Over-expression of the stress-induced OsWRKY45 enhances disease resistance and drought tolerance in Arabidopsis

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\textbf{Abstract}

The WRKY transcriptional factor superfamily regulates diverse functions, including processes such as plant development and stress response. In this study, we have shown that the rice WRKY45 (OsWRKY45) expression is markedly induced in response to stress-related hormone abscisic acid (ABA) and various stress factors, e.g., application of NaCl, PEG, mannitol or dehydration, treatment with 0°C and 42°C as well as infection by \textit{Pyricularia oryzae} Cav. and \textit{Xanthomonas oryzae} pv. oryzae. Together, these results indicate that the OsWRKY45 may be involved in the signal pathways of both biotic and abiotic stress response. Further analyses of 35S::OsWRKY45 Arabidopsis plants have shown that ectopic, constitutive over-expression of the OsWRKY45 transgene confers a number of properties to transgenic plants. These properties include significantly increased expression of PR genes, enhanced resistance to the bacterial pathogen \textit{Pseudomonas syringae} tomato DC3000, enhanced tolerance to salt and drought stresses, decreased sensitivity toward ABA signalling during seed germination and post-germination processes, and modulation of ABA-stress-regulated genes during drought induction. In addition, higher levels of OsWRKY45 expression in transgenic plants correlate positively with the strength of the abiotic and biotic responses mentioned above. More specifically, the decreased ABA sensitivities, the enhanced disease resistance and drought tolerances may be attributed, in part, to stomatal closure and induction of stress-related genes during drought induction. The relationship between OsWRKY45 expression and ABA signalling is discussed.

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1. Introduction

Plants are constantly bombarded with various environmental signals, some of which cause stress and restrict growth and development. In response to those adversities, plants have developed a number of strategies that increase tolerance or adaptation to stress conditions, e.g., by altering gene expression profiles, leading to adaptive responses at the cellular or systemic levels (Bray, 1993; Ingram and Bartel, 1996; Thomashow, 1999; Hasegawa et al., 2000; Xiong et al., 2001). One such phytohormone mediator that results in the alteration of gene expression in plants is abscisic acid (ABA). More specifically, ABA has been shown to mediate plant responses to many abiotic stresses, such as cold, drought, and high salinity, in addition to the regulation of other growth and developmental processes, such as embryogenesis, seed dormancy, shoot/root growth, and leaf transpiration (Leung and Giraudat, 1998; McCourt, 1999; Rock, 2000; Koornneef et al., 2002; Finkelstein et al., 2002). One critical model system for studying ABA response in plants is ABA-regulated gene expression. As such, products of plant ABA response are generally considered to confer protective or adaptive roles under stress conditions (Skriver and Mundy, 1990). Some of the genes induced by ABA comprise many regulatory factor genes, including various kinases/phosphatases and transcriptional factor genes, which are also induced by abiotic stress (Kang et al., 2002; Cheong et al., 2003; Villalobos et al., 2004; Yang et al., 2005; Pandey et al., 2005; Reyes et al., 2006). Although not all stress-inducible genes are regulated by application of exogenous ABA, a large number of them are responsive to it and, in many cases, their induction is impaired in ABA-deficient mutants (Wang et al., 1996; Weatherwax et al., 1996; Kang et al., 2002).

The expression of one class of transcriptional regulators, the WRKY superfamily of transcriptional factor proteins, has been shown to be induced by ABA (Xie et al., 2005, 2006). The WRKY transcription factor superfamily is a large family of proteins composed of 74 members in Arabidopsis (Dong et al., 2003), with at least an additional 97 members in the rice genome (Qiu et al., 2004). Further subdivisions of the WRKY superfamily can be accomplished by using both the number of highly conserved N-terminal amino acid sequence(s) WRKYQK, and the Cys2His2 or Cys2HisCys zinc-finger-like motif(s) (Eulgem et al., 2000; Dong et al., 2003).
al., 2003). These characteristic WRKY and zinc-finger domains have resulted in further subdivision of the WRKY superfamily into three distinct subgroups (Eulgem et al., 2000; Qiu et al., 2004). Moreover, it has been assumed that the WRKY domain constitutes a DNA-binding domain, due to the clear binding preference of all characterized WRKY proteins for the same DNA motif, as well as the fact that the WRKY domain is the only conserved structural feature within the WRKY superfamily.

WRKY proteins have been shown to regulate the expression of target genes by binding specifically to the (T)TGAC(C/T) (W box) DNA sequences located in target promoter regions (Maleck et al., 2000, 2001; Yu et al., 2001, 2002; Cormack et al., 2002; Yoda et al., 2002; Dong et al., 2003; Kalde et al., 2003; Turck et al., 2004; Xu et al., 2006), wounding (Dellagi et al., 2000; Harra et al., 2000; Yoda et al., 2002), trichome development (Johnson et al., 2001), and senescence (Hinderhofer and Zentgraf, 2001; Robatzek and Somssich, 2001, 2002; Johnson et al., 2002). For example, pathogen-induced and salicylic acid (SA)-induced Arabidopsis WRKY18 can positively modulate defence-related gene expression and enhance disease resistance in transgenic Arabidopsis plants (Chen and Chen, 2002). Further analysis has shown that three Arabidopsis members of the WRKY subgroup IIG, WRKY18, WRKY40 and WRKY60 (Dong et al., 2003), all interact both physically and functionally in complex patterns of distinct overlapping and antagonistic roles in plant defensive pathways (Xu et al., 2006). Group III Arabidopsis WRKY proteins have also been involved in an array of defence signalling pathways with unique patterns of expression occurring after being inoculated with different pathogens (Kalde et al., 2003). Another Arabidopsis WRKY protein, WRKY44, is involved in the development of leaf trichome and seed coat (Johnson et al., 2002), while Arabidopsis WRKY70 protein acts as a node of converging for different signal pathways in plant defense by mediating cross-talk between SA and jasmonic acid (JA) antagonistic pathways (Li et al., 2004). WRKY proteins are involved in the regulation of plant responses to abiotic stress and hormones (Pnueli et al., 2002; Rizhsky et al., 2002; Sanchez-Ballesta et al., 2003; Mare et al., 2004; Xie et al., 2005; Zhang et al., 2004; Zou et al., 2004). Recent reports have revealed that Rice WRKY24, WRKY51, WRKY71 and WRKY72 proteins may have a regulatory function in rice aleurone cells and the rice WRKY71 gene encodes a transcriptional repressor of GA signalling in aleurone cells (Xie et al., 2005, 2006).

Despite the fact that rice is one of the most important crops in the world and the obvious significance of WRKY proteins in various developmental, biotic and abiotic processes in rice, there are few reports about functional analysis of rice WRKY genes. To address these concerns, we have described research detailing the identification of OsWRKY45 via the screening of a cold (4°C)-treated cDNA library (Qiu et al., 2004). Moreover, a recent study showed that OsWRKY45 had a crucial role in BTH-inducible defence responses and markedly enhanced resistance to blast diseases (Shimon et al., 2007). However, no further information pertaining to the biological function of OsWRKY45 in abiotic stress signalling has been reported. To understand the endogenous biological function of the OsWRKY45 gene in abiotic stress signalling, we described an over-expression approach in Arabidopsis to ascertain the biological role of the OsWRKY45 protein in the defence against dehydration and pathogen infection. Transgenic plants with constitutive expression of the OsWRKY45 gene under the cauliflower mosaic virus 35S promoter displayed a phenotype with reduced sensitivity toward ABA signalling during seed germination and post-germination processes. In addition, OsWRKY45-over-expressing plants exhibited the expression of ABA-induced or abiotic-related stress factors (e.g. high temperature, high salinity and high osmotic pressure). Furthermore, 35S:OsWRKY45 transgenic plants demonstrate markedly enhanced drought resistance. Analysis of the 35S:OsWRKY45 transgenic plants showed that OsWRKY45 is able to potentiate the expression of PR genes as well as resistance to the bacterial pathogen Pseudomonas syringae. Together, these results suggest a dual role for OsWRKY45 protein, acting as a regulator and as a protective molecule upon water deficit and pathogen attack.

2. Materials and methods

2.1. Plant materials, stress treatment, and RNA analysis

Rice seeds (O. sativa ssp. japonica cv. Nipponbare, our own seed stock) were kindly provided by the Rice Research Institute, Yunnan Agriculture University. Rice seedlings grown in water at 28°C for 14 d were used for treatments with different abiotic stress factors. Seedlings were exposed to abiotic stress: low temperature (0°C), high temperature (42°C), high salinity (250 mM NaCl), and high osmotic pressure (300 mM mannitol and 25% PEG8000). For treatment with ABA, 12d-old seedlings were submerged in 100 μM ABA. For dehydration experiments, 3-week-old seedlings were placed on the laboratory bench to dry. Plants were harvested at time zero and 1, 2, 4 h, 8 h and 12 h after abiotic stress treatment, then frozen in liquid nitrogen and stored at –80°C. Plants at the booting stage were inoculated with Xanthomonas oryzae pv. oryzae by the leaf clipping method (Kauffman et al., 1973). The preparation of bacterial suspension and the inoculation procedure were as described (Lin et al., 1996). Fungal inoculation of rice seedlings at the four-leaf stage was done by spraying with virulent Pyricularia oryzae Cav. at a concentration of 2 × 10³ spores per ml in 0.5% (√/√) Tween 20. After inoculation, plants were kept under conditions of high humidity for 24 h. The pathogen is virulent.

DNA fragment for OsWRKY45 probe was PCR amplified from genomic DNA with the following primers: 5′-TTGCTAGCATGTCGACAGC-3′ and 5′-TTTCTGATCTGACGACATT-3′. Total RNA was isolated as described (Logemann et al., 1987), separated on formaldehyde/agarose gels, and blotted onto a nylon membrane. Blots were hybridized with ³²P-labeled specific probes.

Arabidopsis thaliana ecotype Columbia (Arabidopsis Biological Resource Centre at Ohio State University) were grown in pots under greenhouse conditions. For culture in vitro, seeds were surface-sterilized by treatment with 20% (√/√) bleach for 15 min followed by four consecutive washes with sterile distilled water. Stratification of the seeds was accomplished at 4°C for 2 d.

2.2. Constructs and Arabidopsis transformation

To produce transgenic plants, the coding region of the OsWRKY45 cDNA was cloned into the pOCA30 vector (Du and Chen, 2000), which contains the modified CaMV 35S promoter (35S:OsWRKY45). Arabidopsis plants (Col-0 ecotype) were transformed by the floral-dip method (Clough and Bent, 1998) using Agrobacterium tumefaciens strain LBA4404. Transgenic seedlings were selected on kanamycin medium (50 μg/ml) to identify T₁ transgenic plants.
2.3. Pathogen inoculation

Vector control and transgenic 4-week-old plants were used for treatment with *P. syringae* tomato DC3000 (P.s. tomato DC3000). Pathogen inoculations were performed by infiltrating leaves of at least five plants per treatment with *P. syringae* pv. tomato DC3000 strain (OD$_{600}$ = 0.001 in 10 mM MgCl$_2$). Inoculated leaves were harvested 3 d after infiltration and homogenized in 10 mM MgCl$_2$. Diluted leaf extracts were plated on King's B medium supplemented with rifampicin (100 μg·ml$^{-1}$) and kanamycin (25 μg·ml$^{-1}$) and incubated at 28°C for 2 d before counting the colony-forming units (cfu). All experiments were repeated in triplicate.

2.4. Germination assay and measurements of root

Vector control and 35S:OsWRKY45 seeds (at least 50 of each) were sterilized and kept for 4 d in the dark at 4°C to break dormancy. The seeds were then placed directly onto the surface of filter paper soaked with 1/2 strength Murashige and Skoog basal salt mixture (Murashige and Skoog) and supplemented with B5 vitamins, followed by incubation at 23°C with a 16 h light/8 h dark cycle. Various concentrations of mannitol or ABA, or 200 mM NaCl were added as indicated. The number of seeds that germinated was expressed as a percentage of the total number of seeds plated. Unless indicated otherwise, three replicate plates were used for each treatment.

For the root growth assay, transgenic and vector control seeds were placed onto MS agar plates for germination. At 2 d after germination, seedlings from each line were transferred carefully to a cDNA library of cold-treated rice seedlings (Qiu et al., 2004). The OsWRKY45 cDNA has confirmed the identity of this cDNA to be the same, at least in part, as cDNA clones BK005048 (Xie et al., 2005), DQ298181 (Ryu et al., 2006), AK105939, AK066255 and AK063697 (Kikuchi et al., 2003). The OsWRKY45 protein belongs to the subgroup III, and contains a single WRKY domain as well as the zinc finger-like motif C-X$_7$-C-X$_{23}$-H-X$_1$-C (Qiu et al., 2004).

In order to address the biological function of OsWRKY45 gene, we examined its expression pattern in different plant organs and at different developmental stages with Northern blot analysis. As shown in Fig. 1A, the OsWRKY45 gene was expressed in all organs and developmental stages, including root, young leaf (15 d), mature leaf (45 d), flag leaf and inflorescence. It started to express after 4 days' sowing, the strength of subsequent expression increased with time (Fig. 1B).

2.5. Drought and salt treatment and measurement of transpiration rate

For drought treatment, water was withheld from 4-week-old, soil-grown plants for the specified lengths of time. After subsequent addition of water, plants were transferred to an incubation room and incubated for 3 d at 23°C under 16 h light/8 h dark to determine whether the plants were dead as judged by their colouring. Plants exhibiting green on >50% of their tissue were counted as surviving plants. The entire test was repeated at least four times, with consistent results.

For the salt tolerance assays, 4-week-old plants growing in the same pots (20 plants each) were exposed to 400 mM NaCl solution every 3 d in triplicate followed by placement in greenhouse for 2 weeks.

The transpiration rate of detached leaves was measured by weighing freshly harvested leaves placed abaxial side up on open weighing trays and allowed to dry slowly at a constant temperature (20°C) and humidity (50%). Leaves of similar developmental stages (third to fifth true rosette leaves) from 4-week-old soil-grown plants were used for each treatment, and the percentage of fresh weight loss was calculated.

2.6. Guard cell examination

To examine guard cells, leaves were excised from 4-week-old soil-grown plants with or without drought treatment. Leaves of similar developmental stages (third to fifth true rosette leaves) from 20 different plants of either vector control or transgenic 35S:OsWRKY45 lines were placed on slides, abaxial side up, and photographed immediately after excision. Rosette leaves under drought treatment were also excised from both the vector control and transgenic 35S:OsWRKY45 lines as described above. The number of guard cells was then counted in randomly chosen fields (usually seven to nine).

3. Results

3.1. Constitutive expression of OsWRKY45 in different rice tissues and developmental stages

In an earlier study, we isolated a full-length, 978 bp long cDNA clone encoding a predicted protein of 326 amino acid residues for the OsWRKY45 gene (Genbank Accession number AY870611) from a cDNA library of cold-treated rice seedlings (Qiu et al., 2004). Sequence analysis of OsWRKY45 cDNA has confirmed the identity of this cDNA to be the same, at least in part, as cDNA clones BK005048 (Xie et al., 2005), DQ298181 (Ryu et al., 2006), AK105939, AK066255 and AK063697 (Kikuchi et al., 2003). The OsWRKY45 protein belongs to the subgroup III, and contains a single WRKY domain as well as the zinc finger-like motif C-X$_7$-C-X$_{23}$-H-X$_1$-C (Qiu et al., 2004).

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3.2. Expression profiles of OsWRKY45 in response to ABA, osmotic stress, temperature and pathogen infection

The ABA and stress induction of OsWRKY45 expression was examined by RNA gel-blot analysis. As shown in Fig. 2A, a rapid accumulation of the OsWRKY45 transcript was observed at 1 h, 2.5 μg/ml, 6.25 μg/ml, and 12.5 μg/ml ABA, and a similar level of expression was observed at 7 d. The OsWRKY45 gene expression was apparent at 4 d after sowing, followed by a dramatic increase. Each lane was loaded with 20 μg of total RNA. rRNAs are shown as equal loading controls.

![Fig. 1. Constitutive expression of OsWRKY45 gene in different organs and developmental stages of rice plants.](Image 194x145 to 495x206)}

![Fig. 2. Time-course of the OsWRKY45 expression during rice seed germination.](Image 194x145 to 495x206)
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Fig. 2. RNA blot analysis for the expression profiles of OsWRKY45 in rice under ABA application, abiotic stress and pathogen infection. The densitometric quantification of bands after normalization against RNA loaded according to ImageJ software from NIH (http://rsb.info.nih.gov/ij/download/) is shown at the right. Rice seedlings were treated with: (A) ABA (100 μM); (B) dehydration; (C) PEG (25%); (D) mannitol; (E) NaCl (250 mM); (F) 0°C; (G) 42°C; (H) Pyricularia oryzae Cav.; (I) Xanthomonas oryzae pv. oryzae (Xoo). All samples were analyzed by RNA gel-blot hybridization with gene-specific probe for OsWRKY45. OsWRKY45 expression was induced by ABA and each stress factor. As a control for equal loading, EtBr staining of RNA is shown at the bottom; each control lane was loaded with 20 μg of total RNA (ABA, P. oryzae, Xoo), the others were loaded with 15 μg of total RNA.

followed by a decrease of transcripts to the background level at 4 h after treatment with ABA, a decrease that continued unabated thereafter. In addition to induction by ABA, the OsWRKY45 gene is highly induced by several stress conditions, including high salt, PEG, mannitol, and dehydration. Similarly, a rapid accumulation of transcripts was observed at 1 h after treatment (Fig. 2B–E).

To elucidate the potential involvement of the OsWRKY45 gene in plant defence, we assessed its expression in response to infection by the pathogens Pyricularia oryzae Cav. (P. oryzae) and X. oryzae pv. oryzae (Xoo). Expression of the OsWRKY45 gene was induced strongly by Pyricularia oryzae Cav. during the first day of treatment, followed by a decline of transcripts until 5 d after the treatment, followed by another burst of OsWRKY45 expression (Fig. 2H). In response to infection of X. oryzae pv. oryzae, as shown in Fig. 2I, OsWRKY45 gene expression was repressed during the first day of treatment, and subsequently increased gradually until 3 d after treatment, followed by a decrease. Thus, the results of these pathogen challenge experiments indicate the possibility that the OsWRKY45 gene has an important role in response signalling of pathogen infection. This broad responsiveness of OsWRKY45 to different types of environmental stress suggests that it is a component of both biotic and abiotic stress responses.

3.3. Disease resistance for Arabidopsis over-expressing OsWRKY45

Earlier work illustrated that the WRKY proteins possess regulatory roles in plant response to pathogen infection (Eulgem et al., 1999; Chen and Chen, 2000, 2002; Yu et al., 2001; Li et al., 2004; Xu et al., 2006). With this information in mind, we assessed the function of OsWRKY45 in vivo by using transgenic plants displaying ectopic over-expression of OsWRKY45. We generated transgenic plants expressing the cDNA of OsWRKY45 in Arabidopsis under the control of the constitutive CaMV 35S promoter (35S:OsWRKY45). Twenty-one independent transgenic lines were identified by kanamycin selection, and two of these, line-6 (moderate expression) and line-11 (strong expression), were selected for further analysis by RNA gel blot analysis (Fig. 3A). Except the level of ectopic OsWRKY45 expression, no other obvious difference in morphological or growth phenotype was observed between plants from the vector control line and the 35S:OsWRKY45 transgenic lines (data not shown).

Induction of OsWRKY45 gene expression by two pathogens (Fig. 2H–I), prompted us to test whether OsWRKY45 over-expression in Arabidopsis would affect disease resistance. Initial examination of the expression of PR genes in the transgenic plants (Fig. 3B) revealed that the vector control line plants produced no detectable mRNA for PR1, a reliable molecular marker of systemic acquired resistance (SAR) (Benedetti et al., 1998). However, 35S:OsWRKY45 plants consistently exhibited significantly increased levels of PR1 expression. A pattern of gene expression similar to that of the PR1 gene was found for the PR2 gene in both...
independent transgenic lines were identified by kanamycin selection, from which two transgenic lines, line-6 (moderate expression) and line-11 (strong expression), were selected for further analysis. (B) SAR-inducible genes (PR1, PR2) expression patterns in transgenic plants (line-6 and line-11) and vector control plants by RNA gel-blot analysis. PR genes (PR1 and PR2) were found in transgenic 35S:OsWRKY45 plants but not detected in vector control plants. Total RNA was isolated from 4-week-old untreated vector control plants and two transgenic 35S:OsWRKY45 lines, and probed with Arabidopsis PR1, PR2 genes, respectively. Each lane contained 20 μg of total RNA. (C) Resistance differences between vector control plants and 35S:OsWRKY45 transgenic plants (line-6 and line-11) to virulent bacterial pathogens P. syringae tomato DC3000. Plants were inoculated with P. syringae tomato DC3000 (OD600 = 0.001) for 0 or 3 d, after which leaves were sampled and assessed for growth of the bacterial pathogen. Data are given as the mean ± S.E. The experiment was repeated in triplicate with similar results.

vector control line plants and in 35S:OsWRKY45 transgenic plants. In contrast, the PR5 gene did not appear to be expressed in either the vector control line plants or transgenic OsWRKY45 plants (data not shown).

To determine whether the constitutive PR gene expression correlated with the disease resistance, we examined the response of both the vector control and the transgenic lines to P. syringae tomato DC3000 (P. s. tomato DC3000), a virulent in the Arabidopsis ecotype Columbia (Whalen et al., 1991). In this assay, 4 weeks old plants were inoculated with the bacteria and the growth of the pathogen was monitored 3 d later. As shown in Fig. 3C, over-expression of OsWRKY45 gene bestowed the 35S:OsWRKY45 plant lines with a markedly enhanced resistance to P. s. tomato DC3000. Compared with the control plants, the transgenic plants exhibited an enhanced resistance to P. s. tomato DC3000 that was correlated positively with the level of OsWRKY45 expression (line-6, ~5×; line-11, ~9×). These results suggest that constitutive activation of OsWRKY45 expression can contribute directly to disease resistance.

3.4. Response of 35S:OsWRKY45 seeds to treatment with ABA

Increased OsWRKY45 gene expression in response to exogenous ABA (Fig. 2A) led us to speculate that OsWRKY45 may have a role in plant response to the ABA signal. This hypothesis was tested by the use of several assays, including seed germination and plant tolerance to abiotic stress conditions. Under appropriate conditions, the 35S:OsWRKY45 seeds germinated and the seedlings grew as well as the vector control seeds. In medium supplemented with ABA, the transgenic seeds exhibited less sensitivity to the ABA signal than the vector control seeds (Fig. 4C). In the presence of 0.3 μM ABA, the germination percentage of vector control seed was decreased to 50%, compared to 60% and 88% germination for the line-6 and line-11 35S:OsWRKY45 transgenic seeds, respectively. In the absence of 0.4 μM ABA, the two 35S:OsWRKY45 lines showed 34% (line-6) and 50% (line-11) germination after 3 d, compared to only 20% of the vector control seeds, indicating that the germination of 35S:OsWRKY45 transgenic seeds was partially insensitive to ABA. Similarly, primary root elongation was less sensitive to ABA inhibition in 35S:OsWRKY45 transgenic plants than it was in the vector control plants (Fig. 5A). At 0.5 μM ABA, root growth in the vector control was only 29% of its control rate, whereas that of the line-6 and line-11 plants was 58% and 60% of their control rates, respectively (Fig. 5B). These results indicated that OsWRKY45 could affect the response to ABA at the germination stage and during post-germination developmental processes that are mediated by ABA.

3.5. Response of 35S:OsWRKY45 seeds to salt and osmotic stress

High concentrations of salt inhibit germination of Arabidopsis seed (Werner and Finkelstein, 1995; Leon-Kloosterziel et al., 1996; Quesada et al., 2000; Zhu, 2000; Kang et al., 2002; González-García et al., 2003). Several studies have shown that ABA has a role in the process governing seed germination under high salt condition (Yamaguchi-Shinozaki and Shinozaki, 1993; Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001; Finkelstein et al., 2002; Kang et al., 2002; González-García et al., 2003; Xiong and Zhu, 2003). It is believed that ABA, whose level increases under high salt conditions, promotes the inhibition process (Zhu, 2002). Furthermore, because OsWRKY45 expression was induced by high salinity (250 mM NaCl) and mannitol (300 mM) (Fig. 2D–E), it is conceivable that the OsWRKY45 gene may participate in the osmotic stress response. To test whether the reduced sensitivity of ABA results in 35S:OsWRKY45 plants with reduced sensitivity to ABA-mediated conditions, we analysed seed germination responses of the 35S:OsWRKY45 transgenic lines with various concentrations of mannitol and a high concentration of NaCl (200 mM). With 1/2 MS medium containing 200 mM NaCl, the two 35S:OsWRKY45 transgenic lines showed germination rates of 19% (line-6) and 31% (line-11) after 4 d, whereas only 9% of the vector control seeds germinated (Fig. 4A). With 1/2 MS medium containing various concentrations of mannitol, germination rates were similar to those with the medium containing 200 mM NaCl (Fig. 4B). At a concentration of 400 mM mannitol, the percentage of the vector control seeds that germinated declined to 10%, whereas the transgenic seeds retained 25% (line-6) and 39% (line-11) germination (Fig. 4B). Likewise, root growth of the 35S:OsWRKY45 transgenic lines was insensitive to NaCl and mannitol (Fig. 5A and B). We investigated the transgenic 35S:OsWRKY45 plants response to treatment with glucose. A high concentration of sugar inhibits the development of young seedlings, e.g. by inhibiting cotyledon greening/expansion, root growth and shoot growth. Glucose exerts more severe growth inhibition than other sugars (Jang et al., 1997). The vector control and 35S:OsWRKY45 seeds were germinated in the presence of 4% glucose. The vector control was
sensitive to glucose and became arrested. Compared with the vector control, the transgenic plants displayed a reduced sensitivity to the inhibition of seedling growth, and in particular root growth (Fig. 5A and B). The 35S:WRKY45 and vector control plants were examined in a growth chamber for resistance to high salinity by immersion of roots in 400 mM NaCl every 3 d for 2 weeks (Fig. 6). Growth and development were reduced in the vector control and the 35S:WRKY45 plants. However, the reduction of growth and development was much more severe for the vector control plants.
than the transgenic 35S:WRKY45 lines. Under the same salt treatment, vector control plant leaves exhibited much more bleaching and wilt than the transgenic 35S:WRKY45 plants, even to the point where some of the transgenic 35S:WRKY45 plants could blossom. The decreased sensitivity to osmotic stress in the transgenic plants suggested that ABA might have a crucial role in these physiological processes (Leung and Giraudat, 1998).

3.6. Drought tolerance of 35S:OsWRKY45 plants

One of the key ABA-controlled functions is stomatal closure under conditions of water deficit, which minimizes water loss through transpiration (Leung and Giraudat, 1998; Pei et al., 1998; Assmann and Wang, 2001; Schroeder et al., 2001; Wang et al., 2001; Kang et al., 2002). Thus, we assumed that OsWRKY45 over-expression lines should respond favourably to water deficit if OsWRKY45 is involved with ABA/stress signalling. To address this possibility, we examined the drought tolerance of 35S:OsWRK45 plants by withholding water for 16 d. As shown in Fig. 7A, the growth and development of 35S:OsWRKY45 plants appeared normal compared to the vector control in soil under greenhouse conditions and without stress. However, once drought stress was simulated, about 70% of the vector control plants wilted, and only 9.2% of them recovered once water was restored. Under the same conditions, only 40% of the 35S:OsWRKY45 plants wilted, and >50% survived the drought treatment (Fig. 7D). These results suggested that the OsWRKY45-over-expressing plants have a much higher level of survival during drought conditions than the vector control plants.

The control of water loss by ABA is an important survival tactic for plants during drought periods. Enhanced drought tolerance of the transgenic 35S:OsWRKY45 could be attributed, at least in part, to their lower transpiration rates. The rate of water loss of the 35S:OsWRKY45 transgenic lines was less than that of the vector control plants when measured by the fresh weight loss of detached rosette leaves (similar in size and age) (Fig. 7C). Consistent with this result, the stomata of the transgenic plants had smaller openings than those of the vector control plants when closed. Under normal and light stress conditions, no difference in size or density of the stomata was observed between the vector control and 35S:OsWRKY45 plants (data not shown). However, in response to drought for 6 d, about 25% (24/96) of the stomata in vector control plants were closed, compared to the closure of nearly all (98.2%) (107/109) of the 35S:OsWRK45 stomata. When water was withheld for 8 d, all stomata of the developing/mature leaves from vector control plants and transgenic plants were closed; however, the stomata from over-expressing plants were visibly smaller than those of vector control plants (Fig. 7B). Again, the level of drought tolerance was correlated positively with the expression level of the OsWRKY45 gene in transgenic plants (Fig. 7B and C). These results indicated that the constitutive over-expression of OsWRKY45 is partly responsible for the stomatal closure and consequent reduced transpiration. The closure of stomata in the 35S:OsWRKY45 lines allows these plants to maintain a more favourable water balance compared to the vector control plants, effectively imparting enhanced drought tolerance.

3.7. Expression of ABA/stress-responsive genes in 35S:OsWRKY45 plants

We have shown that the OsWRKY45 gene is regulated strongly by stress conditions (high salinity, dehydration and high osmotic pressure) and exposure to ABA (Fig. 2). In addition, germination assays revealed a decreased sensitivity of the 35S:OsWRKY45 plants when exposed to ABA and osmotic stress conditions (Fig. 4). Since WRKY is a transcription factor, OsWRKY45 might modulate the expression of genes involved in these processes. To test this hypothesis, gene expression analysis of possible gene targets of OsWRKY45 protein were tested in 35S:OsWRKY45 plants by RNA gel blotting (Fig. 8). We selected the following genes for expression analysis: RD29A, RD22, COR47, COR15A, KIN1 and ABA2. More specifically, 4 weeks old vector control, 35S:OsWRKY45 line-6 and line-11 transgenic plants grown in soil under greenhouse conditions were exposed to drought by withholding water before the RNA-gel blot analysis. As shown in Fig. 8, plants that were drought-stressed for 6 d exhibited elevated levels of transcripts for RD29A, KIN1, COR47 and COR15A in 35S:OsWRK45 plants compared to vector control plants; a trend that was much more pronounced at 8 d. Additionally, both of the 35S:OsWRK45 lines showed strong enhancement of ABA2 RNA levels compared with the control vector plants. Interestingly, expression of RD22 in transgenic 35S:OsWRK45 plants were similar to that of vector control plants. Thus, the results described here are consistent with the expression profiles of gene OsWRKY45 in response to the abiotic stress and the stress tolerance phenotypes observed during simulated conditions of drought, high salinity and high osmotic pressure (Figs. 2, 4, 5, 6 and 7).

4. Discussion

The WRKY proteins have been shown to possess multiple functions in plants. Chen and Chen (2002) showed the WRKY proteins regulate plant responses to pathogen attack and physical wounding. In addition, the WRKY proteins are involved in the various protective signal transduction pathways that establish disease resistance and response (Xu et al., 2006). WRKY proteins have
Fig. 7. Enhanced drought tolerance in 35S:OsWRKY45 transgenic plants. (A) Increased drought tolerance. The 4-week-old plants were denied water for 16 d before photography. (B) Micrographs showing the stomatal closure regulation of 35S:WRKY45 and vector control plants under drought stress for 6 d and 8 d, respectively. All pictures were taken at the same scale (the scale bar represents 30 μm). (C) Water-loss percentages from leaves of vector control plants and 35S:OsWRKY45 transgenic plants. Excised leaves from vector control or OsWRKY45-over-expressing plants were assayed for water loss. One asterisk indicate that the values are significant at $p < 0.05$ and two asterisks indicate $p < 0.01$ compared with the vector control based on SPSS analysis. (D) Percentage of plants (from 48 plants of each line and three replicates) scored as wilted and of plants scored as surviving (fully regained turgor and resumed growth) after watering was resumed for an additional 4 d and is represented as the percentage of the wilted plants.
Fig. 8. Time-course of ABA/stress-responsive genes expression in vector control plants and 35:OsWRKY45 transgenic plants during drought. (A) Vector control plants and 35S:OsWRKY45 transgenic plants were cultivated in soil and were denied water when the plants were 4 weeks old. Expression of RD29A, KIN1, COR47 and COR15A in 35S:OsWRKY45 plants were higher than that of vector control plants at 6 d of drought; this trend is much more significant at 8 d of drought. Additionally, both of the 35S:OsWRKY45 lines showed strong enhancement of ABA-biosynthesis gene ABA2 RNA levels compared to the control vector plants. Total RNA was isolated from the samples that were collected at the indicated time-points. Each lane was loaded with 20 μg of total RNA. Ethidium bromide stained rRNAs were used as a loading control (bottom section). (B) Graphics show the densitometric quantification of the bands shown in A after normalization against loaded RNA according to ImageJ software from NIH (http://rsb.info.nih.gov/ij/download/). Transcript levels are shown for COR47, COR15A, KIN1, RD22, and RD29A under normal conditions (filled bars) or drought for 8 d (open bars).

also been shown to be involved in regulating plant response to abiotic stress (Zou et al., 2004), and plant morphologic establishment (Robatzek and Somssich, 2002; Hinderhofer and Zentgraf, 2001; Johnson et al., 2002). We report here that OsWRKY45 was up-regulated in response to stress-related hormone and various stress factors as well as pathogens. Consistently, the over-expression of the OsWRKY45 in transgenic Arabidopsis resulted in an increased expression of PR genes, and the enhanced resistance to the bacterial pathogen as well as the enhanced tolerance to salt and drought stresses.

4.1. Over-expressed OsWRKY45 in Arabidopsis increased pathogen defence

The OsWRKY45 protein belongs to the subgroup III of WRKY superfamily, and contains a single WRKY domain and a zinc finger-like motif C-X7-C-X23-H-X1-C (Qiu et al., 2004). Xie et al. (2005) showed that the OsWRKY45 protein contains the consensus coactivator motif LXXLL, where L is Leu and X is any amino acid (Savkur and Burris, 2004), which can function as an activator of many different biological processes (de Pater et al., 1996; Eulgem et al., 1999;
Asai et al., 2002; Chen and Chen, 2002) or as activators in one pathway but as repressors in another (Robatzek and Somssich, 2002; Xie et al., 2005). The OsWRKY45 gene was a constitutive gene, expressed in various organs and all lifetime of plant we detected (Fig. 1). Like many other members of the WRKY super-family of transcription factors implicated in processes associated with environmental response (Pnueli et al., 2002; Rizhsky et al., 2002; Sanchez-Ballesta et al., 2003; Mare et al., 2004; Zhang et al., 2004; Zou et al., 2004; Xie et al., 2005), a rapid increase in OsWRKY45 expression was observed in rice after pathogen infection, which is in agreement with the data reported by Shimono et al. (2007), indicating the possibility that the OsWRKY45 gene has an important role in response signalling of pathogen infection (Fig. 2H–I).

Further analysis showed that increased resistance to P. s. tomato DC3000 is significantly enhanced in transgenic plants in which the OsWRKY45 gene is over-expressed (Fig. 3C). The degree of resistance established in the various over-expression plant lines can be attributed, in part, to the constitutive activation of pathogen-related genes, such as PR1 and PR2 (Fig. 3B), several of which are known to be responsive to SA and associated with SAR. Three days after inoculation, bacterial density was 9-fold lower in line-11 and 5-fold lower in line-6 (Fig. 3C) than vector transgenic plants; whereas, the expression level of the PR genes was lower in line-11 (Fig. 3B). Similar results have been obtained in Arabidopsis for AtWRKY11 and AtWRKY17 proteins; both act as negative regulators of the plant defence response toward P. s. tomato DC3000 (Journot-Catalino et al., 2006). The enhanced resistance phenotype of double mutants is not correlated with a constitutive or increased activation of defence-response genes. No difference in the expression level of the marker genes was detected in the double mutants compared with wild-type or the single mutants (Journot-Catalino et al., 2006).

In addition, in the transgenic plants over-expressing AtWRKY18 there was a constitutive expression of PR1 and enhanced resistance to P. s. tomato DC3000. However, the enhanced PR1 gene expression and resistance to P. syringae DC3000 in the transgenic plants is apparently not consistent with the expression level of WRKY18 in plant defence, and the similar phenotypes of its T-DNA insertion mutant (Xu et al., 2006; Marchive et al., 2007). So, it is not surprising that the expression of PR genes is stronger, but the resistance to P. s. tomato DC3000 is lower in line-6 than it is in line-11.

Although it has been shown that the defence pathway in rice is largely different from that in Arabidopsis, some observations suggest that rice has a signalling pathway for disease resistance that is similar to the SA-dependent pathway in dicots (Chern et al., 2001, 2005; Yang et al., 2004; Shimono et al., 2007). OsWRKY45 transcript levels responded strongly to SA, indicating that it acts in the SA signalling pathway (Ryu et al., 2006; Shimono et al., 2007). SA is necessary for SAR, which is characterized by an increase in endogenous SA, transcriptional activation of PR genes, and enhanced resistance to pathogens in dicots (Rylas et al., 1996; Li et al., 2004; Shimono et al., 2007). Our data here shows that a set of SAR marker genes is activated in the OsWRKY45-over-expressing plants, which may account for the observed enhanced resistance phenotypes. OsWRKY45 confer improved resistance to water deficit (Fig. 7). As to the actual mechanism of OsWRKY45 modulation of stresses of water deficit response, our data indicated that the over-expressed OsWRKY45 results in enhanced transduction of stress–response signals. It is well known that during vegetative growth, ABA is essential in plant responses to environmental stress factors, particularly in regulation of stomatal aperture and gene expression (Skriver and Mundy, 1990; Chandler and Robertson, 1994; Leung and Giraudat, 1998), and an increased ABA content is beneficial for plants under environmental stress as a result of ABA-induced changes at the cellular and whole-plant levels (Xiong and Zhu, 2003). In response to drought-induced stress, ABA is synthesized and triggers a calcium-activated signalling cascade in guard cells, resulting in stomatal closure (Schroeder et al., 2001). The data described here indicated that the OsWRKY45 protein may be involved in this process, since its over-expression resulted in significant reduction of transpiration and increased drought tolerance (Fig. 7C) in transgenic plants. Consistent with this result, the stomatal openings of 35S:OsWRKY45 transgenic plants were smaller than those of vector control plants during drought-induced stress for 6 d (Fig. 7B). Again, the level of drought tolerance was correlated positively with the expression level of the OsWRKY45 gene in transgenic plants (Fig. 7D). These results indicated that the constitutive over-expression of OsWRKY45 was partly responsible for the stomatal closure and reduced transpiration that allowed these plants to maintain a more favourable water balance compared to the vector control plants, effectively imparting enhanced drought tolerance. On the other hand, the enhancement of drought tolerance in the over-expressing OsWRKY45 transgenic plants was due to an increased expression of a gene(s) involved in stress response and ABA biosynthesis or signalling (Fig. 8), including COR47, COR15A, KIN1, RD29A and ABA2, RD29A is responsive to desiccation (Yamaguchi-Shinozaki and Shinozaki, 1993, 1994); COR47 and COR15A are induced by cold (Lin and Thomashow, 1992; Baker et al., 1994; Gilmour et al., 1992); and KIN1 responds to ABA, drought, cold, and salinity (Kurkela and Franck, 1990; Kurkela and Borgan-Frang, 1992; Tahtiharju et al., 1997). These genes were up-regulated in 35S:OsWRKY45 plants under normal conditions or drought (Fig. 8). ABA2/GIN1 encodes a short-chain dehydrogenase/reductase (SDR) gene in Arabidopsis which has a unique and specific role in the ABA biosynthesis pathway (Cheng et al., 2002; Rook et al., 2001; González-Guzmán et al., 2002), and is a late stress-responsive gene with a fine-tuning function in response to stress (Lin et al., 2007). Transgenic plants over-expressing ABA2 showed increased ABA levels, on average ∼44.7% higher than wild-type (Cheng et al., 2002). With or without the drought stress, transcriptional up-regulation of a key ABA biosynthesis gene, ABA2, was much stronger in 35S:OsWRKY45 plants compared with vector control plants (Fig. 8). The increased level of expression of ABA2 might result in the increased content of ABA, which is beneficial for plants under drought stress. Thus, the enhanced drought tolerance in transgenic plants might depend, in part, on changes in the expression of those genes.

Over-expressing OsWRKY45 in Arabidopsis also enhanced the tolerance to salt stress (Fig. 6). ABA is a central regulator of plant adaptation to salt stress during vegetative growth (Zhu, 2002; Kariola et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). The physiological mechanisms governing the plant responses to salinity and drought show a high degree of similarity, in that both stresses must bring the deprivation of water, and salt tolerance is often accompanied by drought tolerance, and both adopt ABA-dependent and ABA-independent pathways (Xiong and Zhu, 2002; Zhu, 2002; Jakab et al., 2005). High concentrations of salt in the soil lead to a decrease in water potential, which affects water availability (Hasegawa et al., 2000). In addition, there is much overlap in the expression pattern of stress genes after exposure to drought and
high salt (Leung and Giraudat, 1998; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein et al., 2002; Jakab et al., 2005; Yamaguchi-Shinozaki and Shinozaki, 2005, 2006; Qin et al., 2007). Usually, the salt tolerance reflects the decreased sensitivity to high osmolarity, because high salt concentration accompanies hypersensitivity to ABA during seed germination and early seedling development (Figs. 4 and 5). Germination is determined by growth potential of the embryo and by the restrictive properties of the seed coat (i.e. the sensitivity of the embryo to ABA, in situ ABA synthesis in the embryo, and surrounding structure of the embryo) (Bewley, 1997; Debeaupuij and Koornneef, 2000), and it is subject to control by the combined effects of an assortment of positive and negative regulatory signals (Finkelstein and Lynch, 2000). The plants response to a hormone is established, at least in part, by the interplay of several hormones (Trewavas, 1992). For example, a high concentration of glucose or sucrose inhibits seed and seedling development by increasing the level of ABA, whereas ethylene is a negative regulator of ABA action during seed germination and root growth, and impacts the glucose signal pathway (Chassemirian et al., 2000), suggesting a complex interaction of hormone signalling in plants. Several studies indicated that the pleiotropic phenotypes of ABA-deficient mutants are the result of auxin and ethylene production and/or an indirect effect (Sharp et al., 2000). Furthermore, the suggested physiological function of the OsMYB3R-2 gene was supported by their expression patterns (Dai et al., 2007), OsWRKY45 is expressed 4 d following sowing with subsequent expression increasing dramatically during germination and post germination growth (Fig. 1B), suggesting that the OsWRKY45 gene might have a positive role in seed germination and post-germination developmental processes. Although the precise mechanism is unknown, this phenotype exists in other genes, such as AtHD2C, CoXTH3, AtTPS1 and OsMYB3R-2 (Avonce et al., 2004; Cho et al., 2006; Sridha and Wu, 2006; Dai et al., 2007).

Xie et al. (2005) proposed that OsWRKY45 induced the HVA22 promoter-β-glucuronidase construct and Shimono et al. (2007) reported the level of OsWRKY45 expression was not affected by ABA. Our data showed that the mRNA level of the OsWRKY45 was enhanced in response to ABA treatment at 1 h time point, followed by a decrease of transcripts to the background level at 4 h (Fig. 3A). This observed difference may be due to variations of the experimental set-ups used, such as different tissues, different time points of sampling. Several lines of evidence have hinted to the possibility (Liu et al., 2007; Olker et al., 2007).

Some recent researches show that biotic and abiotic signalling pathways share multiple nodes and have significant functional overlap (Mani-Mauch and Mauch, 2005). With few exceptions, ABA was a negative regulator of disease resistance (Mohr and Cahill, 2003; Anderson et al., 2004). However, there are also reports of a positive correlation between ABA levels and disease resistance (Whenham et al., 1986; Chini et al., 2004). In addition, recent research work reveals that stomata also have an important role in host defence, which close upon detection of potential microbial pathogens to prevent the infection of the leaf interior (Melotto et al., 2006; Schulze-Lefert and Robatzek, 2006). Although we do not know the exact mechanism of OsWRKY45 dual functions, the fact that OsWRKY45 expression is up-regulated both by biotic- and abiotic-associated signals acting in a coordinated manner suggests that OsWRKY45 function may be required to integrate signals that link both pathways.

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