OsWRKY03, a rice transcriptional activator that functions in defense signaling pathway upstream of OsNPR1

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ABSTRACT

WRKY family proteins are a class of plant specific transcription factors that involve in many stress response pathways. It has been shown that one Arabidopsis WRKY protein, AtWRKY29/22, is activated by MAP kinase signaling cascade and confers resistance to both bacterial and fungal pathogens. However, little is known about the biological roles of WRKY proteins in rice. In this study, we investigated the expression patterns of rice *AtWRKY29/22* homolog, *OsWRKY03*, under different conditions, and also its possible role involved in plant defense. Our results showed that *OsWRKY03* was up-regulated by several defense signaling molecules or different treatments. Further analysis revealed that the expression of *OsWRKY03* was light dependent. Transcriptional activation activity of *OsWRKY03* was also demonstrated by yeast functional assay. Transient expression of *OsWRKY03*-GFP fusion protein in onion epidermis cells showed that *OsWRKY03* was a nuclear localized protein. *OsNPR1* as well as several other pathogenesis-related genes, such as *OsPR1b*, phenylalanine ammonia-lyase (*ZB8*) and peroxidase (*POX22.3*), were induced in *OsWRKY03*-overexpressing transgenic plants. These results indicated that *OsWRKY03* is located upstream of *OsNPR1* as a transcriptional activator in salicylic acid (SA)-dependent or jasmonic acid (JA)-dependent defense signaling cascades.

Keywords: defense response, *OsWRKY03*, transcription factor, rice.

INTRODUCTION

Plants have developed a number of inducible defense systems against pathogen attacks. It was shown that plants are capable of differentially activating distinct defense pathways depending on the type of pathogen encountered. Plant signaling molecules, such as salicylic acid (SA), jasmonic acid (JA) and ethylene, play important roles in defense signaling networks [1-3].

Systemic acquired resistance (SAR) is a classic inducible defense response activated by a necrotizing pathogen [4]. Typical SAR results in an increment of endogenous SA and transcriptional activation of pathogenesis-related (*PR*) genes (e.g., *PR1*, *PR2*, *PR5*) [5]. It finally leads to an enhanced resistance to a broad spectrum of pathogens in distant and uninfected plant parts [6-9]. Inhibition of SA accumulation can effectively block the activation of SA-dependent defense response [10]. Exogenous application of SA or its synthetic functional analog BTH can activate *PR*-gene expression and enhance host resistance to pathogens [11]. It has been shown that SA is an essential signal for SAR across a wide range of plant species although the mechanism of SA induced SAR might differ from one to another [12-14].

In addition to SA, other signaling molecules, such as JA and ethylene, are also able to regulate various plant defense responses. JA- and ethylene-mediated signaling pathways are distinct from the classic SA-mediated SAR pathways [15]. For example, induced systemic resistance (ISR),

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Abbreviations: ACC (1-aminocyclo-propane-1-carboxylic acid); BTH (benzo-1,2,3- thiadiazole-7-carbothioic acid S-methyl ester); CHS (chalcone synthase); GP (glutathione peroxidase); GST (glutathione S-transferase); ISR (induced systemic resistance); MeJA (methyl jasmonate); ORF (open reading frame); PAL (phenylalanine ammonia lyase); PR (pathogenesis-related); SA (salicylic acid); JA (jasmonic acid); SAR (systemic acquired resistance); X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

which is triggered by nonpathogenic root-colonizing bacteria, is JA and ethylene dependent [16, 17] and is independent of SA and *PR*-gene activation [18, 19]. The JA response mutant *jar1* and the ethylene response mutant *etr1* lose ISR upon treatment with *Pseudomonas fluorescens* WCS417r, but remain normal level of pathogen-induced SAR [20, 21], indicating that JA and ethylene responses are critical for the ISR-signaling pathway [22, 23]. Analysis of signaling molecules involved in rhizobacteria-mediated ISR suggests that ISR might be associated with another pathogen-induced jasmonate and ethylene defense response, in which a subset of *PR* genes, such as *PR3*, *PR4*, and *PR12*, were co-regulated by jasmonate and ethylene [24, 25]. However, there is no increment in transcript abundance of jasmonate-inducible gene *AtVSP*, ethylene-inducible gene *HEL*, and jasmonate- and ethylene-inducible plant defense gene *PDF1.2* in rhizobacteria-mediated ISR in Arabidopsis [22]. Thus, rhizobacteria-mediated ISR signaling pathway does not coincide with pathogen-induced jasmonate and ethylene response [22].

More than a decade ago, additional components downstream of signaling molecules SA, JA and ethylene were characterized in several mutant plants, such as the jasmonate response mutant *jar* [26], the ethylene response mutant *etr* [27] and the SAR regulatory mutant *npr1* [28]. In a genetic screening for plants failed to express *PR* genes after SAR induction, *npr1* mutant was isolated in Arabidopsis [28] and *NPR1* gene was cloned [29]. Further results revealed that *NPR1* was a key regulator of SA-mediated SAR in Arabidopsis [30–32]. Arabidopsis *NPR1/NIM1* gene encodes a novel protein with ankyrin repeats [29], which is essential for *NPR1* binding to the TGA basic leucine zipper transcription factors [33]. The interaction between *NPR1* and TGA factors facilitates the binding of TGA proteins to the SA-responsive *as-1* or *as-1*-like promoter elements, and leads to the activation of downstream genes during defense response [34, 35].

The activation of *NPR1* is not only required for the expression of *PR* genes in SA-dependent SAR, but also for the jasmonate- and ethylene-dependent rhizobacteria-mediated ISR [22, 36]. SAR and ISR can be activated simultaneously, leads to synergetic additive protection against plant pathogens, such as *P. syringae* [37]. However, *NPR1* is not required for the expression of the *PDF1.2* during JA/ethylene-dependent defense responses induced by some necrotrophic pathogens [24, 25]. It has been shown that the SA-inducible *NPR1*-independent pathway also exist [38, 39].

Furthermore, *NPR1* mediates cross-talk between the SA, JA and ethylene signaling pathways, generating resistance to insects and some necrotrophic pathogens [40]. Therefore, *NPR1* is a key regulatory component which

locates at the crossroads of multiple defense pathways. The functions of *NPR1* were mainly characterized in Arabidopsis, rice *NPR1* was identified just recently [42]. It has been shown that overexpression of *OsNPR1* gene in rice leads to constitutive activation of defense response and hypersensitivity to light [42]. Transgenic rice overexpressing *OsNPR1* gene displayed enhanced resistance to rice bacterial blight pathogen *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*) (Jia Xun FENG, personal communication, unpublished data). These results suggested that rice *NPR1* gene might have similar function as their Arabidopsis homolog and a disease-resistance pathway similar to Arabidopsis *NPR1*-mediated signal transduction pathway also exists in rice [41].

Except for the defense inducible gene products immediately downstream of pathogen perception, such as protein kinases, phosphatases and G-proteins, other signal molecules, such as ROS, lipid peroxides, SA, JA, and ethylene, were generated during defense response [43]. Once the earliest defense response starts, enormous biochemical pathways within the responding cells are activated, followed by the induction of a number of defense-related genes, such as *PRs*, phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), glucanase, chitinase and also those protectant genes, such as glutathione S-transferase (*GST*), glutathione peroxidase (*GP*), peroxidase [4], to activate cell protection mechanisms.

As mentioned above, transcriptional activation of defense-related genes plays a central role in plant defense. Such process requires the expression of related transcription factors. Recently, the plant-specific transcription factors, WRKY family proteins containing highly conserved WRKY domain [44], have been found to be involved in the regulation of plant defense response. It has been shown from a number of plants that *WRKY* genes are rapidly induced by pathogens, pathogen elicitors, or SA treatment [45–47]. Arabidopsis WRKY transcriptional factor (*AtWRKY52*), containing a LRR motif of TIR-NBS-LRR subclass *R* gene products, has been shown to enhance disease resistance to several strains of *Ralstonia solanacearum* [48]. It was also demonstrated that some *WRKY* genes are necessary for inducible expression of defense-related genes in SAR, such as *PR1* in Arabidopsis and *PR10* in parsley [49, 50]. In Arabidopsis, WRKY29 has been identified as an important downstream component of a MAPK pathway that confers resistance to both bacterial and fungal pathogens [51]. Recently, Arabidopsis WRKY70 was also identified as a common regulatory component of SA- and JA- dependent defense response, mediating cross-talk between antagonistic pathways downstream of *NPR1* [54]. It has been shown that pathogen- and SA-induced WRKY proteins also regulate genes en-

coding proteins with regulatory functions (e.g. receptor protein kinases and NPR1) [52, 53]. Besides participating plant defense response, WRKY transcription factors also involve in the regulation of several other developmental and physiological processes such as trichome initiation [55], senescence [50], carbohydrate anabolism and secondary metabolism [56, 57].

Although several *OsWRKY* genes have been isolated so far, little is known about their functions in defense signaling pathways in rice. In this work, we demonstrated that one of the *WRKY* genes, *OsWRKY03*, functions in JA- or SA-defense signaling pathways by regulating *NPR1* and defense-related genes.

MATERIALS AND METHODS

Analysis of Arabidopsis and rice *WRKY* genes

The predicted WRKY family protein sequences of *Oryza sativa* rice (*japonica*) and *Arabidopsis thaliana* were downloaded from TIGR rice databases (<http://www.tigr.org/>) and TAIR (The Arabidopsis Information Resource) *A. thaliana* genome annotation database release 5 (<http://www.arabidopsis.org/>) respectively. WRKY proteins sequences of other species were extracted from NCBI. The phylogenetic tree of WRKY proteins was constructed using MEGA3 [58] with pairwise distance computed and the implementation of neighbour-joining algorithm.

Plant materials and treatments

Rice (*O. sativa* L. ssp. *Indica* cv. Guangluai) seedlings were grown in greenhouse at 28°C for 6 weeks until it is ready for different treatments. For hormone treatments, 6-week-old seedlings were sprayed with 2 mM SA (Sigma), 2 mM BTH (Sigma), 2 mM ACC (Sigma), and 100 μM MeJA (Sigma), respectively until liquid dripped off the leaves. Mock plants were treated in the same way with 0.1% (v/v) ethanol solution in which these compounds were dissolved. Infections with bacterial blight pathogen (*Xoo*) were performed by the scissors-dip method [59]. The wound treatment was carried out by cutting the rice leaves with clean scissors. The treated leaves were harvested at 0, 0.5, 2, 6, 12 and 48 h post-treatment, frozen in liquid nitrogen immediately, and stored at -80°C for RNA extraction.

Cloning of *OsWRKY03* and other defense marker genes

Clones of the rice *NPR1* homologue (*OsNPR1*, acc No. AY923983), *NB8* (acc No. X87946), *OsPR1b* (acc No. U89895) and *POX22.3* (acc No. AF014467) cDNAs were kindly provided by Prof. Jia Xun FENG (Guangxi University, Guangxi, China). *OsWRKY03* full-length ORF was amplified from total RNA extracted from SA-treated rice leaves by RT-PCR using a pair of special primers: 5'-ATG GAG CAC TTC AAC GAC TGG GAC CTG-3' and 5'-CTA TAG CGA GAG CCG TCG CCC CAG CAC-3'. The 1011 bp ORF of *OsWRKY03* was cloned into pENTRY TOPO vector (pENTRY Directional TOPO Cloning Kit, Invitrogen) according to the manufacturer's instruction, and was confirmed by following sequencing and the GenBank accession number of *OsWRKY03* is AY676924.

RNA isolation and Northern blot analysis

Total RNAs were isolated as described by Chomazynski and

Sacchi [60]. For Northern blot analysis, 20 μg of total RNAs were denatured and separated on a 1.3% agarose-formaldehyde gel. Ethidium bromide was included in the loading buffer to confirm equal sample loading. After blotting to positively-charged nylon membranes (Amersham Pharmacia Biotech, UK), RNAs were crosslinked by UV irradiation (GS GENE Linker UV Chamber, BIO-RAD). Prehybridization and hybridization buffer contains 0.33 mol/L sodium phosphate buffer (pH 8.3), 7% Na-dodecyl sulfate (SDS) and 20 mmol/L EDTA (pH 8.0). α -³²P-dCTP-labelled cDNA probes were used and hybridized at 65°C for 16-20 h. After hybridization, the membranes were washed at room temperature in 2×SSC, 0.1% SDS for 10 min followed by 15 min washing with 2×SSC, 0.1% SDS at 65°C twice. Then, the filters were exposed to Storage Phosphor Screen (Amersham Biosciences, USA) and scanned with Phosphorimager (Molecular Dynamics, USA). For rehybridization, the membranes were stripped off by agitation in boiling 0.1% SDS and 2 mM EDTA (pH8.0) until the solution was cooled down to room temperature. The membranes were then used for rehybridization as described above. About 300 bp long fragment of 3'-specific region of *OsWRKY03* ORF digested by *Pst*I was used as specific probe.

Subcellular localization of *OsWRKY03*

The full-length ORF of *OsWRKY03* without the termination codon was amplified by PCR with primers containing *Spe*I site (underlined): 5'-GAACTAGT ATG GAG CAC TTC AAC GAC TGG GA-3', 5'-CAACTAGT TAG CGA GAG CCG TCG CCC CA-3'. After verified by sequencing, the fragment was introduced into the *Spe*I site of the pCambia1302 vector, creating an in-frame fusion between the *OsWRKY03* cDNA and green fluorescent protein (*GFP*) gene. The fusion construct (35S:*OsWRKY03*-GFP) and control (35:*GFP*) were transformed into onion epidermis cells by particle bombardment using PDS-1000/He (BIO-RAD) [61]. After incubation of transformed onion epidermis cells for 24 h at 28°C, GFP was detected by a confocal fluorescence microscopy (Olympus FV5000).

Yeast transcriptional activation assay

The full-length ORF of *OsWRKY03* was amplified by PCR with a pair of primers containing *Eco*RI site (underlined): 5'-TCGAATTC ATG GAG CAC TTC AAC GAC TG-3', 5'-TCGAATTC CTA TAG CGA GAG CCG TCG CC-3' After cloned into T-vector and sequenced, the fragment was introduced into the *Eco*RI site of pGAL4-BD vector (Stratagene) and was in-frame fused with the GAL4 DNA binding-domain to generate the resultant plasmid pGAL4-BD-03. According to the manufacturer's protocol (Stratagene), the plasmids pGAL4-W (positive control: wild-type full-length pGAL4), pGAL4-BD (negative control) and pGAL4-BD-03 were transformed into yeast YRG-2 cells. The transformed yeast cells were then analyzed on selective medium plates without His (SD-His) to test the expression of the reporter gene *HIS3*. The expression of another reporter gene *lacZ* was analyzed by detection of β-galactosidase activity with a solution containing a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) substrate on filter paper. All the tests were performed according to the manufacturer's instruction (Stratagene).

Generation of *OsWRKY03*-overexpressing transgenic rice

The full-length ORF of *OsWRKY03* was introduced into the binary vector pGW001 (provided by Prof. Kang CHONG, the Institute of Botany, CAS) under the control of the maize ubiquitin pro-

motor by LR reaction (Gateway LR Clonase plus Enzyme Mix, Invitrogen) generating the resultant plasmid pG03. The pG03 were introduced into *Agrobacterium tumefaciens* strain AGL1. For rice transformation, mature seed-derived rice callus (*O. sativa* L. cv. Nipponbare) was used and the *Agrobacterium*-mediated gene-transfer was described by Hiei *et al* [62] and the calli were selected under 50 mg/L of hygromycin. After differentiation, twenty independent lines were obtained and confirmed by PCR and histochemical GUS assay.

RESULTS

Isolation of *OsWRKY03* cDNA

Hundreds of *WRKY* genes from numerous plant spe-

Fig. 1 The phylogenetic tree of WRKY family proteins (A) and partial subtree of the subgroup IIe (B). The phylogenetic tree was constructed using 185 WRKY proteins from rice, Arabidopsis, parsley and tobacco (A). The locations of *OsWRKY03* and its Arabidopsis homologs (*AtWRKY29* and *AtWRKY22*) on the phylogenetic tree were marked with blank square and black dots, respectively (A).

Fig. 2 Alignment of *OsWRKY03* with related WRKY proteins from Arabidopsis. The deduced amino acid sequence of *OsWRKY03* was aligned with *AtWRKY22* (NM_116355) and *AtWRKY29* (NM_118486) using Vector NTI with default parameters. Black and gray shadings indicate conserved amino acid residues. Predicted domains are indicated above the sequences. The acidic residues "E" and "D" and putative NLS were underlined.

cies are available in public databases when both of the Arabidopsis and rice genome sequencing projects were completed [63, 64]. To better understand *OsWRKY* gene family, a phylogenetic tree of 185 WRKY proteins, including 72 from Arabidopsis, 105 from rice and 7 from other species was constructed (Fig. 1A). Based on the predicted Open Reading Frames (ORFs), ten *OsWRKY* genes were isolated by RT-PCR from the SA-treated rice seedlings. One of them, *OsWRKY03*, is the closest homolog of *AtWRKY29* in the phylogenetic analysis (Fig. 1B). Since *AtWRKY29* was identified as an important downstream component of MAPK pathway involved in resistance to both bacterial and fungal pathogens [51], we wanted to investigate whether *OsWRKY03* also plays important roles in rice defense response. The *OsWRKY03* was predicted to encode a 336-amino acid protein that contains a single WRKY domain and a zinc finger-like motif (C_2H_2) at its C-terminus. *OsWRKY03* can be categorized into group IIe of the WRKY superfamily [44] (Fig. 2). *OsWRKY03* has a putative basic nuclear localization signals at its NH₂ terminus, and an acidic amino acid-rich region at its C terminus, which may have transcriptional activation activity (Fig. 2). Alignment of the protein sequences of *OsWRKY03*, *AtWRKY29* and *AtWRKY22* revealed that the WRKY domains were highly conserved, whereas the rest amino acid sequences are rather diverse (Fig. 2).

Expression patterns of *OsWRKY03* under different treatments

Plant defense response is regulated by multiple signal

Fig. 3 Expression profiles of *OsWRKY03* transcripts in rice. Expression patterns of *OsWRKY03* in rice seedlings treated with 2 mM SA, 2 mM BTH, 100 μ M MeJA, 2 mM ACC, rice bacterial blight pathogen (*Xoo*), wounding respectively, and mock control was included (A). About 20 μ g of total RNA was loaded each lane, equal loading was monitored by ethidium bromide staining of the gels. The [α - 32 P] dCTP-labeled *OsWRKY03* 3'-specific cDNA was used for hybridization. The signals were then quantitated and the ratio of *OsWRKY03* band intensities to 28S rRNA band intensities represented the relative expression level of the *OsWRKY03* gene (B).

transduction pathways, in which SA, JA and ethylene function as key signaling molecules [65]. To study the potential functions of *OsWRKY03* in plant defense response, the expression profiles of rice *WRKY03* gene under wounding, rice bacterial blight pathogen (*X. oryzae* pv.

Fig. 4 The expression of *OsWRKY03* in dark and light. (A) Northern analysis of RNA from rice seedling treated with 50 μ M MeJA and non-treatment in a time-dependent manner, under both continuous light and continuous dark incubation. Equal loading was monitored by ethidium bromide staining of the gels. The [α - 32 P] dCTP-labeled *OsWRKY03* 3'-specific cDNA was used as probe. (B) The signals were quantitated and the ratio of *OsWRKY03* band intensities to 28S rRNA band intensities represented the relative expression level of the *OsWRKY03* gene.

Fig. 5 Nuclear localization of OsWRKY03 and the transactivation activity assay for OsWRKY03 in yeast. **(A)** The OsWRKY03 protein was localized in nucleus. The photographs were taken in the dark field for green fluorescence (a and d), under bright light for the morphology of the cell (b and e) and in combination (c and f), respectively for *OsWRKY03-GFP* plasmid (a, b, and c) and *35S::GFP* control plasmid (d, e, and f). **(B)** The OsWRKY03 protein has transactivation activity in yeast. The yeast carrying *pGAL4-BD-OsWRKY03* and positive control can grow on the medium without histidine (SD-His) and YAPD, and turn blue on the filter paper with a solution containing an X-gal substrate, while negative control can neither grow on SD-His medium nor turn blue on the filter assay.

Oryzae, *Xoo*) infection and treatments with different exogenous signal molecules, such as SA, BTH, ACC and MeJA, were investigated. A basal expression of *OsWRKY03* was detected in mock-treated plants and is up-regulated in all tested treatments within the first 6 h after treatment (Fig. 3). However, at 12 h (even with mock control) the expression level of *WRKY03* was significantly decreased, and re-increased at 48 h (Fig. 3). Since the 12 h sample was collected from the plants grown in dark, this result suggested that the expression of *WRKY03* might be light dependent. To verify this hypothesis, we monitored the abundance of *OsWRKY03* transcripts under both light and dark conditions. After cultivated at 25°C and an 18-h light period per day for two weeks, MeJA was added at 50 μM final concentration and the plants in light period were transferred to continuous dark or continuous light. Northern blot analysis showed that *OsWRKY03* expression was up-regulated by MeJA only in light, whereas the transcript was reduced in dark even under MeJA treatment (Fig. 4).

Furthermore, plants growing in dark period were transferred to continuous dark and continuous light incubation without any further treatment. The expression of *OsWRKY03* in these plants maintained low level when it was in the dark, but was highly induced when plants were transferred to light (Fig. 4). These results suggested that *OsWRKY03* was involved in plant defense response pathways, in which plant hormones SA, JA and/or ethylene were also participated. We have also demonstrated that the expression of *OsWRKY03* was light-dependent.

OsWRKY03 is nuclear-localized and has transcriptional activation activity in yeast

To determine the subcellular localization of OsWRKY03, the ORF of *OsWRKY03* was fused to the *GFP* in pCAMBIA1302 vector. The construct was introduced into onion epidermal cells by particle bombardment. After incubation for 24 h, GFP fluorescence was detected exclusively in the nucleus, suggesting that OsWRKY03 is a

nuclear localized protein (Fig. 5A, a-c). In contrast, onion cells transformed with 35S:GFP vector (control) showed fluorescence throughout the cells (Fig. 5A, e-f).

To determine whether OsWRKY03 protein has transcriptional activation activity, a fusion protein containing a GAL4 DNA binding domain (GAL4-BD) and the whole ORF of *OSWRKY03* was constructed (pGAL4-BD-OsWRKY03). The fusion plasmid pGAL4-BD-OsWRKY03, pGAL4-W (positive control) and pGAL4-BD (negative control) were all transformed into yeast strain YRG-2 containing upstream activating sequence (UAS), which could be specifically bound by GAL4 binding domain. All transformants containing pGAL4-W, pGAL4-OsWRKY03 and pGAL4-BD grew well on rich medium YAPD (Fig. 5B). The results revealed that yeast transformed by pGAL4-BD-OsWRKY03 can grow on selective medium without histidine (SD-His) and also β -galactosidase activity can be detected, indicating that the expression of reporter genes (*lacZ* and *HIS3*) was activated, whereas neither *lacZ* nor *HIS3* reporter genes could be activated in yeast transformants by negative control (pGAL4-BD) (Fig. 5B). These results indicated that pGAL4-WRKY03 indeed could transactivate the expression of both reporter genes *lacZ* and *HIS3* in the same way as the positive control in yeast (Fig. 5B), suggested that OsWRKY03 was a transcriptional activator.

Fig. 6 Phenotypes of *OsWRKY03*-overexpressing transgenic rice. The phenotype of the transgenic rice and the control line three months after regeneration. S3 and S14 represent the transgenic lines and CK stands for the plants transformed with empty vector.

Fig. 7 Expression of *OsWRKY03* and some marker genes in defense pathways in *OsWRKY03*-overexpressing transgenic rice. Northern blot was hybridized to the [α - 32 P] dCTP-labeled *OsWRKY03* 3'-specific cDNA probe, three different bands were detected (band a, b and c). According to RNA marker labeled left, band b (2 kb > band b > 1 kb) is *OsWRKY03* transcript only detected in *WRKY03*-overexpressing lines. Band a (3 kb > band a > 2 kb) and band c (1 kb > band c > 0.5 kb) is likely to be produced by cross hybridization. And the same filter was stripped and rehybridized subsequently with *OsNPR1*, *ZB8*, and *POX22.3* cDNA probes. Equal loading was monitored by ethidium bromide staining of the gels. S3 and S14 are two independent transgenic lines and CK represents the plant transformed with empty vector.

Overexpression of *OsWRKY03* gene in transgenic rice

To elucidate OsWRKY03 function, transgenic rice overexpressing *OsWRKY03* gene was generated, and about 20 independent transgenic lines were obtained. The regenerated transgenic plants containing *OsWRKY03* construct showed overall shoot and root abnormality, such as severe dwarfism, dark green and curly leaves, and root initiation and elongation was greatly reduced (Fig. 6). The plants died eventually after transferring into soil.

To examine *OsWRKY03* expression level, Northern blot analysis was carried out with probes derived from *OsWRKY03* sequence. The results showed that *OsWRKY03* transcripts, with a size of about 1 kb, were substantially elevated in transgenic lines compared with the control (Fig. 7, band b). Besides the expected *OsWRKY03* transcript, two other bands were also detected in our Northern blot hybridization results. Band c had a size smaller than *OsWRKY03* and was detected in all samples including the

control lines transformed by empty vector. Band a migrated slower than *OsWRKY03* on the agarose gel and was only present in *OsWRKY03* transgenic lines (Fig. 7). We did not know yet what the band a and band c represent. Because band a could only be detected in transgenic plants, we supposed it probably is another induced WRKY family gene, which has high sequence similarity to *OsWRKY03*, and is *OsWRKY03*-dependent. Because band c could be detected in both the control and transgenic lines, it would be produced by an *OsWRKY03* homologues gene whose expression is independent of *OsWRKY03*. Further experimental verifications are needed to support this hypothesis. RT-PCR was used to further confirm that the band b detected in Northern blot hybridization was indeed the transcript of *OsWRKY03*, strong signals were achieved by *OsWRKY03* specific primers in transgenic plants whereas no detectable band was found in control samples (data not shown). These results suggested that the expression of *OsWRKY03* was really elevated in the *OsWRKY03* transgenic lines compared to the control.

Overexpression of *OsWRKY03* in rice leads to induced expression of defense-related genes

To determine whether OsWRKY03 functions in NPR1-mediated defense pathways in rice, we investigated the expression level of *OsNPR1* in *OsWRKY03* transgenic plants. The results demonstrated that *OsNPR1* expression was constitutively induced in transgenic plants but not in control plants (Fig. 7), suggesting that OsWRKY03 functions upstream of NPR1 in defense signal pathway.

The expression of several defense-related genes, such as *OsPR1b*, *NB8*, and *POX22.3* were also analyzed in *OsWRKY03*-overexpressing transgenic plants. Pathogenesis-related proteins (PR-proteins) were suggested to inhibit pathogen growth, multiplication and/or spread, and are responsible for the state of SAR in plants [66]. Among known PRs genes, the PR1 class is a dominant group induced by pathogens, and is commonly used as a marker for SAR. In 2000, rice PR1 genes including rice acidic PR1 (*OsPR1a*) and rice basic PR1 (*OsPR1b*) were firstly characterized by Ganesh Kumar *et al* [67, 68]. To determine whether OsWRKY03 is involved in the regulation of expression of *OsPR1b*, we studied the transcripts level of *OsPR1b* in *OsWRKY03*-overexpressing transgenic rice and an increased expression of *OsPR1b* gene was observed in transgenic plants comparing to control, indicating that *OsWRKY03* is located upstream of *OsPR1b*.

Phenylalanine ammonia-lyase (PAL) catalyzes the first reaction in the biosynthesis of a wide variety of phenylpropanoid natural products including lignin, flavonoid, pigments and phytoalexins, and is the entry point enzyme of the phenylpropanoid and flavonoid pathways. PAL has been

proven to be a key enzyme in pathogen defense and stress response [69, 70]. *ZB8* is a rice PAL gene and has been shown to be induced under wounding and fungal cell wall elicitor treatment [71]. Peroxidase genes are also a class of protective genes that are induced in plant defense response [5]. One rice peroxidase gene, *POX22.3* has been shown to be predominantly induced upon *X. oryzae pv. Oryzae* infection [72]. To determine whether defense-related genes, *ZB8* and *POX22.3* genes are regulated by OsWRKY03, we examined their expression levels in *OsWRKY03* over-expressing transgenic lines. The results showed that both *ZB8* and *POX22.3* genes were induced by OsWRKY03 (Fig. 7).

DISCUSSION

Transcriptional regulation of plant defensive genes plays a central role in the activation of plant inducible defense response. Therefore, an important step toward understanding the regulation of plant defense systems is to identify regulatory components and establish defense response pathways. In the past years, several families of sequence-specific DNA binding transcription factors involving in the transcriptional regulation of plant defense genes have been identified, including ethylene-responsive element binding factors, myb-like factors, bZIP factors and W-box binding WRKY proteins [72]. Since the first WRKY protein (SPF1) was identified in sweet potato [73], much progress has been made in studying the functions of WRKY family transcriptional factors. However, most researches were conducted in dicots plants, such as Arabidopsis, tomato, tobacco and potato. Although many *OsWRKY* genes have been isolated from different species and about one hundred WRKY family members have been computationally identified in rice, very limited information about the functions of rice WRKY genes was available. Considering the importance of rice as a model crop genera and a valuable crop species, elucidation of the functions of WRKY proteins in plant defense response signaling pathways has important significance both in basic research and agricultural application. In this paper we provided evidence that *OsWRKY03*, a rice WRKY gene, functions in JA- or SA-defense signaling pathways by regulating *NPR1* and other downstream defense-related genes.

Increased expression was observed for *OsWRKY03* upon treatments of different defense related molecules, suggesting that *OsWRKY03* plays a role in rice defense systems. Interestingly, the expression of *OsWRKY03* reduced significantly to almost undetectable level under all treatments at 12 h post-treatment and was recovered at post-treatment of 48 h (Fig. 3). In our experiment, all the treatments were undertaken under the natural daytime light condition. The 12 h post-treatment was exactly in the midnight,

whereas all other time points were during daytime. This result suggested that the expression of *OsWRKY03* has a circadian rhythm and is light dependent. This hypothesis has been proved by further experiment (Fig. 4). It has been reported that rice *PR1b* gene is highly induced by JA, protein phosphatase inhibitors catharidin (CN) and endothall (EN) [67] in a light-dependent manner. Furthermore, overexpression of *OsNPR1* gene leads the plants to be hypersensitive to light [42]. Because the expressions of *PR1b*, *OsNPR1* and their upstream regulator *OsWRKY03* are all correlated with light, we hypothesis that the light-dependent expression patterns of *OsPR1b* is likely to be caused by expression pattern of *OsWRKY03*.

Ectopic expression can provide much information for gene function [74]. Identification of downstream target genes of WRKY factors will be crucial for the understanding of their biological functions. Currently, our knowledge of *WRKY* genes relies mainly on the strong ectopic expression of *WRKY* genes in transgenic plants or protoplasts [46, 50, 51, 54]. In this work, we used *OsNPR1*, which is a central regulator of plant defense signaling pathways, and several defense-related genes, such as *OsPR1b*, *NB8* and *POX22.3*, as markers to monitor the functions of *OsWRKY03* in its overexpressing transgenic rice. Both of the two classes marker genes: *OsNPR1* and *OsPR1b*, *NB8* and *POX22.3* were induced in *OsWRKY03*-overexpressing rice, which suggests that *OsWRKY03* locates upstream of *NPR1* and *PR1* genes in JA- and/or SA-defense response pathway. But the details about functions of *OsWRKY03* in the regulation of disease defense signaling pathway are still unclear. For example, how does *OsWRKY03* regulate defense-related genes? Is *OsNPR1* a mediator? Which phytohormone, SA or JA or both play a role? What is the regulator upstream of *OsWRKY03* and the ones downstream of JA and SA? Is there a MAPK cascades upstream of *WRKY03* similar to *WRKY29* in *Arabidopsis* [51]? Discovery and analysis of mutants involved in the pathway would provide us more information.

It has been suggested that disease resistance is associated with fitness costs and that plants have evolved inducible defense mechanism because it is too costly to have defense responses switch on all the time [5, 75]. For instance, overexpression of *AtWRKY6*, *AtWRKY18*, *AtWRKY53* or *AtWRKY70* always resulted in small stunted transgenic plants [50, 54, 76]. Our *OsWRKY03*-overexpressing transgenic rice showed more serious phenotype with stunted growth, loss of apical dominance, curly leaves, and finally died after transferred to greenhouse. This is most likely due to the constitutive expression of *NPR1* gene and defense-related genes, such as *PR1*, *PAL* and *POX22.3*, which allocate much more plant's resources. From this point of view, the transgenic lines

with inducible expression of *OsWRKY03* will be much helpful for the further elucidation of its biological function *in vivo*.

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