Rice WRKY11 Plays a Role in Pathogen Defense and Drought Tolerance

Heyoung Lee†, Jooyoung Cha†, Changhyun Choi, Naeyoung Choi, Hyun-So Ji, Sang Ryeol Park, Seungbum Lee and Duk-Ju Hwang*

Abstract

Background: Plants are frequently subjected to abiotic and biotic stresses, and WRKY proteins play a pivotal role in the response to such stress. OsWRKY11 is induced by pathogens, drought, and heat, suggesting a function in biotic and abiotic stress responses.

Results: This study identified OsWRKY11, a member of WRKY group IIc. It is a transcriptional activator that localized to the nucleus. Ectopic expression of OsWRKY11 resulted in enhanced resistance to a bacterial pathogen, Xanthomonas oryzae pv. oryzae; resistance was compromised in transgenic lines under-expressing OsWRKY11. Ectopic expression of OsWRKY11 resulted in constitutive expression of defense-associated genes, whereas knock-down (kd) of OsWRKY11 reduced expression of defense-associated genes during pathogen attack, suggesting that OsWRKY11 activates defense responses. OsWRKY11 bound directly to the promoter of CHITINASE 2, a gene associated with defense, and activated its transcription. In addition, ectopic expression of OsWRKY11 enhanced tolerance to drought stress and induced constitutive expression of drought-responsive genes. Induction of drought-responsive genes was compromised in OsWRKY11-kd plants. OsWRKY11 also bound directly to the promoter of a drought-responsive gene, RAB21, activating its transcription. In addition, OsWRKY11 protein levels were controlled by the ubiquitin-proteasome system.

Conclusion: OsWRKY11 integrates plant responses to pathogens and abiotic stresses by positively modulating the expression of biotic and abiotic stress-related genes.

Keywords: OsWRKY11, Disease resistance, Drought tolerance, Cross-talk, Rice

Background

Plants in the field are often subjected to abiotic and biotic stresses simultaneously or successively and have therefore evolved elegant mechanisms to respond precisely to individual or combined stresses. Cross-talk between individual stress response signaling pathways is well documented (reviewed in Sharma et al. 2013; Kissoudis et al. 2014; Takatsuji 2017), and evidence has accumulated that the signaling pathways involved in abiotic and biotic stress responses interact either antagonistically or synergistically via the stress hormones salicylic acid, ethylene, jasmonic acid, and abscisic acid (Sharma et al. 2013; Kissoudis et al. 2014; Takatsuji 2017). In rice, many genes are involved at the points of intersection of abiotic and biotic stress signaling, including OsMPK5, OsMPK6, OsEIN2, and transcription factors (TFs) (Xiong and Yang, 2003; Sharma et al. 2013; Ueno et al., 2015).

Bioinformatics and functional analyses demonstrated that TFs, including NAC, MYB, AP2/ERF, and WRKY, are involved in biotic and abiotic stress responses (Atkinson and Urwin, 2012; Shaik and Ramakrishna, 2014). Of these, WRKY TFs are involved in various biological processes, including growth and development (Han et al. 2014; Dai et al. 2016), abiotic stress responses (Kim et al. 2016; Raineri et al. 2015; Yokotani et al. 2013), and biotic stress responses (Abbruscato et al. 2012; Choi et al. 2015; Han et al. 2013; Hwang et al. 2016; Lan et al. 2013; Shimono et al. 2007; Wang et al. 2015; Zhang et al. 2008). WRKY TFs are therefore likely candidates for proteins involved in the cross-talk between abiotic and biotic stresses in rice. Some of these proteins play opposing roles, either positive or negative, during biotic and abiotic...
stress responses. Involvement of OsWRKY13 gene in cross-talk between disease resistance and abiotic stress tolerance pathways has been intensively studied. OsWRKY13 enhances resistance to Xanthomonas oryzae pv oryzae (Xoo) and Magnaporthe oryzae and decreases tolerance to cold and salt stresses (Qiu et al. 2007, 2008). More recently, a molecular mechanism has been proposed by which the suppression of SNAC1 by OsWRKY13 enhances drought tolerance (Xiao et al. 2013). The OsWRKY45–2 TF confers broad-spectrum disease resistance in rice but reduces adaptation to salt, cold, and drought stresses (Tao et al. 2009, 2011). OsWRKY45–1 confers rice resistance to M. oryzae but reduces resistance to the bacterial pathogens Xoo and Xanthomonas oryzae pv oryzicola (Xoc) and to cold and drought stresses (Shimono et al. 2007; Tao et al. 2009, 2011; Goto et al. 2015). Interestingly OsWRKY62, one of the group IIA WRKY TFs, plays a positive role in pathogen defense together with OsWRKY45–1 but plays a negative role in pathogen defense under hypoxia stress by self dimerization (Fukushima et al., 2016). OsWRKY76 enhances susceptibility to M. oryzae and Xoo but increases cold tolerance (Seo et al. 2011; Yokotani et al. 2013).

Some OsWRKY TFs play positive roles in biotic and abiotic stresses. OsWRKY89 [renamed 104 by the Committee on Gene Symbolization, Nomenclature and Linkage (CGSNL)] enhances resistance to M. oryzae and UV-B irradiation (Rice WRKY working group, 2012; Wang et al. 2007). It is reported that over-expression of OsWRKY30 increases resistance to fungal pathogens and drought stress by two independent research groups (Peng et al. 2012; Shen et al. 2012). For OsWRKY71 two independent reports showed that OsWRKY71 enhances resistance to Xoo and tolerance to cold, respectively (Liu et al. 2007; Chudo et al., 2008; Kim et al. 2016).

Although evidence for cross-talk between biotic and abiotic stress responses is rapidly accumulating, the molecular mechanisms remain largely unknown, particularly with respect to positive cross-talk. We describe here the role played by OsWRKY11 in positive cross-talk between biotic and abiotic stress responses. OsWRKY11 is induced by heat, drought, combined heat/drought, and pathogen stresses (Shiroto et al. 2004; Ryu et al. 2006). Ectopic expression of OsWRKY11 under the control of the heat shock protein (HSP) 101 promoter enhances drought tolerance (Wu et al. 2009). These expression patterns suggest that OsWRKY11 is involved in biotic and abiotic stress responses. We analyzed the severity of disease in response to Xoo, drought tolerance, and expression of stress-related genes in OsWRKY11 overexpression (ox) and RNA interference (RNAi) lines, and demonstrated direct binding of OsWRKY11 to promoters of both biotic and abiotic stress-related genes.

Results
Generation of transgenic plants over-expressing and under-expressing OsWRKY11
We were most interested in investigating genes involved in the response to combined stresses such as drought and Xoo infection. The nine OsWRKY TFs (OsWRKY7, −10, −11, −30, −32, −67, −70, 83 (renamed 94 by CGSNL), −85 (renamed 96 by CGSNL) are previously reported on their induction upon the infection of an incompatible race of Xoo (Ryu et al. 2006). In the current study, we focused on OsWRKY11 (Os01g43650), whose expression enhances drought tolerance (Wu et al. 2009). More recently we reported that OsWRKY11 expression is increased in compatible and incompatible interaction but the level of its expression is more pronounced in the incompatible interaction than in the compatible interaction (Choi et al., 2017).

To elucidate the function of OsWRKY11 in defense signaling, we generated OsWRKY11-ox lines and OsWRKY11-knock-down (kd) lines by RNA interference approach in the rice cultivar, Nipponbare. We confirmed that OsWRKY11 was expressed at higher levels in transgenic lines #73 and #80 than in non-transgenic wild-type (WT) control plants (Fig. 1a; Additional file 1: Figure S2), and was compromised in OsWRKY11-kd lines #45 and #91 during pathogen attack (Fig. 1b; Additional file 1: Figure S2). Wu et al. (2009) reported that the heights of OsWRKY11-ox plants were similar to those of WT plants. Our results, however, were not consistent with this previous report. The heights of the OsWRKY11-ox plants, generated in this study and grown in a culture box (Fig. 1c) and a greenhouse (Fig. 1d), were about 60% those of the controls at the seedling stage, but had reached 80% of the controls’ height at the tillering stage (Fig. 1d). As plant height is an important agronomic trait, we examined heights of OsWRKY11-ox and OsWRKY11-kd lines grown in paddy fields. The heights of OsWRKY11-ox and OsWRKY11-kd plants were approximately 80% of the height of control plants under these conditions (Fig. 1e).

OsWRKY11 reduces the susceptibility to a bacterial pathogen, Xoo
To examine an effect of OsWRKY11 on bacterial blight disease caused by Xoo 10 plants from OsWRKY11-ox lines #73 and #80 and OsWRKY11-kd lines #45 and #91, respectively, were challenged with a compatible race of Xoo using the leaf-clip method. The areas of lesions on the OsWRKY11-ox lines #73 and #80 were smaller than those on the WT controls (Fig. 2a, b). By contrast, the areas of lesions on the OsWRKY11-kd lines were larger than those on the WT controls (Fig. 1a). This suggests that enhanced expression of OsWRKY11 results in a reduced susceptibility to the bacterial pathogen Xoo.
OsWRKY11 increases the expression of defense-related genes

To elucidate the mechanism of OsWRKY11-mediated resistance to pathogens, we used RT-PCR to analyze the expression of defense-related genes. Levels of CHITINASE 2 (CHIT2), PATHOGENESIS-RELATED 10 (PR10), and Betv1 transcripts were higher in the OsWRKY11-ox plants than in WT plants before pathogen infection and increased transcript levels of these genes were maintained upon Xoo infection (Fig. 3a).

By contrast, induction of defense-associated genes during Xoo infection was more highly compromised in OsWRKY11-kd line #45 than in line #91. This indicated that OsWRKY11 regulated the induction of defense-associated genes such as CHIT2, PR10, and Betv1. Together with the data obtained from OsWRKY11-ox lines, these results show that OsWRKY11 regulates the expression of defense-related genes, resulting in reduction of disease susceptibility to Xoo.

OsWRKY11 trans-activates the CHIT 2 promoter by direct binding in vivo

Since subcellular localization of transcription factor is important to predict its function subcellular localization of OsWRKY11 was assessed. 35S::YFP-OsWRKY11 was generated and introduced into rice protoplasts along with 35S::NLS-RFP, a nuclear localization marker that contains a classical NLS (Choi et al. 2015). YFP::OsWRKY11 was localized exclusively in the nucleus, together with the nuclear marker NLS-RFP (Additional file 1: Figure S1a), suggesting that OsWRKY11 functions in nucleus.

Since defense-related genes were highly expressed in OsWRKY11-ox lines but their induction by pathogen infection was compromised in OsWRKY11-kd lines, we predicted that OsWRKY11 was a transcription activator. To test this hypothesis, a yeast vector (BD-OsWRKY11) was generated and transformed into yeast cells. Activity of a reporter gene was assessed (Additional file 1: Figure S1b).
**Fig. 2** Analysis of OsWRKY11-ox and RNAi (kd) plants in response to Xoo infection. Wild-type (WT) and transgenic lines (T2) over-expressing or under-expressing OsWRKY11 were challenged with Xoo using the leaf-clip method and photographed at 14 dpi (a). Lesion lengths were measured at 14 dpi, and disease incidences for OsWRKY11-ox (b) and OsWRKY11-kd lines (c) were expressed as the percentage of lesion length/leaf length. Asterisks indicate significant differences from the WT (**: P < 0.01; *: P < 0.05).
We found that OsWRKY11 was a transcription activator in yeast. To test this in plants, a promoter-reporter construct containing the CHIT 2 promoter upstream of a GFP-GUS fusion gene (pCHIT2::GFP-GUS) was generated. Agrobacterium-mediated transient assays were performed in N. benthamiana leaves (Fig. 4a; Additional file 1: Figure S2). Trans-activation activity of OsWRKY11 at the CHIT 2 promoter was assessed using GUS staining. GUS activity was stronger in leaves co-infiltrated with pCHIT2::GFP-GUS and 35S::OsWRKY11 than in leaves infiltrated with either pCHIT2::GFP-GUS or 35S::OsWRKY11 alone. We also performed a transient assay of promoter activity in rice protoplasts using PEG-mediated transformation (Additional file 1: Figure S3). Luciferase activity was about 2-fold higher in samples co-transformed with pCHIT2::LUC and 35S::OsWRKY11 than in those transformed with pCHIT2::LUC alone. These results suggest that OsWRKY11 trans-activates the CHIT 2 promoter in plants.

To examine direct binding of OsWRKY11 to the CHIT 2 promoter in vivo, transgenic lines over-expressing HA-OsWRKY11 were generated. In vivo binding of OsWRKY11 to the CHIT 2 promoter was assessed by chromatin immunoprecipitation (ChIP) with anti-HA antibody followed by qPCR with primers binding near the W-box or WLE1 (Fig. 4b; Additional file 1: Figure S4). DNA binding was higher in IP samples treated with anti-HA antibody than in no-antibody samples, indicating that OsWRKY11 directly binds the CHIT 2 promoter in vivo.

**OsWRKY11 positively regulates drought-responsive genes**

OsWRKY11 was initially reported to be induced following infection with pathogens such as M. grisea and Xoo (Ryu et al. 2006). Later, Wu et al. (2009) reported that over-expression of OsWRKY11 under the control of a heat-inducible promoter such as HSP101 conferred enhanced tolerance of heat and drought stresses. Therefore, we performed a drought tolerance assay with the transgenic lines over-expressing OsWRKY11 under the control of the 35S constitutive promoter (Additional file 1: Figure S5a). Water was withheld from 6-week-old plants for 10 days to induce drought conditions. Transgenic lines #73 and #80, which over-expressed OsWRKY11, showed reduced leaf wilting after 8 days of drought treatment compared with WT plants (Additional file 1: Figure S5a). After 5 days of re-watering, OsWRKY11-ox plants showed a better recovery from wilting than WT plants. Our results are consistent with a previous report (Wu et al. 2009); however, those authors did not investigate the drought tolerance of OsWRKY11-kd lines. We performed a drought tolerance assay of OsWRKY11-kd lines by withholding water from 6-week-old plants for 7 days to induce drought conditions (Additional file 1: Figure S5b). After 5 days of re-watering, OsWRKY11-kd plants showed a lower recovery from wilting than WT plants. Tolerance to drought was compromised in OsWRKY11-kd lines (Additional file 1: Figure S5b). Our kd approach therefore further confirmed the effect of OsWRKY11 on drought tolerance.

The expression of drought-responsive genes in transgenic plants over-expressing OsWRKY11 has not been previously investigated (Wu et al. 2009). We therefore analyzed expression of drought-responsive genes, including dehydrins such as DIP1, DHN1, and RAB21 (Fig. 5a). These three dehydrins were constitutively expressed in transgenic OsWRKY11-ox plants. By contrast, induction

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**Fig. 4** OsWRKY11 directly binds to and activates the CHITINASE 2 promoter. a Nicotiana benthamiana leaf discs were infiltrated with *Agrobacterium* carrying pCHIT2::GFP-GUS alone or with a mixture of *Agrobacterium* carrying 35S::OsWRKY11 and pCHIT2::GFP-GUS. Promoter activities in each sample were visualized 2 days post-infiltration using β-glucuronidase (GUS) activity staining. b Transgenic rice leaves expressing 35S::HA-OsWRKY11 were used for chromatin immunoprecipitation with anti-HA antibody. ChiP-PCRs were performed on genomic DNA fragments using promoter-specific primers against CHIT2 (Table S1). To normalize qPCR values, the value obtained from the sample with pre-immune serum (no antibody) was divided by the value obtained from the sample with 10% input of each sheared chromatin sample and arbitrarily set at 1. The value resulting from OsWRKY11 binding to the promoter was expressed as a relative ratio of the pre-immune serum sample. Asterisks indicate significant differences between treatments from the no-antibody control sample (**: P < 0.01; *: P < 0.05)**.
of dehydrins following drought stress was compromised in OsWRKY11-kd lines (Fig. 5b). All these results indicate that OsWRKY11 positively regulates the expression of drought-responsive genes.

**OsWRKY11 trans-activates the RAB21 promoter by direct binding in vivo**

We demonstrated that OsWRKY11 was a transcription activator and drought-responsive genes were up-regulated in OsWRKY11-ox lines but these effects were compromised in OsWRKY11-kd lines. To examine whether OsWRKY11 directly regulated drought-responsive genes as it did defense-related genes, we performed transient assays of promoter activity in N. benthamiana.

A promoter-reporter construct containing the RAB21 promoter upstream of a GFP-GUS fusion gene (pRAB21::GFP-GUS) was generated and transformed into Agrobacterium. Agrobacterium-mediated transient assays were performed in N. benthamiana leaves. Trans-activational activity of OsWRKY11 at the RAB21 promoter was assessed using GUS staining (Fig. 6a; Additional file 1: Figure S2). GUS activity was stronger in leaves co-infiltrated with pRAB21::GFP-GUS and 35S::OsWRKY11 than in leaves infiltrated with either pRAB21::GFP-GUS or 35S::OsWRKY11. This suggested that OsWRKY11 trans-activated the RAB21 promoter in planta.

In vivo binding of OsWRKY11 to the RAB21 promoter was also assessed in transgenic plants over-expressing HA-OsWRKY11 using ChIP with anti-HA antibody (Fig. 6b; Additional file 1: Figure S4). The signal obtained by PCR with primers binding sites near the W-box or WLE1 of the RAB21 promoter was higher in IP samples treated with anti-HA antibody than in no-antibody samples, suggesting that OsWRKY11 binds directly to the promoter.

**OsWRKY11 protein is controlled by the Ubiquitin-Proteasome system**

To prevent untimely activation of immune response transcription factors such as OsWRKY6 and OsWRKY45 are often controlled by the ubiquitin-proteasome system (UPS) (Choi et al., 2015; Matsushita et al., 2013). To examine whether OsWRKY11 was regulated by the UPS, we monitored the effect of the proteasome inhibitor MG132 on OsWRKY11 protein accumulation. GFP florescence was monitored in protoplasts transformed with 35S::YFP-OsWRKY11 (Fig. 7a). GFP florescence was stronger in protoplasts transfected with 35S::YFP-OsWRKY11 in the presence of MG132. This is suggesting that the level of OsWRKY11 may be controlled by the UPS. YFP-OsWRKY11 protein was detected by the immuno-blot analysis with a GFP antibody (Fig. 7b). OsWRKY11 protein levels were higher in the MG132-treated sample than in the mock-treated sample, while PAT protein levels were similar in both samples, again suggesting that the UPS controls levels of OsWRKY11 protein.

**Discussion**

A number of studies show that WRKY TFs are involved in the integration of signaling pathways in biotic and abiotic stress responses negatively or positively (reviewed in Sharma et al. 2013; Kissoudis et al. 2014; Takatsuji 2017). WRKY TFs involved in positive cross-talk are thought to be good candidates for proteins conferring tolerance to abiotic and biotic stresses; however, there has been no consideration of the potential uses of these TFs in improving tolerance to abiotic and biotic stresses. This may be because the positive effects of those TFs were reported by independent research groups. Here, we report the involvement of OsWRKY11 in positive cross-talk between abiotic and biotic stress responses.
OsWRKY11 is induced by heat and drought stresses, and over-expression of OsWRKY11 under the control of the HSP101 promoter enhances drought tolerance (Shiroto et al. 2004; Wu et al. 2009). In addition, OsWRKY11 expression is induced by pathogens such as M. grisea and Xoo (Ryu et al. 2006). There are, however, no further reports on the function of OsWRKY11 in pathogen-mediated defense signaling beyond these studies of OsWRKY11 expression. The expression pattern of OsWRKY11 prompted us to consider it a good candidate for playing a role in positive cross-talk between abiotic and biotic stresses.

OsWRKY11 is a member of group IIc (Xie et al. 2005), which contains 18 OsWRKY proteins (Berri et al. 2009). An Arabidopsis protein in this group, AtWRKY75, is involved in Pi stress as well as pathogen defense (Devaiah et al. 2007; Encinas-Villarejo et al. 2009; Choi et al. 2014). OsWRKY31 (OsWRKY23, according to CGSNL) confers enhanced resistance to Pseudomonas syringae when expressed heterologously in Arabidopsis (Jing et al. 2009; Berri et al. 2009). To assess the involvement of OsWRKY11 in pathogen-mediated defense signaling transgenic lines over- or under-expressing OsWRKY11 were therefore generated. OsWRKY11-ox lines displayed reduced susceptibility to bacterial leaf blight caused by a bacterial pathogen Xoo, while this reduced susceptibility was compromised in OsWRKY11-kd lines.

Defense-associated genes, such as PR10, Betv1, and CHIT2, were constitutively expressed in OsWRKY11-ox lines, while their expression was compromised in OsWRKY11-kd lines, indicating that OsWRKY11 was a positive regulator of expression of defense-associated genes (Peng et al. 2008). There are 125 WRKY TFs in rice (Rice WRKY Working Group, 2012), and, of these, OsWRKY03 (12 by CGSNL), 6, 13, 31 (23, by CGSNL), 45, 53, 62, 71, and 89 (104, by CGSNL) are known to be positive regulators of defense-associated genes (Liu et al. 2005; Li et al. 2006; Choi et al. 2015; Chujo et al. 2007;
Liu et al. 2007; Shimono et al. 2007; Qiu et al. 2007; Zhang et al. 2008; Chujo et al. 2009; Hwang et al. 2011). Out of the OsWRKY proteins, only the functions of OsWRKY6, OsWRKY13, OsWRKY45, and OsWRKY62 (Fukushima et al., 2016) have been thoroughly investigated using kd or knock-out strategies. Our analysis of OsWRKY11-kd lines confirmed that OsWRKY11 functioned as a positive regulator in the expression of defense-associated genes, thereby conferring disease resistance, in a similar manner to the other WRKY factors described above.

A number of studies show that WRKY TFs act as transcriptional activators or repressors in pathogen-mediated defense signaling. Our trans-activation assay in yeast demonstrated that OsWRKY11 functioned as a transcriptional activator. The protein contains the consensus coactivator motif LXLL (L: leucine; X: any amino acid) (Xie et al. 2005). This motif may contribute to transcriptional activation of reporter genes. TFs mostly localize to the nucleus, either by themselves or with help from other protein(s). OsWRKY11 was localized in nuclei. The putative NLS may contribute to nuclear localization of OsWRKY11 (Xie et al. 2005). In addition, we used ChIP and transient assays of promoter activity to confirm that OsWRKY11 could bind directly to the CHIT2 promoter and induce transcriptional activation in vivo. We demonstrated that OsWRKY11 trans-activated and bound around the W box or WLE1 of the CHIT2 promoter in vivo. There was no report yet that OsWRKY TF directly regulates the expression of the chitinase in vivo in our knowledge. Previously we reported that OsWRKY6 directly regulates OsPR10a promoter (Choi et al. 2015).

It was previously reported that OsWRKY11 is induced by heat and enhances drought tolerance (Wu et al. 2009). However, this study did not investigate the downstream target genes of OsWRKY11 in terms of drought tolerance. We demonstrated that over-expressing OsWRKY11 under the control of the 35S promoter resulted in enhanced tolerance of drought, as previously reported (Wu et al. 2009). Moreover, we observed constitutive expression of drought-responsive genes in OsWRKY11-ox lines and that induction of drought-responsive genes, such as RAB21, DHN1, and DIP1, was compromised in OsWRKY11-kd lines during drought stress. Our ChIP and promoter transient assays showed that direct binding of OsWRKY11 to the RAB21 promoter led to transcriptional activation in vivo. Taken together, our data indicate that OsWRKY11 is a positive regulator of drought-responsive genes, and thereby enhances drought tolerance. Each TF positively or negatively regulates a subset of stress-related genes. In line with this concept, OsWRKY11 positively regulates drought-responsive genes, including several dehydrin genes.

Positive regulators of rice immunity, such as OsNPR1, OsWRKY6, and OsWRKY45, are often subject to control by the UPS to prevent untimely activation of the immune response (Choi et al. 2015; Liu et al. 2017; Matsushita et al. 2013). OsWRKY11 was also controlled by the UPS. Plant defense responses to abiotic and biotic stresses theoretically impart a cost of reduced growth and reproduction (recently reviewed in Karasov et al. 2017; Takatsuji 2017), and thus plants are likely to develop various means to minimize growth/defense trade-offs. Degradation of immune proteins by the UPS is considered one such strategy, and therefore degradation of OsWRKY11 by the UPS may underlie the beneficial phenotypes for aspects of plant performance observed in transgenic lines over-expressing OsWRKY11, such as plant height and yield.

Conclusions

Plants are normally exposed to abiotic and biotic stresses in their natural environment. Drought stress and diseases are major causes of reduced crop yields. To overcome both stresses efficiently, plants must establish elaborate cross-talk between signaling pathways at various levels. We found that OsWRKY11 was a positive regulator of plant defense responses to drought and pathogens, including the bacterium, Xoo. Many transgenic approaches have been performed to increase biotic and abiotic stress tolerance (Kissoudis et al. 2014). Transgenic rice cultivars with improved drought tolerance and resistance to two severe diseases can increase and stabilize crop yields in stressful environments. Our data indicated that OsWRKY11 is a good candidate for improving the yield of transgenic crops. Identification of upstream factor(s) regulating OsWRKY11 is required to elucidate OsWRKY11-mediated cross-talk between the abiotic and biotic stress response pathways.

Methods

Plant materials and treatments

Transgenic rice seedlings (Oryza sativa L. ssp. japonica cv. Nipponbare) were grown in a greenhouse for 3 weeks for RT-PCR analysis, and for 6 weeks for studies of disease severity. Xoo KACC10331, a compatible strain to the Nipponbare cultivar, were grown in peptone-sucrose-agar medium (10 g peptone, 10 g sucrose, 1 g sodium-glutamate, and 15 g agar per liter) at 28 °C for 2 days and resuspended in 10 mM MgCl2 to an OD600 of 0.5.

Transgenic rice seedlings (Nipponbare cultivar) were inoculated with Xoo using a toothpick at approximately 10 mm intervals. Samples were taken at the times indicated and stored at –80 °C prior to RNA isolation and RT-PCR analysis. For RT-PCR analysis of gene expression under drought conditions, 6-week-old seedlings were drought-treated, and samples were collected approximately 6 days after treatment (dat).
Vector construction and generation of transgenic rice plants
A full-length OsWRKY11 cDNA clone was obtained from cDNA reverse transcribed from total RNA extracted from rice leaves infected with Xoo by PCR (Additional file 1: Table S1). The PCR product was amplified using primers OsWRKY11-F and R, and cloned into pDONR221 using BP clonase (Invitrogen, Carlsbad, CA) to make an entry clone. This entry clone was confirmed by sequencing.

The 35S::OsWRKY11 and 35S::HA-OsWRKY11 constructs were produced using an LR reaction between the entry clone and the destination vectors pB2GW7 (Karimi et al. 2002) and pEarleygate201 (Earley et al. 2006), respectively. The 35S::OsWRKY11 RNAi construct was produced using an LR reaction between the entry clone pDONR201-OsWRKY11-RNAi and pB7GWIW2 (II) (Karimi et al. 2002).

The three constructs were introduced into rice using Agrobacterium—mediated transformation as previously described (Kim et al., 2009) with minor modifications. Agrobacterium LBA4404 carrying each construct was used to infect rice callus generated from Oryza sativa cv. Nipponbare. Transgenic lines were generated by transforming rice with the 35S::OsWRKY11-ox construct or the 35S::OsWRKY11 RNAi construct.

RT-PCR analysis
Leaf samples were ground to powder in liquid nitrogen, and total RNA was isolated for RT-PCR, as described (Hwang et al. 2008). RT-PCR analysis using M-MLV RTase (Promega, Madison, WI, USA) was performed using 1 μg total RNA according to the manufacturer’s instructions. Subsequent PCR was performed using 25–30 cycles and gene-specific primers (Additional file 1: Table S1). OsActin (XM469569) (Hwang et al. 2008) primers were used as a loading control. All gel images are generated by a program (Quantity one-4.5.2) in gel documentation system (Bio Rad Gel Doc XR system US170–8170) and trimmed around corresponding band for each gene in size.

Disease assays
For Xoo inoculation, the bacteria were grown on peptone-sucrose-agar plates at 28 °C for 2 days and resuspended in 10 mM MgCl2 to an OD600 of 0.5 (Hwang et al. 2011). OsWRKY11-ox lines (T1) and OsWRKY11 RNAi lines (T1) were challenged with a compatible strain of Xoo strain KXO85 (KACC10331) using the leaf-clip method (Kauffman et al. 1973). Lesion lengths in each plant were measured at 14 days post-inoculation (dpi). Disease severity was expressed as a percentage of lesion length (cm)/leaf length for individual plants (Tao et al. 2009).

Promoter transient expression assay
A 1.5 kb region of the RAB21 (Os11g26790) promoter were amplified using PCR with specific primers (Additional file 1: Table S1) and introduced into an entry vector, pENTR/d-TOPO (Invitrogen, Carlsbad, CA, USA). pRAB21::GFP-GUS constructs were made by an LR reaction between the entry clone containing the pRAB21 promoter and the destination vector pBGWFS7 (Karimi et al. 2002). A 2.0 kb region of the CHITINASE 2 (CHIT2; Os04g41620) promoter were amplified using PCR with specific primers in Additional file 1: Table S1 and used for entry vector. pCHIT2::GFP-GUS constructs were made by an LR reaction between the entry clone containing the pCHIT2 promoter and the destination vector pBGWFS7. Transient expression assays in Nicotiana benthamiana were performed using the protocol reported previously (Li, X. 2011). N. benthamiana was infiltrated with Agrobacterium carrying pCHIT2::GFP-GUS and pRAB21::GFP-GUS constructs alone, or with a mixture of Agrobacterium carrying 35S::OsWRKY11 and either pCHIT2::GFP-GUS or pRAB21::GFP-GUS. Infiltrated leaves were collected 2 days post-infiltration, and promoter activities in each sample were visualized using β-glucuronidase (GUS) activity staining. Leaf disks were immersed in a solution of 1 mM 5-bromo-4-chloro-3-indolyl-b-glucuronic acid in 100 mM sodium phosphate pH 7.0 plus 0.1% Triton X-100 and incubated at 37 °C before clearing in 70% ethanol.

Chromatin immunoprecipitation (ChIP)
Chromatin immunoprecipitation (ChIP) assays were generally followed as previously described (Haring et al. 2007). Transgenic rice leaves over-expressing 35S::HA-OsWRKY11 were harvested and fixed with formaldehyde under a vacuum. The chromatin was sheared by sonication and mixed with anti-HA antibody (Abcam, Cambridge, UK). Protein A agarose (Thermo Scientific, Rockford, IL, USA) was added to precipitate the DNA fragments.

ChIP-PCRs were performed using promoter-specific primers against CHIT2 and RAB21 (Additional file 1: Table S1, see Supporting Information). qPCR values were normalized with the value obtained from the sample with pre-immune serum (no antibody) divided by the value obtained from the sample containing 10% input of each sheared chromatin was arbitrarily set at 1, and subsequent values resulting from OsWRKY11 binding to the promoter were expressed as relative ratios of the pre-immune serum sample. Chromatin samples precipitated with pre-immune serum were used as negative controls in all ChIP-qPCR reactions. Similar results were obtained in three independent experiments.
Protein accumulation assay for OsWRKY11 in rice protoplasts

Protoplasts were prepared from dark-grown rice seedlings for 10 days. PEG-mediated transformation was performed as previously described (Bart et al. 2006). The vector 35S::YFP-OsWRKY11 was introduced into rice protoplasts as described previously. After 4 h, 100 μM MG132, a proteasome inhibitor, was added to the protoplasts and incubated for 1 day. Green fluorescent protein (GFP) fluorescence was examined under a confocal laser scanning microscope (Olympus FV300; Olympus, Germany). Proteins extracts were prepared in ice-cold extraction buffer [150 mM NaCl, 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.5% NP-40, 1 mM PMSF, 50 μM MG132, and one protease inhibitor cocktail tablet per 10 ml; Roche, Mannheim, Germany]. Proteins were separated using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblot analysis was performed with GFP antibody (Abcam; code: ab6556). Immunoblot analysis with phosphinothricin acetyltransferase (PAT) antibody (Abcam; code: ab1791) was used as a loading control.

Additional file

Additional file 1: Table S1. Primers used in this study. Figure S1. Subcellular localization and transcriptional activity of OsWRKY11. Figure S2. Schematic maps of constructs used for transient assays of promoter activity. Figure S3. Activation of the CHIT2 promoter by OsWRKY11 in rice. Figure S4. W-box and W-box-like element 1 (WLE1) depicted in CHIT2 and RAB21 promoter. Figure S5. Drought response assays for plants over (ox)- or under-expressing (RNAi-knock-down; kd) OsWRKY11. Supplementary experimental procedures. (DOCX 130 kb)

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Availability of data and materials

All relevant data are provided as Tables within the paper and in the Supporting information files.

Authors’ contributions

DJH developed ideas, coordinated the experiments, and wrote the manuscript. HL, JL, CC, and CC are carried out the experiments that analyzed transgenic plants. SRP and HJ carried out disease assays. SL produced transgenic rice plants. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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