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

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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 *Pyricularia oryzae* Cav. and *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:*OsWRK45 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 *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 comprised 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) WRKYGQK, and the Cys2His2 or Cys2HisCys zinc-finger-like motif(s) (Eulgem et al., 2000; Dong et

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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; Yu et al., 2001). For instance, a number of studies have shown that WRKY transcription factors bind and promote the expression of several well characterized *PR* and *NPR1* genes, all of which contain W box elements in their promoter regions (Yang et al., 1999; Yu et al., 2001). Not surprisingly, WRKY proteins have been implicated in the regulation of various response processes, including pathogen infection (Eulgem et al., 1999; Chen and Chen, 2000, 2002; Du and Chen, 2000; Robatzek and Somssich, 2001, 2002; Yu et al., 2001; 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; Hara et al., 2000; Yoda et al., 2002), trichome development (Johnson et al., 2002), 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 convergence for different signal pathways in plant defence 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 ABA and gibberellin (GA) signalling pathways, since the expression of these rice *WRKY* genes is induced by ABA signal 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 (Shimono 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*-overexpressing plants exhibited the expression of ABA-induced or abiotic-related stress factors (e.g. high temperature, high salinity and high osmotic pressure). Furthermore, 35S:*OsWRK45* transgenic plants demonstrate markedly enhanced drought resistance. Analysis of the 35S:*OsWRK45* 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 h, 2 h, 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 2×10^5 spores per ml in 0.5% (v/v) 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′-TTGCTAGCATGTCTGCAGC-3′ and 5′-TCTTCGATCTGGACGACATT-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 ^{32}P-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% (v/v) 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:*OsWRK45*). *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 T_1 transgenic plants.

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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:*OsWRK45* 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 new MS agar plate or supplemented with 150 mM NaCl, 300 mM mannitol and 0.5 μM ABA. The plates were placed vertically on a rack and the root length of the seedlings was measured after 6 d growth in treatment media. For glucose response, seeds were germinated and grown for 10 d in the vertical position on MS medium supplemented with 4% glucose, and representative seedlings are shown.

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).

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).

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,

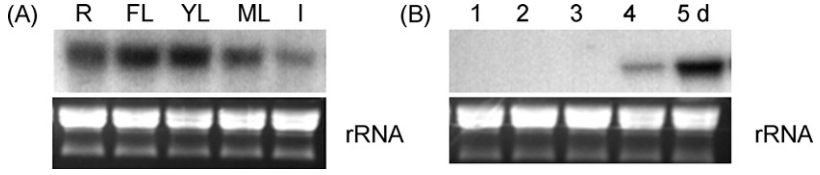

Fig. 1. Constitutive expression of *OsWRKY45* gene in different organs and developmental stages of rice plants. (A) *OsWRKY45* gene expression in various organs and developmental stages of rice (R, root; FL, flag leaf; YL, young leaf (15 d); ML, mature leaf (45 d); I, inflorescence). Northern blot analysis indicated that *OsWRKY45* is highly expressed in both young leaves and flag leaves. (B) Time-course of the *OsWRKY45* expression during rice seed germination. Total RNA was isolated from germinating rice seeds at 1 d, 2 d, 3 d, 4 d and 5 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.

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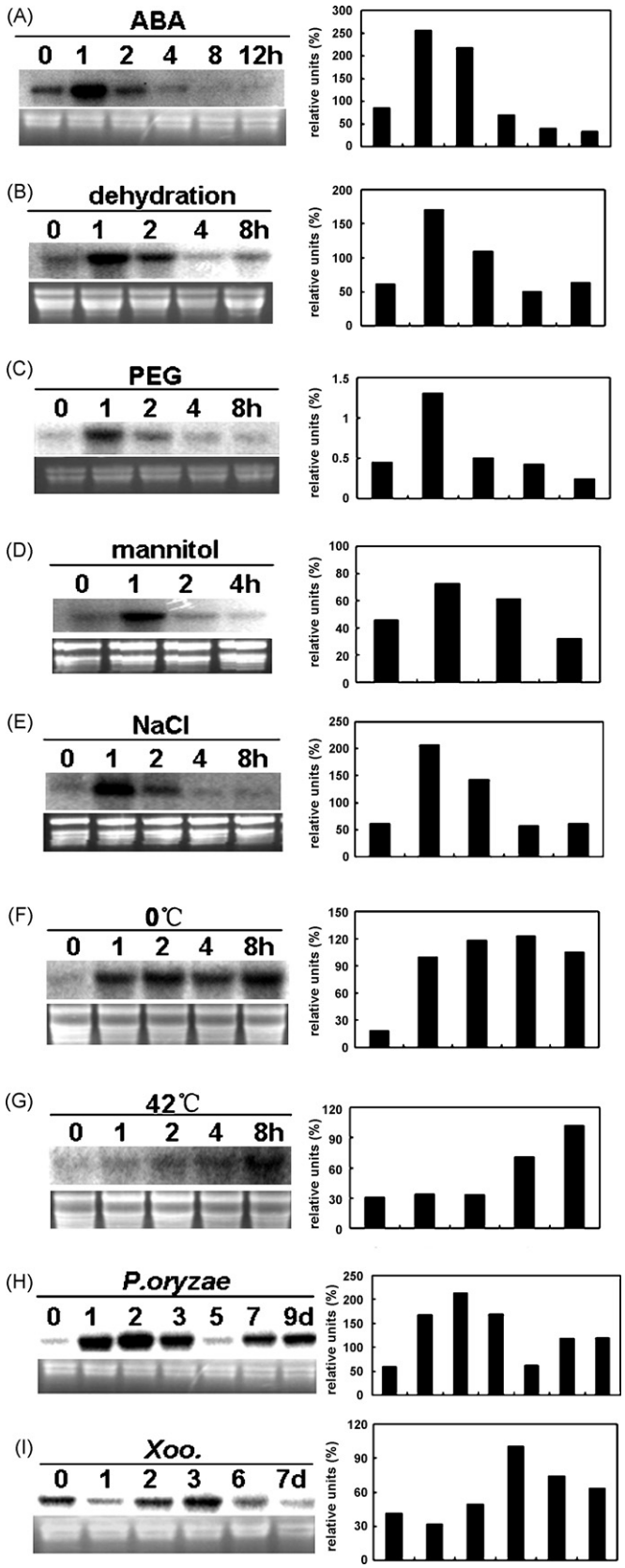

Fig. 2. RNA blot analysis for the expression profiles of *OsWRKY45* in rice under ABA application, abiotic stress and pathogen infection. The densitometric quantification of bands after normalization against RNA loaded according to ImageJ software from NIH (http://rsb.info.nih.gov/ij/download/) is shown at the right. Rice seedlings were treated with: (A) ABA (100 μM); (B) dehydration; (C) PEG (25%); (D) mannitol (300 mM); (E) NaCl (250 mM); (F) 0 °C; (G) 42 °C; (H) *Pyricularia oryzae* Cav. I, *Xanthomonas oryzae* pv. *oryzae*. 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). The induction of *OsWRKY45* at cold (0 °C) and high temperature (42 °C) followed slightly different patterns. Induction by cold was more rapid and more robust as compared to the induction by high temperature, with a maximum production of transcripts after treatment for 4 h (Fig. 2F–G).

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-expresssing 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:*OsWRK45*). 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

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