressor lines still exhibited dwarfism and fewer roots in comparison with the control plants at the heading stage (Figure 6G and 6H), which exhibited noticeably reduced lateral root formation and elongation in seed roots and crown roots (Figure 6I). A similar examination was performed on the seedlings harboring the hairpin construct; however, the difference

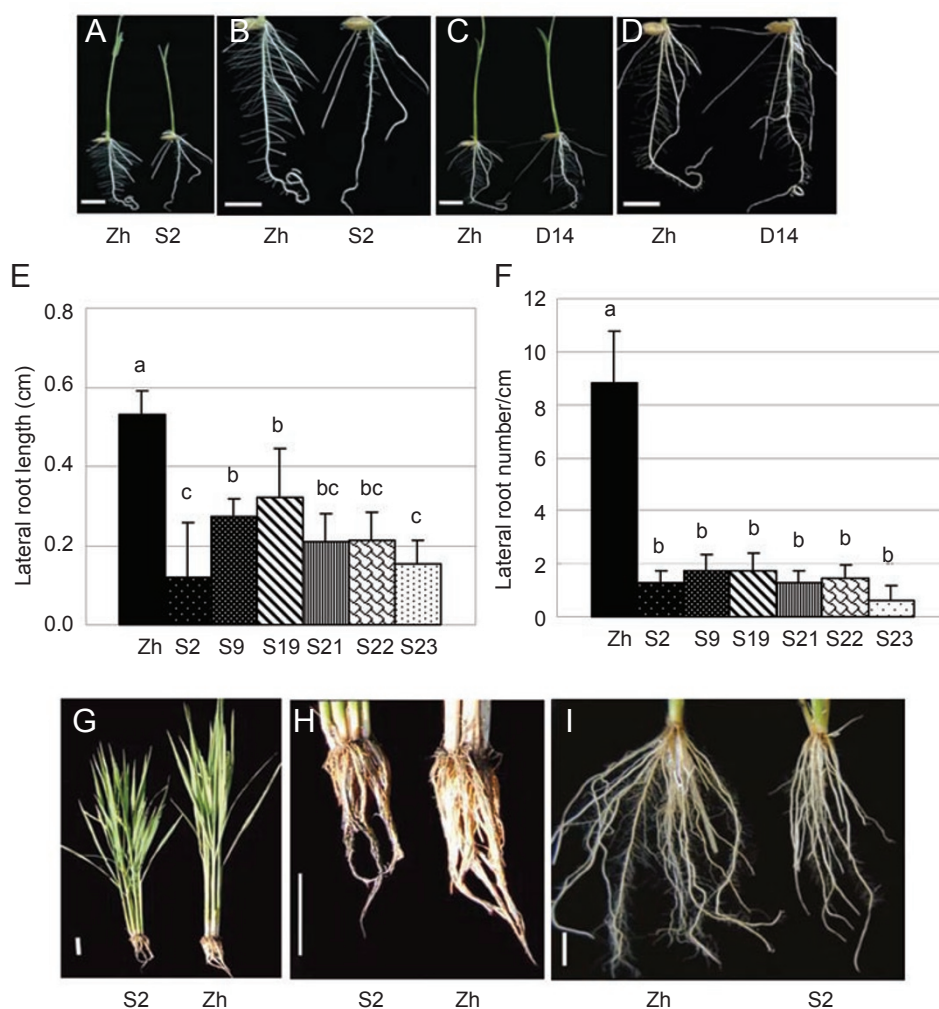


Figure 6 Reduced lateral root formation and elongation by ectopic expression of *OsWRKY31*. (A-D) Overexpression plants showed a decrease in lateral root formation and elongation, whereas the RNAi line was not apparently different from the control plant. Pictures were taken from 7-day-old seedlings. (E and F) Histograms showing the changes in lateral root length (E) and number (F) in overexpression plants compared with control plants. Figures represent the means ± SD from at least 15 plants for each line. (G-I) Images showing the differences in plant height and the root formation between overexpression (S2) and control (Zh) lines at their heading stage. Scale bars indicate 5 cm in (H) and 1 cm in other parts. Different letters (a, b and c) indicate statistically significant differences as analyzed by the SAS software (Duncan's multiple range test; $\alpha = 0.01$).

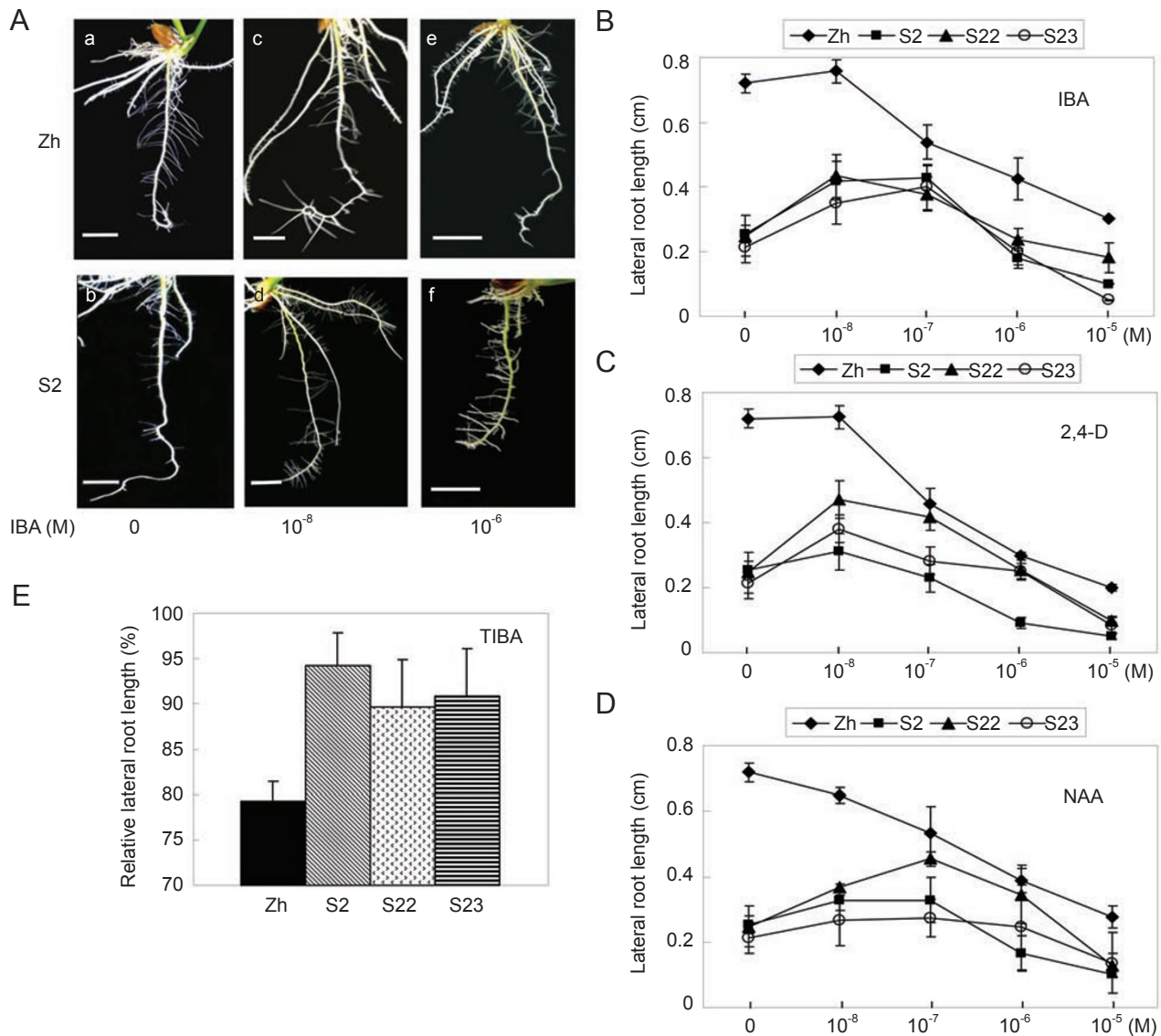


Figure 7 Effect of exogenous auxin on lateral root elongation. **(A)** The phenotypes of control (Zh: a, c and e) and overexpression lines (S2: b, d and f) in lateral root elongation after treatment with 10^{-8} M IBA (c, d), 10^{-6} M IBA (e, f) or without IBA (a, b). **(B-D)** Plants were treated with different concentrations of IBA, NAA and 2,4-D. The lengths of lateral roots were measured 6 days after the auxin treatments. The means \pm SD of the 20 longest lateral roots are given for each line. **(E)** Plants were treated with 10^{-6} M of TIBA or water. The relative lateral root length is given as a percentage of untreated plants against the control and overexpression lines. Scale bars indicate 1 cm.

between the RNAi lines and the control plants was not statistically significant (data not shown).

Responses to exogenous auxin in the lateral root elongation of OsWRKY31 overexpressing plants

In general, the initiation and later development of lateral roots are related to auxin. Therefore, a dose-response assay was conducted to examine the response of the transgenic plants to exogenous auxin. Both control and transgenic plants were treated with different types

of auxin and the length of lateral root was determined according to the method described by Debi *et al.* [18]. Similarly, we observed that treatment with IBA promoted slight lateral root elongation at a lower concentration (10^{-8} M) and exhibited inhibitory effects at a higher concentration (up to 10^{-7} M) in the control plants (Figure 7A and 7B). In the *OsWRKY31* overexpressing lines, the length of lateral roots nearly doubled following treatment with IBA at 10^{-7} M compared with non-treatment, and was inhibited when the concentration of IBA was 10^{-6}

M. Similar phenomena were observed in the treatment of transgenic plants with NAA or 2,4-D (Figure 7C and 7D), although the response to the NAA treatment varied between individual transgenic lines. Furthermore, the effect of auxin transport inhibitors on root elongation was examined. The results indicate that the *OsWRKY31* overexpressing lines exhibit slightly more resistance to the auxin transport inhibitor TIBA in comparison with the response of the control seedlings (Figure 7E).

OsWRKY31 is an auxin inducible gene

To examine whether *OsWRKY31* is regulated by auxin, we investigated the expression of the *OsWRKY31* gene following application of the hormone. As shown in Figure 8A, induction of *OsWRKY31* was observed through the treatment of rice roots with IBA (1 μ M) and 1-NAA (an active form of auxin, 1 μ M), whereas the accumulation of *OsWRKY31* mRNA was not induced by treatment with the inactive 2-NAA (1 μ M). This suggests that *OsWRKY31* is an auxin-inducible gene.

Moreover, the expression of *OsWRKY31* at early time points was studied after the treatment of exogenous auxin using an RT-PCR method. Induction of *OsWRKY31* in rice roots was observed as early as half an hour after the treatment with 1 μ M IBA and its level gradually increased with time (Figure 8B). To investigate the relationship of *OsWRKY31* with other auxin-induced genes, we also determined the expression of *OsIAA4*, a member of the early auxin response *Aux/IAA* gene family, and the *OsCr11* gene, an essential gene for crown root formation in rice [16]. The induction profiles for both genes, also described by Inukai *et al.* [16], were similar to the induction profile of *OsWRKY31* (Figure 8B). Interestingly, constitutive expression of the *OsIAA4* and *OsCr11* genes was observed in the *OsWRKY31* overexpressor lines of T₂ progenies and their expressions were not increased further by the treatment of the overexpressor plants with IBA (Figure 8C). These results suggest that *OsWRKY31* may be involved in auxin signaling.

Discussion

The WRKY transcription factors have been shown to have important roles in biotic and abiotic stresses. However, most studies have focused on dicot plants such as *Arabidopsis* and tobacco, with only a few focusing on rice WRKYs. In a previous study, we phylogenetically classified the rice *WRKY* gene family and compared them with their counterparts in *Arabidopsis* [40]. In rice the genes in the IIIb subgroup are considered to have been generated sometime after the divergence of monocot and dicot plants, and possibly have a special role in

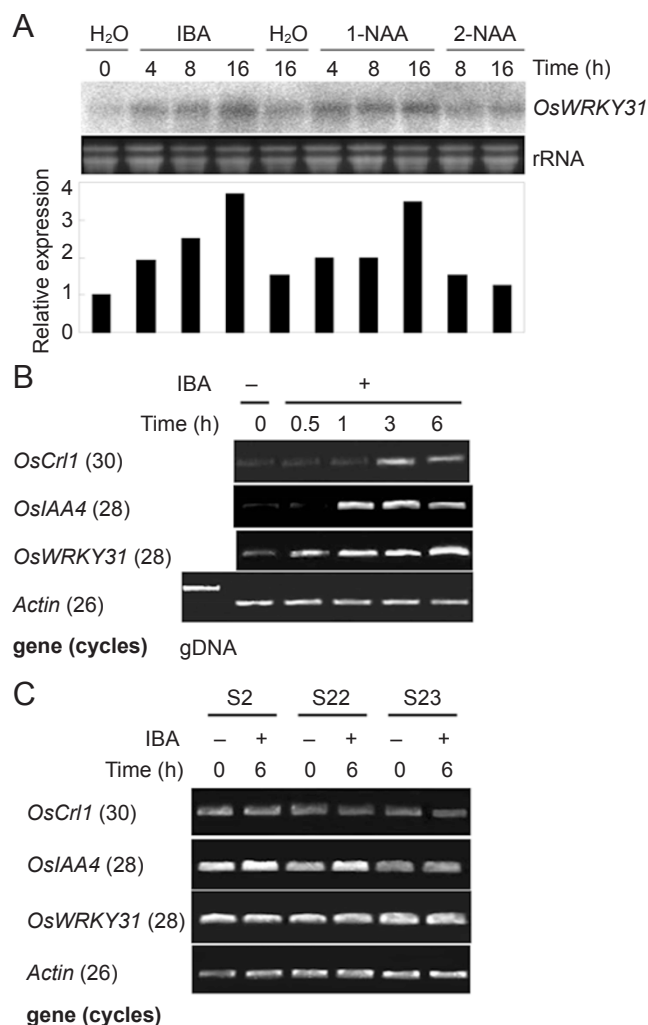


Figure 8 *OsWRKY31* induction with auxin treatments and constitutive expression of the auxin-response genes in *OsWRKY31* overexpressing lines. **(A)** *OsWRKY31* expression was analyzed in rice roots that were treated with 1 μ M of 1-NAA, its inactive analog 2-NAA and IBA, using H₂O as a control, at different time points. Relative levels are shown. **(B and C)** Analyses of gene expression by semiquantitative RT-PCR. Four-day-old seedlings of the control **(B)** and overexpressing **(C)** plants were treated with or without 1 μ M IBA. The actin gene was used as an internal standard and was amplified using genomic DNA (gDNA) as a template to check the gDNA contamination in the transcribed RNAs.

rice plants. In this study, we have characterized the *OsWRKY31* gene, a member of rice IIIb subgroup.

OsWRKY31 contains regions that are rich in Ser and in acidic amino acids in its C terminus, which act as transcription activation domains in yeast (Figure 3). However, this activity seems to be affected by complicated factors. Deletion of several amino acids from the N terminus

eliminates transcriptional activity, which is in contrast with the corresponding plasmid harboring the N-terminal sequence (Figure 3). Further deletion of the N terminus, which includes the WRKY domain, resulted in the restoration of some transactivation, suggesting that the WRKY domain has a suppression effect in the absence of the very N-terminal amino acids. However, deletion of the WRKY region did not increase the transcriptional activity of the rest of the proteins. On the contrary, transcription decreased. In a search of the OsWRKY31 protein sequence, we did not find the LxLxL motif, which is typically present in the suppression domains of Aux/IAA, WRKY proteins and ethylene response factors [35, 41, 42]. A possible explanation (at least in yeast) could be that the N terminus, which includes the WRKY domain, has a special conformation that affects the transactivation of OsWRKY31 and so any deletion of the sequence in this region would lead to a possible conformation change and a decrease or elimination of transcriptional activity in the rest of the protein.

The transcription of *OsWRKY31* was induced in rice seedlings as early as 4 h post-treatment with the rice blast fungus *M. grisea* (Figure 4). This induction was much faster than that of a PR gene, *OsSci2*. At this stage, *M. grisea* fungi were in the process of penetrating the epidermal cell layers of the rice leaves, suggesting that OsWRKY31 might be involved in early defense against the infection. In addition, overexpression of the *OsWRKY31* gene significantly decreased lesion sizes and the number of infection sites of *M. grisea* fungi on transgenic rice plants (Figure 5A), indicating that pathogen invasion was blocked in the early stages as they spread. Constitutive expression of defense-related genes, such as *OsSci2* and *PBZ1*, may contribute to the enhanced disease resistance of the *OsWRKY31* overexpressor lines (Figure 5F) because their expressions were not induced by *M. grisea* in RNAi lines examined by RT-PCR (data not shown). In combination with the targeting of OsWRKY31-eGFP to the nucleus (Figure 2), the results indicate that OsWRKY31 may function as a transcription activator in the rice defense response.

Aux/IAAs encode the rapidly turned over transcriptional repressors of auxin-inducible genes [11]. Previous analyses of *Arabidopsis* mutants have revealed that gain-of-function *aux/iaa* mutants have altered auxin sensitivity and pleiotropic defects in growth and development, including the reduction or increase in lateral root formation [43-47]. In *OsWRKY31* transgenic plants, we noticed that lateral root formation and elongation were reduced (Figure 7). In addition, exogenous treatments with different types of auxins or auxin transport inhibitors indicated that the overexpressing plants are also less sensitive to

high concentrations of auxin (Figure 7), results that are similar to those on the reported auxin-resistant mutants such as *aux1*, *axr1*, *axr2*, *axr4*, *axr5*, *axr6* and *tir1* [13-15, 48]. In *Arabidopsis*, production of auxin-insensitive plants has been obtained by introducing gain-of-function *aux/iaa* mutants, which show typical auxin-insensitive phenotypes, such as fewer lateral roots, shorter hypocotyls and suppressed root inhibition with exogenous auxin at high concentrations [49, 50]. We also found that the induction of *OsWRKY31* was even faster than the expression of auxin-inducible *OsIAA4* and *OsCr11* genes following treatment of rice seedlings with IBA (Figure 8B). The early auxin response of *OsWRKY31* expression suggests that *OsWRKY31* is a member of the early auxin response family. However, constitutive expression of auxin-inducible *OsIAA4* and *OsCr11* genes in *OsWRKY31* overexpressor lines did not exhibit corresponding phenotypes, such as more or longer lateral roots (Figure 8). An explanation for this dissimilarity may be that the constitutive expression in some of the rice *Aux/IAA* genes, such as *OsIAA4*, might lead to the stabilization of these proteins at some specific stage or condition of development. In support of this idea, some *SLR1/IAA14* overexpressing *Arabidopsis* plants exhibit an *slr* (*solitary-root-1*-like phenotype, which was interpreted by Fukaki *et al.* [47] to be the accumulation of IAA14 proteins in these 35S::*IAA14* plants at the CaMV35S promoter, which is constitutively active in most plant tissues. Furthermore, even though rice *Cr11* is a positive regulator of crown and lateral root formation, its expression has been found to be insufficient to initiate crown or lateral root development [16]. Nevertheless, further studies on the stability of rice IAA proteins in ectopic *OsWRKY31*-expressing plants are necessary to clarify this issue.

Auxin is known to negatively regulate several defense genes [51, 52], but some researches have demonstrated that *in vitro* IAA has some antimicrobial activity at relatively high concentrations [53, 54]. For example, potato meristems and young leaves are more resistant than old leaves to the late blight agent *Phytophthora infestans*, which is probably due to a relatively high level of auxins in these tissues that provide a natural defense against pathogenic spread and disease development [53]. Furthermore, IAA or other indole-related compounds can have a protective role against *M. grisea* infection in barley leaves [54]. In our study, overexpressing *OsWRKY31* led to enhanced disease-resistance and auxin-related phenotypes in rice. Taking into account the known function of the SGT1b factor, this result indicates the existence of common components in certain aspects of the auxin signaling and defense response pathways [6-9]. Furthermore, we propose that OsWRKY31 might be such

a component in this crosstalk between auxin and the defense signal transductions.

Functional redundancy is a common occurrence with structurally related transcription factors. For example, in *Arabidopsis* the structurally related WRKY18, WRKY40, WRKY60 and WRKY6 have been shown to have functional redundancy [28, 33]. In this study, the RNAi lines of *OsWRKY31* did not show opposite phenotypes to the overexpressing lines, implying the functional redundancy of this gene with its homologous member. Nevertheless, the identification of *OsWRKY31* might help to elucidate the interlaced signaling pathways of the auxin response and disease resistance in rice.

Materials and Methods

Isolation of *OsWRKY31* and bioinformatics analysis

The primers designed for *OsWRKY31* amplification were based on public databases of the rice genome and expressed sequence tags. *OsWRKY31* was amplified from a rice (cultivar IR 72) cDNA library induced by elicitors from *M. grisea* using LA Taq DNA polymerase (Takara, Dalian) with the primers 31BIF 5'-ttggatcct ttc tct ctg gaa aca atg tc-3' (*Bam*HI site underlined) and 31R 5'-ttc att tac aaa aaa tgg aag aat ctg ata gaa cc-3'. The PCR product was cloned into a pUCm-T vector and sequenced from both ends.

A BLAST (Basic Local Alignment Search Tool) search of the National Center for Biotechnology Information (NCBI) database was used to carry out bioinformatic analysis. DNAMAN software (<http://www.lynonn.com/download/index/html/>) was applied to establish the primary structure, including nucleotide and amino-acid sequences. The ClustalW program with BOXSHADE (<http://bio-web.pasteur.fr>) was then used to align the protein sequences of the WRKY proteins.

Subcellular localization of *OsWRKY31*

A cDNA clone of *OsWRKY31* was obtained through amplification with the primers of 31BIF and 31XhR 5'-tta ctcgag aaa atg gaa gaa tct gat aga acc-3' (*Xho*I site underlined). The PCR products were digested with the restriction enzymes *Bam*HI and *Xho*I, and inserted in frame with *gfp* at its 5' end in pCaMV35S::eGFP ('e' stands for 'enhanced') linearized with *Bam*HI and *Sall*. The constructed vector pCaMV35S::*OsWRKY31*-eGFP was confirmed by DNA sequencing.

The inner epidermis cells of onion (*Allium cepa*) were prepared for bombardment with pCaMV35S::*OsWRKY31*-eGFP or the pCaMV35S::eGFP plasmid on a PDS-1000/He system (Bio-Rad, USA) according to the manufacturer's instructions. After bombardment, the epidermises were cultured in the dark for 18 h at 28 °C. GFP fluorescence was examined using confocal fluorescence microscopy (Olympus, Japan).

A yeast one-hybrid assay

In the transcription activation assay *OsWRKY31* and a series of its deletion mutants were amplified by PCR and the products were inserted into the GAL4 DNA-binding domain of the pGBKT7 vector. The plasmids obtained were transformed into AH109 yeast cells according to protocols provided by Clontech (USA). The

transformed yeast cells were streaked on selective SD-Trp and SD-Trp-Ade-His medium plates to observe yeast growth at 30 °C for 3-4 days. An assay of β -galactosidase activity was performed using *O*-nitrophenyl β -D-galactopyranoside as a substrate.

The pBD-W31 plasmid for the *OsWRKY31* encoding region was constructed from a PCR product amplified with primers of 31EcoF and 31XhR (Table 1). The plasmids of pBD-C1, pBD-C2 and pBD-C3 contained fragments of C1 (aa 1-150, indicating the region of amino acids), C2 (aa 1-100) and C3 (aa 1-70), amplified using the primers C1R, C2R and C3R, respectively, paired with 31EcoF. Similarly, the plasmids of pBD-N1, pBD-N2 and pBD-dC1 harbored fragments of N1 (aa 94-211), N2 (aa 37-211) and dC1 (aa 151-211), obtained using the primers N1F, N2F and dC1F, respectively, paired with 31XhR. Fragments of N3 (aa 8-150), N4 (aa 16-150) and N5 (aa 28-150) were amplified using the primer C1R paired with the primers N3F, N4F and N5F, respectively, and were used to construct pBD-N3, pBD-N4 and pBD-N5 plasmids. The primers N1F and C1R were applied to amplify the truncated NIC1 (aa 94-150) for pBD-NIC1. The plasmid pBD-WD, which does not contain the WRKY domain, was constructed using the product of the aa 1-37 fragment from primer pairs of 31EcoF and WDBamR, fused with the aa 94-211 fragment amplified by the primers of WDBamF and 31XhoR. Finally, the plasmid pBD-WC1 was obtained using an amplification product of 31EcoF and 31C1 from the pBD-WD plasmid.

Plant materials and treatments

Rice plants (*Oryza sativa* L. ssp. *japonic* cv. Zhonghua 17) were grown in a greenhouse at 28 °C. To study abiotic stresses, 25-day-old rice plants were sprayed with 100 μ M MeJA or nipped with a hemostat to cause wounding. To study pathogenic infection, 25-day-old rice seedlings were inoculated with conidiophores of the pathogen *M. grisea* (incompatible strain P131 and compatible strain MS220) at 10⁵ spores/ml containing 0.01% (v/v) Tween-20. *M. grisea* cultures and conidiophore preparation were carried out according to the procedures described previously in Peng and Shishiyama [55]. The inoculated plants were kept in the dark for 24 h at 25 °C with 100% relative humidity and then moved to a normal culture environment. To serve as a control, seedlings

Table 1 Primers for *OsWRKY31* cDNA amplification

Primer	Sequence (restriction enzyme site)	
31EcoF	5'-ccg aat tca tgt ctc ctg tgc cga gt-3'	<i>Eco</i> RI
N1F	5'-cag aat tca ata gtg ctt ttc ttc ctc tt-3'	<i>Eco</i> RI
N2F	5'-cag aat tcc agt gga gga agt acg gcg ag-3'	<i>Eco</i> RI
N3F	5'-ccg aat tcc atc aat cac acc atc tag gcc at-3'	<i>Eco</i> RI
N4F	5'-ccg aat tcc gct caa gga aag aga agc gca t-3'	<i>Eco</i> RI
N5F	5'-ccg aat tca cct ttg cgc cgc aca a-3'	<i>Eco</i> RI
dC1F	5'-ccg aat tcc ctc ttc cac ggc att-3'	<i>Eco</i> RI
WDBamF	5'-agg gat cca ata gtg ctt ttc ttc ctc tt-3'	<i>Bam</i> HI
WDBamR	5'-ttg gat ccc tgg tgg ccg tgc ttg t-3'	<i>Bam</i> HI
C1R	5'-ctg gtc gac ctg att ctt gtt ctc tga agg t-3'	<i>Sall</i>
C2R	5'-cta gtc gac aag agg aag aaa agc act-3'	<i>Sall</i>
C3R	5'-cta gtc gac ctt cgt tgc tgg gca att cat-3'	<i>Sall</i>

were treated with 0.01% Tween-20 and the leaves were sampled at designated time points before storage at -80°C for RNA isolation.

To evaluate the capacity of disease resistance, 3-week-old transgenic and control plants were inoculated with a strong virulent strain of *M. grisea* P140. Disease incidence was investigated 7 days post-inoculation. The lengths and numbers of lesions were averaged from at least five leaves from each line. For northern blot analysis, RNA was isolated from leaves 18 h post-inoculation.

Analysis of RNA

Total RNA was extracted using methods described in Chomczynski *et al.* [56]. RNA (20 μg for each sample) was separated in 1.2% agarose gels containing formaldehyde, and was transferred onto a Hybond N⁺ nylon membrane and cross-linked using UV irradiation. The membrane was hybridized with a fragment of *OsWRKY31* or *OsSci2* (a *PR* gene, GenBank acc. no. AY878694) using methods described in Guo *et al.* [30]. The hybridization signals were scanned using a phosphor screen (Amersham Pharmacia, UK).

For reverse transcriptase PCR (RT-PCR), total RNA was isolated from rice leaves cultured in a greenhouse for 25 days. Total RNA was treated with DNase I (RNase-free) and a ribonuclease inhibitor to remove any possible DNA contamination. The first-strand cDNA was synthesized using AMV transcriptase (TaKaRa, Dalian) in a 25- μl reaction containing 2.5 μM oligo (dT) primers and 2.5 μg RNA according to the manufacturer's instructions. The synthesized cDNAs were used as a template for PCR amplification in a 25- μl reaction mixture containing 1.5 μM MgCl_2 , 200 μM dNTP, 5% dimethyl sulfoxide, 2.5 U of Taq DNA polymerase and 0.4 μM gene-specific primers. The primers used were 5'-gga act ggt atg gtc aag gc-3' and 5'-agt ctc atg gat acc cgc ag-3' for *Actin* (AB047313) as an internal standard, 5'-agt gct ttt ctt cct ctt agc c-3' and 5'-aat gca tcc tga cat tcg gtg-3' for *OsWRKY31*, 5'-acg cgt cca ctt tgc cca ag-3' and 5'-ttc tcc ggc gac agt gag ct-3' for *PBZ1* (D38170), 5'-gag gaa ttc tgt gac cag aat gag-3' and 5'-gag act cga gag cta gac aca gc-3' for *OsSci2*, 5'-ggc att ccc ggt gcc cat ga-3' and 5'-gtc cat cgc cta tgg tgc gac-3' for *OsIAA4* [16], and 5'-agc aac gtg tcc aag ctg ct-3' and 5'-tgt agc cgc cgt acc cta at-3' for *OsCr11* (AB200234) [16].

Generation of *OsWRKY31* transgenic rice plants

For overexpression, the fragment covering the encoding region of *OsWRKY31* was cut from its ligated pUCm-T vector and inserted into the pCoU vector under the control of a maize *ubiquitin* promoter to generate pCo-Ubi::OsWRKY31. To suppress *OsWRKY31* transcription, we constructed a hairpin structure as described by Wesley *et al.* [57]. A 260-bp (283-543 bp) *OsWRKY31* fragment was used to generate the hairpin vector pCo-Ubi::OsWRKY31d after several digestions and ligations. The plasmids were confirmed through DNA sequencing and were transformed into rice calli (Zhonghua 17) using *Agrobacterium*-mediated transformation methods [58]. Rice calli were transformed with an empty vector as a control.

Morphological analysis and hormone treatment of transgenic plants

Seeds of plants overexpressing *OsWRKY31*, as well as RNAi and control plants from the T₂ generation, were grown on 1/2 MS (0.6% agar, containing 30 mg/l hygromycin) at 26 $^{\circ}\text{C}$ in a plant

growth chamber. Morphological data, including plant height, seminal root length, adventitious root length, lateral root length, the number of adventitious roots and the number of lateral roots, were measured using 7-day-old rice seedlings [18]. Statistics from at least 15 plants from each transgenic line were taken.

To investigate the effects of exogenous auxin on root elongation and development, control and transgenic seeds were sown in black cups with an agar medium for 4 days and then transferred to media containing different concentrations of IBA, 2,4-D and NAA. The hormone solutions were changed every day and the lengths of the 20 longest lateral roots were measured after 6 days of treatment. To inhibit auxin transport, seeds were sown on 1/2 MS with or without 1 μM 2,3,5-triiodobenzoic acid (TIBA). The lengths of the lateral roots were measured 6 days after the treatment. For mRNA analyses, 4-day-old seedlings were treated with IBA (1 μM), 1-NAA (1 μM), or 2-NAA (1 μM), and the roots were sampled at designated time points.

Acknowledgments

This research was supported by the National Basic Research Program of China (2006CB101905) and the National Nature Science Foundation of China (30370139 and 30471122).

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