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An ethylene response factor OsWR1 responsive to drought stress transcriptionally activates wax synthesis related genes and increases wax production in rice

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Abstract Increasing evidence has revealed the major enzymes-involved in Arabidopsis and maize wax/cutin synthesis; however, there is limited information about the genes-associated with wax/cutin synthesis in rice. Here we report the characterization of an ethylene response factor gene in rice. This rice *w*ax synthesis *r*egulatory gene 1 (*OsWR1*) is a homolog of Arabidopsis wax/cutin synthesis regulatory gene *WIN1*/*SHN1*. Transcript analysis showed that *OsWR1* is induced by drought, abscisic acid and salt, and is predominantly expressed in leaves. Functional analyses indicated that overexpressing *OsWR1* (Ox-WR1) improved while RNA interference *OsWR1* rice (RI-WR1) decreased drought tolerance, consistent with water loss and cuticular permeability, suggesting that OsWR1-triggered drought response might be associated with cuticular characteristics. In addition, OsWR1 activated the expression of the genes-related to oxidative stress response and membrane stability. Gas chromatograph–mass spectrometry analysis further showed that OsWR1 modulated the wax synthesis through alteration of long chain fatty acids and alkanes, evidencing the regulation of OsWR1 in wax synthesis. Detection with real-time PCR amplification indicated that Ox-WR1 enhanced while RI-WR1 decreased the expression of wax/cutin synthesis related genes. Furthermore, OsWR1 physically interacted with the DRE and GCC box in the promoters of wax related genes *OsLACS2* and *OsFAE1'-L*, indicating that OsWR1 at least directly modulates the expression of these genes. Thus our results indicate that OsWR1 is a positive regulator of wax synthesis related genes in rice, and this regulation, distinct from its homology regulator of WIN1/SHN1 in cutin synthesis, subsequently contributes to reduced water loss and enhanced drought tolerance.

Keywords Rice · Ethylene response factor OsWR1 · Wax synthesis · Cuticle · Drought tolerance

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Introduction

The cuticle of all land plants is a continuous hydrophobic layer on epidermal cells (Post-Beittenmiller 1996; Millar et al. 1999; Raffaele et al. 2008), consisting of two major types of lipids (Schreiber 2010). This layered hydrophobic structure makes the cuticle an efficient barrier that waterproofs and protects internal tissues from environmental stresses including pathogen and insect attack, UV light or water deficit (Jenks et al. 1994; Post-Beittenmiller 1996; Millar et al. 1999; Riederer 2006; Raffaele et al. 2008; Schreiber 2010). The cutin, the core structural polymer, is composed of C16 and C18 hydroxy and epoxy-hydroxy fatty acid monomers. And cuticular wax, a polyester

typically formed by polymerization of C16 and C18 ω-hydroxy fatty acids and partial cross-linking of secondary hydroxyl functions (Kolattukudy 1966), predominantly comprises alcohols, aldehydes, ketones, alkanes, and esters derived from aliphatic very long-chain fatty acids (VLCFAs) with chain lengths ranging from C20 to C34 (Kolattukudy 1966; Liu and Post-Beittenmiller 1995; Kunst and Samuels 2003, 2009; Nawrath 2006).

Increasing evidence indicates that the wax components are ubiquitously produced from VLCFAs mediating two biosynthetic pathways. The first is the alcohol-forming pathway that is involved in the generation of primary alcohols and wax esters; while the alkane-forming pathway confers the increase of aldehydes, alkanes, secondary alcohols and ketones (Xia et al. 1996; Holmes and Keiller 2002; Chen et al. 2003; Aharoni et al. 2004; Islam et al. 2009; Schreiber 2010). The major biosynthetic steps leading to aliphatic wax products have been established using Arabidopsis and maize wax-deficient mutants. Enzymes catalyzing some of the steps in the wax synthesis have been characterized, or their function has been proposed based on the phenotype of the corresponding mutants, including condensing enzymes, reductases and putative decarbonylases (Aarts et al. 1995; Millar et al. 1999; Todd et al. 1999; Pruitt et al. 2000; Rowland et al. 2006). Among these genes, some encode wax biosynthetic enzymes, such as FATB, FAE1, KCS1, KCS2, CUT1, FDH, CER4, CER8, CER10 and GL8 (Millar et al. 1999; Todd et al. 1999; Pruitt et al. 2000; Bonaventure et al. 2003; Rowland et al. 2006; Lu et al. 2009). For example, the *CER4* encodes a fatty acyl-CoA reductase, which catalyzes the two-step reduction of VLCFAs to primary alcohols in Arabidopsis shoots (Rowland et al. 2006), while CER8 as a member of the long chain acyl-CoA synthetase family is identified to join free fatty acids to CoA (Lu et al. 2009). Some genes encode regulatory proteins. For instance, ethylene response factor (ERF) protein WIN1/SHN1 transcriptionally modulates cuticular wax levels (Aharoni et al. 2004; Broun et al. 2004). And more recently, an Arabidopsis abscisic acid (ABA)—responsive R2R3-type MYB transcription factor, MYB96, was reported to transcriptionally activate the expression of very-long-chain fatty acid–condensing enzyme genes and cuticular wax biosynthesis to promote drought tolerance (Seo et al. 2011). In addition, an exosome subunit CER7 controls the expression of CER3 (Hooker et al. 2007). More importantly, though the genes are evidenced to be involved in wax biosynthesis of different plants, mutations in these homologous genes confer to different wax compositions. For example, the mutation of CER1 displays deficient in alkanes and an elevated levels of aldehydes (Aarts et al. 1995); whereas the mutation of GL1 confers deficient in aldehydes but high levels of alkanes (Sturaro et al. 2005), while the *wax2* mutant results in deficient in aldehydes and alkanes (Chen et al. 2003), indicating that wax synthesis has a diverse regulatory mechanism.

Most interestingly, cuticular wax deposition is greatly regulated both developmentally and environmentally and involves the coordinated induction of several pathway genes as well (Suh et al. 2005; Shepherd and Wynne Griffiths 2006). It is possible that transcription factors play an important role in controlling this process, as these regulators have been shown to coordinate the expression of gene networks involved in complex metabolic pathways in plants (Broun et al. 2004; Seo et al. 2011). For instance, ERF proteins, Arabidopsis *WIN1/SHN1* and Medicago *WXP1/2*, have been shown to induce the production of epidermal waxes when overexpressed in plants (Aharoni et al. 2004; Broun et al. 2004; Zhang et al. 2005, 2007). The ERF proteins belonging to the AP2/ERF superfamily that contain a conserved ERF domain identifies many cis-acting elements, such as the GCC box, dehydration-responsive element (DRE) and the jasmonic acid- and elicitor-responsive element, subsequently resulting in the genes' expression and corresponding regulatory roles in abiotic and biotic stress responses (van der Fits and Memelink 2001; Nakano et al. 2006; Wu et al. 2008; Nakashima et al. 2009; Zhang et al. 2009; Wan et al. 2011).

Rice (*Oryza sativa*) is a worldwide staple food crop and has become a model plant of monocot species for functional genomics studies. For instance, 11 putative rice GL1 homologous genes (*OsGL1*) have been analyzed for sequence and expression, and the OsGL1-1 and OsGL1-2 have been revealed to be involved in cuticular wax accumulation and drought tolerance (Islam et al. 2009; Qin et al. 2011), while rice wax crystal-sparse leaf1 (WSL1) and WSL2, homologues of KCS and WAX2/GL1, respectively, confer wax synthesis in leaves and enhanced tolerance to drought (Yu et al. 2008; Mao et al. 2011). In addition, identification of the wax-deficient anther1 (*Wda1*) gene in rice revealed the biosynthesis role of VLCFA in wax biosynthesis (Jung et al. 2006). Although ERF proteins and other proteins have been shown to be involved in wax biosynthesis; however, information about the genes associated with wax synthesis in rice is very limited. In this study, we investigated the regulation of wax accumulation and related gene expression of a homolog gene of *WIN1/SHN1* in rice. Our results suggest that this WIN1-like OsWR1 controls wax formation by direct or collaborative activation of wax synthesis genes, and this regulation, distinct from its homology regulator of WIN1/SHN1 in cutin synthesis, subsequently results in reduced water loss and enhanced drought tolerance.

Materials and methods

Plant materials and growth conditions

All plants were grown in growth chamber at 25°C with a 16/8 h light/dark cycle. Four-week-old rice (*Oryza sativa*) seedlings were used. For ABA treatment, rice plants were sprayed with 0.1 mM ABA for the indicated time; ABA was dissolved in ethanol, and the same concentration of 0.01% (v/v) ethanol only was used for control. For drought and NaCl treatments, the rice plants were removed from soil, and washed with water. Then the seedlings were either put into a beaker containing 40 mL of 200 mM NaCl for the indicated time, or treated in the air without water supply for the indicated time interval.

Subcellular localization of OsWR1

The coding region of *OsWR1* was amplified by PCR. The resulting fragments were digested with *Kpn*I and *Xho*I and cloned into the expression vector pCHF3 (containing GFP-coding region derived from pBI121; Clontech, USA), yielding the plasmids pWR1-GFP. Then, the above constructs were introduced into *Agrobacterium* LBA4404 for further analysis. Rice callus cells were dipped in the prepared *Agrobacterium* solution for 10–20 min, transferred to nutrient broth plates and incubated at 21°C–22°C in darkness for 3 days. The localization of the fusion protein was observed by confocal microscopy (Bio-Rad, USA).

Transactivation assay

The coding region of *OsWR1* and deletion of the predicted activating domain were fused in frame to the DNA-binding domain vector pGBKT7, resulting in pGBKT7–WR1 and pGBKT7–DAD, respectively. The fusion plasmids were transformed into AH109 with lacZ as described by the manufacturer (Clontech, USA). The transformants were selected by growth on selective medium plates at 30°C for 3 days. The colony lift filter assay, using o-nitrophenyl β-D-galactopyranoside as a substrate, was performed subsequently to determine the activation ability of each translation product.

Transcript level analysis with real-time PCR validations

The first strand of cDNA was synthesized by M-MLV reverse transcriptase (Invitrogen, USA) using 2 μg of total RNA as a template. For RT-PCR analysis, obtained cDNAs were used as template in each PCR reaction (25 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C). Real-time PCR amplifications were performed with the IQ5 system (Bio-Rad) using SYBR (Takara, China) with gene-specific primers (Table S1). The relative gene expression was evaluated using the comparative cycle threshold method. *OsActin1* (Os03g0718100) was used as an internal loading control.

Scanning electron microscopy

Scanning electron microscopy was used to study wax crystals on the abaxial and adaxial surfaces of matured leaves. Fragments of leaves were first fixed in 5% glutaraldehyde and mounted on stubs. Samples were coated with grain-size gold particles for 15 min. Coated samples were transferred to an S-3400 N, HIACHI scanning electron microscope for examination.

Generation of transgenic rice

The full-length *OsWR1* cDNA was PCR amplified using specific primers (Table S1) and then fused with HA tag in pCAMBIA1307 (a derivative of pCAMBIA1300 carrying the 2 × CaMV 35S promoter and the OCS terminator), yielding the pOx-WR1. To understand the essential roles of OsWR1 in rice, we used an RNA interference approach to knockdown *OsWR1*. Because *OsWR1* is a member of a large ERF subfamily (Nakano et al. 2006), we used the less conserved region at the C-terminus (localized at amino acids 123–205), to interfere with the expression of *OsWR1* (the primers were listed in Table S1). A hairpin construct driven by the rice *Actin1* promoter (Ding et al. 2007) was named pRI-WR1. Then the resulting plasmids pOx-WR1 and pRI-WR1 were separately introduced into rice (cv. Nipponbare) using *Agrobacterium*-mediated transformation. Transformed plants were selected on the basis of their resistance to hygromycin or G418. After RT-PCR or Q-PCR analyses, the expression levels of *OsWR1* that either decreased by less than half in knockdown lines (named RI-WR1) or obviously increased in overexpressor lines (denoted as Ox-WR1), compared to Nipponbare (WT, Fig. S1A and S1B), were selected for our research. In addition, in order to exclude the potential effect of RNA interference construct that is controlled by the *Actin1* promoter on the expression of the gene, the expression of the *Actin1* using equal RNA samples of WT and transgenic lines was compared, our results showed that the signals in WT and transgenic lines were very similar (Fig. S1B), indicating that *Actin1* promoter in RNAi construct did not affect the expression of the gene. In the present study, transgenic T2 and T3 generations were used.

Drought treatment and water loss

Seeds of WT and transgenic rice were germinated at 30°C, and the germinated seedlings were transferred to pots

containing soil and grown at 28°C and 16/8 h light/dark. For the drought treatment at seedling stage, 4-week-old rice seedlings were exposed to progressive drought by withholding water until the drought phenotype was observed.

The rate of water loss from detached leaves was determined according to the method of Jenks et al. (1994). The second fully expanded leaves were excised and soaked in distilled water for 2 h. After removing excess water, the detached leaves were placed in an open petri dish in the laboratory and water loss determined by weighing the leaf every 30 min. Finally, the detached leaves were dried for 24 h at 80°C to determine the final dry weight. The rate of water loss was calculated as the weight of water lost divided by the initial leaf weight.

Quantification of epidermal traits

Epidermal permeability was measured using a chlorophyll extraction assay. Leaves from six seedlings were collected from 4-week-old rice plants, and immersed in 50 mL tubes with 40 mL of 80% ethanol. Tubes were agitated gently on a rotator platform. Aliquots of 2 mL were taken out for chlorophyll quantification and poured back to the same tube at every time point. The amount of chlorophyll extracted into the solution was quantified using a SPEKOL 1,300 spectrophotometer (Analytic jena, Germany) and calculated from light absorption at wavelength of 647 and 664 nm as described by Lolle et al. (1997). Chlorophyll extracted at each time point was expressed as a percentage of total chlorophyll extracted after 48 h of immersion.

Wax extraction and quantification

For wax extraction, the method was mainly followed as described by Yu et al. (2008). Six leaf blades (each about 10 cm in length) from each plant were immersed in 30 mL of chloroform for 30 s at room temperature. The same leaf blades were then re-extracted with chloroform at 60°C for 30 s, and the two chloroform extracts containing wax were pooled. Chloroform was evaporated by a nitrogen evaporator (Dry N-EVAPTM111; Organomation Associates Inc, USA). To each sample, 5 μg of n-tetracosane (C24) was added as an internal standard. After the samples were transferred to gas chromatograph vials, 10 μL of N,O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) and 10 μL of pyridine were added and the samples heated at 70°C for 1 h. BSTFA and pyridine were then removed under a stream of nitrogen. Then the samples after addition of 100 μL of chloroform were loaded for gas chromatography–mass spectrometry (GC–MS) analysis as previously described (Jung et al. 2006). Wax components were separated using a 30 m × 0.25 mm VF-17MS capillary column and helium as the carrying gas with constant flow of 2 mL/m in a GC–MS-QP2010 (Plus) gas chromatograph (Shimadzu, Japan). Gas chromatography was carried out with on-column injection temperature at 280°C, oven temperature of 50°C for 2 min; increasing at 40°C/min to 200°C, 2 min at 200°C; increasing at 3°C/min to 320°C, and 25 min at 320°C. Wax composition was determined by comparing peak retention times with those of reference standards, and by a GC–MS analysis of representative samples. Wax loads were estimated by quantifying the areas of major peaks in comparison with the internal standard. Wax load per unit leaf area was calculated based on the area of leaves used for wax extraction.

Chromatin immunoprecipitation assay

Rice seedlings (1.5 g) of WT (as a control) and Ox-WR1 lines were used to performed chromatin immunoprecipitation (ChIP) experiments, following the method described previously (Gendrel et al. 2005; Saleh et al. 2008), using HA antibody. The DNA fragments were cleaned up using a Qiaquick PCR DNA purification kit (Qiagen, USA). Promoter fragments were amplified using 1 μL of purified DNA as a template in each PCR reaction. Only PCR amplification products of promoter fragments from *OsLACS1* and *OsFAE1-L'* are shown, other wax synthesis related genes that did not show PCR signatures are not presented.

Electrophoretic mobility shift assay

To construct plasmids for expression of *OsWR1* in *Escherichia coli* BL21, a DNA fragment corresponding to the full coding region was obtained by PCR amplifications and inserted into the multi-cloning sites of the pGEX-6p-1 vector (Amersham, USA). Purification of the fusion protein was conducted according to the Glutathione Sepharose 4B instruction. Electrophoretic mobility shift assay (EMSA) was performed as described (DIG Gel Shift Kit, Roche, USA). The 3′-DIG-labeled probes were prepared by annealing of the synthesis oligonucleotides listed in Table S1. Recombinant protein GST–OsWR1 or GST only (100 ng) and 1 ng of labeled DNA probe were used in the binding reaction (20 μL). The reaction mixtures were loaded onto 5% polyacrylamide gels (29:1) to separate free and bound DNA. The DNA in the gel was then transferred onto nylon membranes. After UV cross-linking, the DNA on the membranes was detected using anti-digoxigenin-conjugated alkaline phosphatase (DIG Gel Shift Kit, Roche).

Result

OsWR1 protein is a homology of Arabidopsis WIN1/SHN1

The ERF protein WIN1/SHN1 was shown to transcriptionally activate the expression of wax synthesis genes and wax production, resulting in drought tolerance in Arabidopsis (Aharoni et al. 2004; Broun et al. 2004). To further investigate the regulation of ERF proteins in rice, we started with homology search for rice genes in the National Center of Biological Informatics using the Arabidopsis WIN1/SHN1 as a probe and found 4 rice orthologues with high amino acid identities in N-terminal region. These rice WIN1-like genes, named rice *wax synthesis regulator* (*OsWR*), all encode ERF proteins that contain a basic N-terminal region that might function as an nuclear localization signal and an acidic C-terminal region that might act as an activation domain for transcription (Fig. 1a). One of the rice WIN1-like genes, *OsWR1* (Os02g0202000), encodes a protein of 205 amino acids with a predicted protein molecular weight of 22.45 kDa. The amino acid of full length OsWR1 shows a high similarity to WIN1/SHN1 (68.8%), SHN2 (58%), SHN3 (57.8%) and OsWR2 (Os06g0604000, 81%), but low similarity to OsWR3 (Os02g0797100, 41.4%) and OsWR4 (Os06g0181700, 41.7%); these all contain the conserved ERF domain (Fig. 1a), suggesting that OsWR1 is a homology of WIN1/SHN1.

In addition, the WIN1-like members are conserved in gene structure because five of them (three from Arabidopsis and two from rice) contain a single intron positioned at 80–83 bp from the start codon, located from 80 bp in WIN/SHN genes, but from 83 bp in rice WIN1-like genes. The three odd nucleotides code the basic amino acid lysine (Fig. 1b). We reasoned that such sequence conservation with WIN/SHN genes, which were shown to participate in wax and cutin syntheses (Aharoni et al. 2004; Broun et al. 2004; Kannangara et al. 2007), might confer a similar function in rice.

It has been accepted that transcription factors of the same family generally have distinct actions due to differences in their regulatory domains that function as either repressors or activators, depending on whether they inhibit

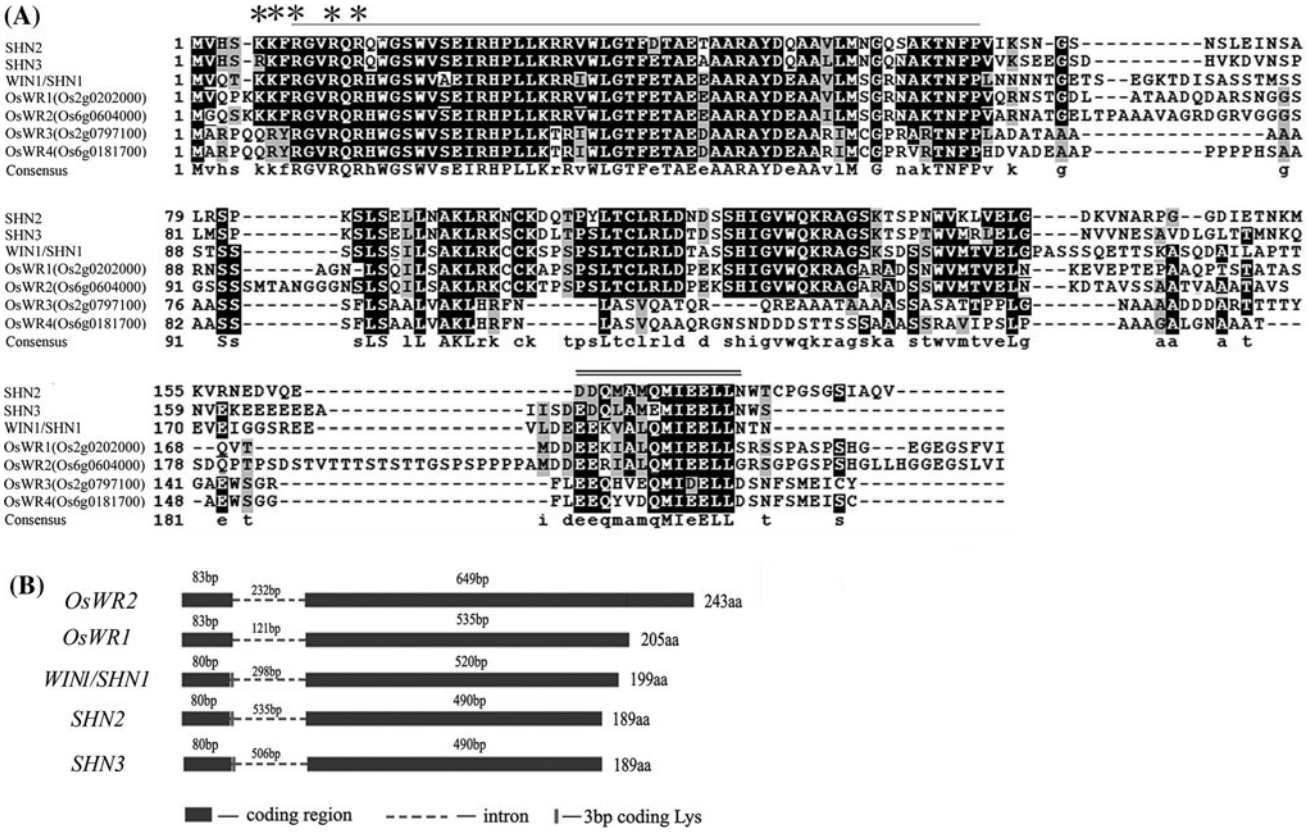

Fig. 1 Sequence analysis of OsWR1 with WIN1-like. **a** Whole amino acid sequence comparison of WIN1-like from Arabidopsis and rice. *Asterisks* represent the putative domain of the nuclear localization signal, the *black line* indicates the highly conserved AP2 domain, and *double black lines* indicate the putative activation domain. The *shaded boxes* indicate the percentage of sequences at this position with the same amino acid identity: *black*, 100%; and *gray*, 75%. Protein names are indicated at the *left*. **b** Nucleotide sequences comparison derived from sequence alignment of ERF whole regions from Arabidopsis and rice

or stimulate the transcription of target genes. OsWR1 has an acidic C-terminal region (Fig. 1a) that might act as a regulatory domain. To investigate the functional role of this region, we fused the coding regions of *OsWR1* and its deletion of the predicted acidic activation (DAD, located at 174–186 AA) region to the DNA-binding-domain expression vector of *GAL4* and examined the behavior of GAL4–OsWR1 and GAL4–OsWR1–DAD constructs as potential transcriptional activators in yeast. In the presence of the activation domain, the OsWR1 protein fused to the DNA-binding domain, e.g. GAL4–OsWR1 (indicated as WR1 in Fig. 2a), activated transcription of the lacZ reporter gene, whereas GAL4–OsWR1–DAD (indicated as WR1–DAD in Fig. 2a) could not, indicating that the OsWR1 protein may act as a transcriptional activator.

Because OsWR1 is believed to act as a transcription factor, nuclear localization should be necessary for the function of OsWR1. To test this possibility, we performed an in vivo targeting experiment by fusing the coding region of *OsWR1* to *GFP*, and the resulting constructs (WR1–GFP and GFP only) were introduced into rice calli by agroinfiltration. These fusion proteins, under the control of the CaMV 35S promoter, were expressed in rice callus cells, and the localization of the fusion protein was determined by visualization with a fluorescence microscope. The WR1–GFP was localized primarily in the nucleus, while GFP-only was observed in cytosol (Fig. 2b).

OsWR1 is responsive to drought, salt and abscisic acid

To elucidate the regulatory role of OsWR1, we first investigated the transcript levels of *OsWR1* in rice plants induced by drought, salt and ABA. Our analysis with real-time PCR (Q-PCR) amplifications showed that the expression of *OsWR1* was quickly responded at 1 h, and then gradually increased to peak at 2.5 h after drought treatment. The transcripts of *OsWR1* induced by ABA treatment were clearly observed at 6 h, and reached maximum induction at 9 h and then decreased, whereas the induction of *OsWR1* by salt treatment was dramatically increased at 3 h and reached a maximum at 6 h, and sustained high induction thereafter (Fig. 3a). These data reveal that *OsWR1* is responsive to drought, salt and ABA in rice.

Because the expression patterns of the gene in different tissues might be associated with its function (Millar et al. 1999; Chen et al. 2003), we further investigated the expression of *OsWR1* in different tissues of rice with Q-PCR. Our data showed that transcripts of *OsWR1* were highly expressed in seedling leaves and stems, compared to these in seedling roots, whereas the expression of *OsWR1* was mainly detected in leaves, blade sheaths, eustipes, internodes, testae and thrumb in the heading stage (Fig. 3b), indicating that the targets of OsWR1 might be expressed, at least partly, in the same tissues as the transcription factor.

OsWR1 is a positive regulator for drought response through affecting water loss

Because *OsWR1* is quickly induced by drought (Fig. 3a), the regulation of OsWR1 in drought response was first investigated using *OsWR1* overexpressors (Ox-WR1) and RNA interference plant (RI-WR1). Analysis with Q-PCR amplifications indicated that the transcripts of *OsWR1* reduced to 35–45% in RI-WR1 lines, but this interference did not affect the expression of *OsWR2–OsWR4*; whereas the transcripts of *OsWR1* in Ox-WR1 lines were obviously enhanced (Fig. S1A and S1B), compared to these in wild type (WT). Physiological tests showed that there were unobvious differences between WT and transgenic rice plants under normal growth conditions at the seedling stage (Control in Fig. 4). However, most WT seedlings (87.8%) were sensitive to drought after drought treatment for 8 days, showing a rolled-leaf phenotype, while much less of Ox-WR1 (18.5–28.6%) seedlings displayed drought-sensitive phenotypes (Fig. 4a), indicating that Ox-WR1 enhances tolerance to drought. Moreover, 71.5–78.9% of RI-WR1 seedlings showed drought sensitive phenotypes, while $> 80\%$ WT seedlings did not, after drought treatment for 5 days (Fig. 4b), suggesting that RI-WR1 is more sensitive to drought than WT.

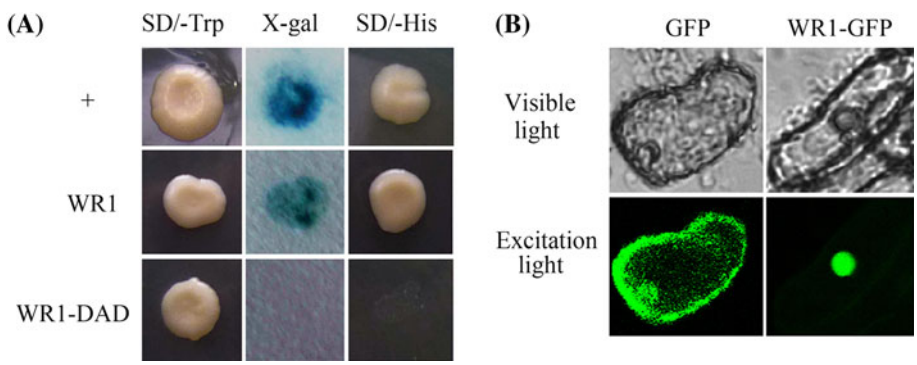

Fig. 2 Nuclear localization and activation assays of OsWR1. **a** Yeast growth and β-galactosidase activity assay; +: the positive control, WR1: AH109 with pGBKT7-WR1, WR1-ΔAD: AH109 with pGBKT7-ΔAD. **b** Cellular localization of OsWR1. GFP: rice callus contained pCHF3–GFP; WR1–GFP: rice callus contained pCHF3–WR1–GFP

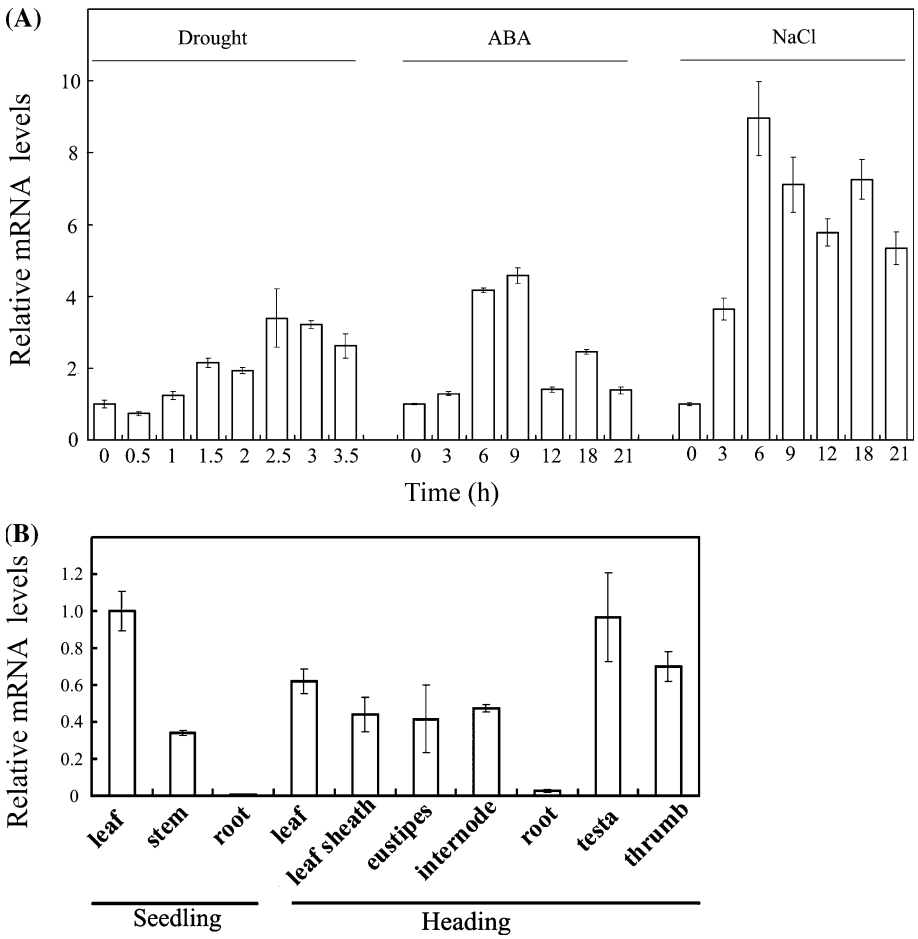

Fig. 3 The expression of *OsWR1* in tissues and in response to drought, ABA and salt. **a** The expression of *OsWR1* in WT after treatments with drought, 100 μmol/L ABA and 200 mmol/L NaCl. Transcripts of *OsWR1* gene are indicated relative to the level of the control (taken as 1), referring to the transcripts of *OsActin1* in the same sample. *Error bars* are based on three independent experiments. **b** The tissue expression pattern of *OsWR1*. Transcripts of *OsWR1* gene are indicated relative to the level of the leaf in seedling stage, referring to the transcripts of *OsActin1* in the same sample. *Error bars* (SD) are based on three independent experiments

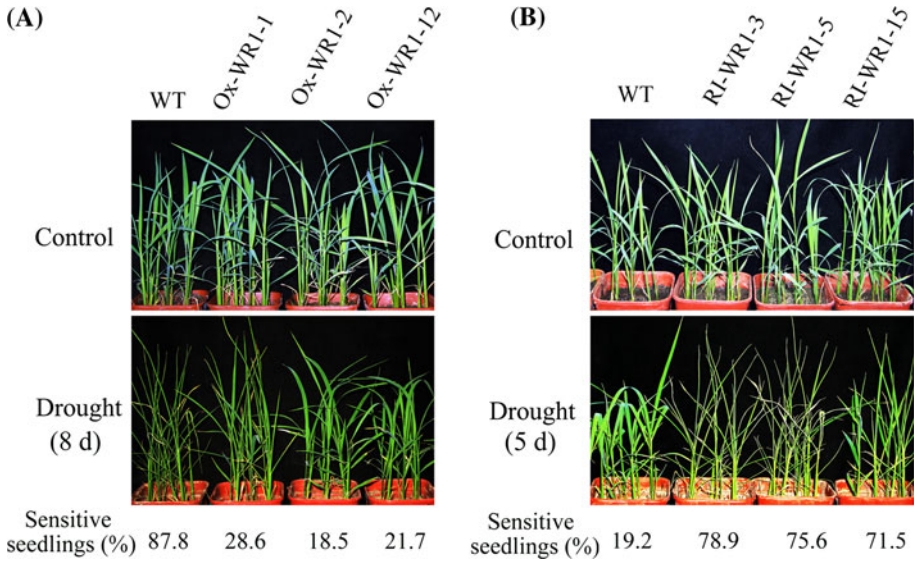

Fig. 4 Phenotypic observations in response to drought. Drought stress response of WT, Ox-WR1 (**a**) and RI-WR1 (**b**) rice seedlings. The sensitive seedlings (%) show the percentage of survival ratio. Control: 4-week-old rice seedlings grown under normal conditions. Drought: 4-week-old rice seedlings were withheld daily water supply for 8 days (**a**) or 5 days (**b**). Each experiment was repeated at least three times, with 10–15 seedlings each of WT and transgenic rice *lines*

To explain how OsWR1 modulates drought response, we then compared water loss using dissected leaves. Our analyses showed that the Ox-WR1 lines retained more water than WT did, whereas RI-WR1 lost more water than WT and Ox-WR1 leaves (Fig. 5a). In order to prove whether the changes of water loss were attributed to the cuticle properties of the transgenic lines, chlorophyll leaching assays were further conducted. Our data showed that Ox-WR1 lines had much less chlorophyll leaching; whereas the RI-WR1 lines showed faster chlorophyll

extraction than the wild type did (Fig. 5b), consistent with observations that Ox-WR1 lines showed enhanced drought tolerance with reduced water loss, while RI-WR1 lines did not.

In addition, we further detected the expression of stress related genes to explain the regulation of OsWR1 in drought response. Because drought triggers accumulation of reactive oxygen species (ROS), which results in cellular damage by oxidative stress, the abundance of intercellular ROS is tightly regulated through complex antioxidant systems in diverse subcellular compartments. Among these ROS-scavenging enzymes, ascorbate peroxidase, superoxide dismutase, and catalase are major proteins that detoxify superoxide and hydrogen peroxide under stressed conditions in plants (Mittler et al. 2004; Lu et al. 2007); whereas LEA proteins are a family of highly hydrophilic proteins that stabilize the membrane structure under dehydrated conditions (Valliyodan and Nguyen 2006; Hirayama and Shinozaki 2010). Our results showed that the transcripts of *CatA*, *CatB*, *LEA3*, *APX1* and *SodA* dramatically increased >fourfold in Ox-WR1 lines, but only two of genes (*CatA* and *CatB*) were obviously decreased in RI-WR1 lines, compared to those in the WT (Fig. S2), suggesting that OsWR1 might participate in the regulation of oxidative stress response and membrane stability, and this regulation might be a compensation for the modulation of OsWR1 in drought response.

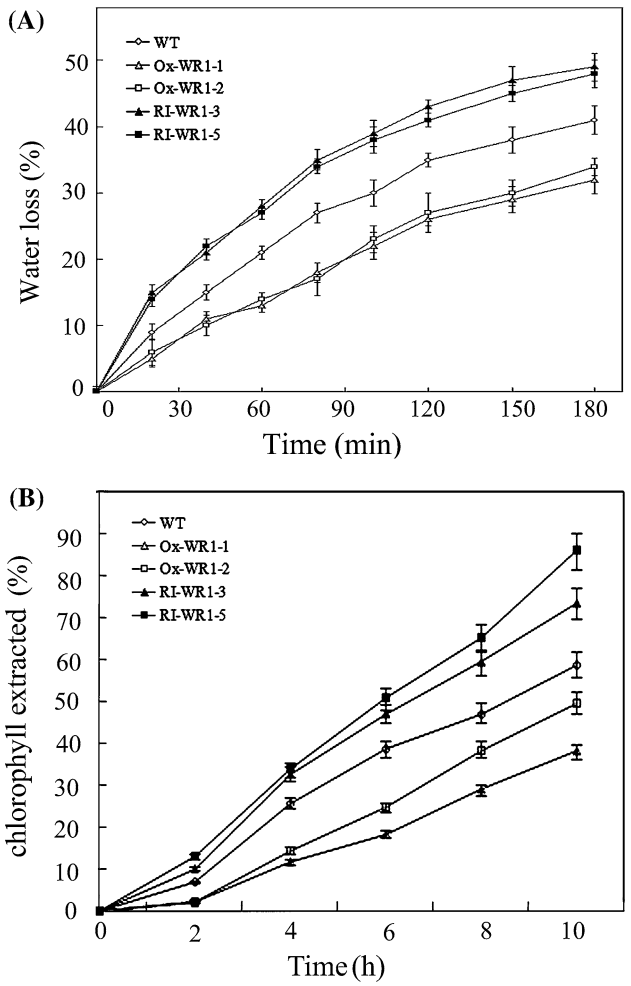

Fig. 5 Comparison of water loss and chlorophyll leach in dissected leaves. **a** 4-week-old rice seedling leaves grown under normal conditions were used to measure leaf-water loss. **b** Chlorophyll leaching from leaves transgenic *lines* and wild type. The results show averages of three replicates, and *error bars* indicate ± SD

OsWR1 mainly affects wax synthesis and composition in rice

For OsWR1 is a homology of WIN1, we were questioned whether the changes of drought response and water loss in transgenic seedlings were associated with wax synthesis. To address this hypothesis, we first checked the wax accumulation on the leaf surfaces observed with scanning electron microscopy. In WT of Nipponbare, the surfaces of adaxial and abaxial matured leaves including the cuticular papillae were densely covered by wax crystals. Carefully observation found that the surfaces in Ox-WR1-1 plants were more densely covered by platelet-type wax crystals, even at the surface of cuticular papillae, than these in WT. Different from the Ox-WR1 plant, fewer and shorter wax crystals were observed on the surfaces including the papillae in the RI-WR1-3 plants (Fig. 6). Then we applied gas chromatograph–mass spectrometry

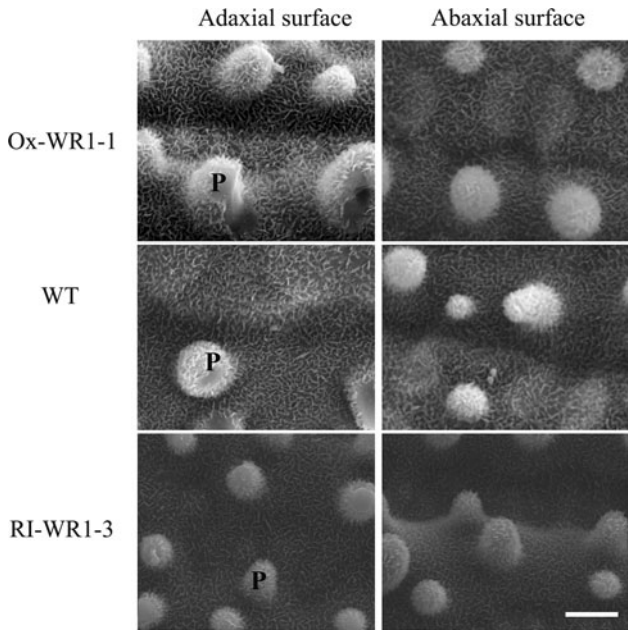

Fig. 6 Analyses of wax crystals in rice leaves. Electron microscopic analysis of wax crystals in the Ox-WR1-1, RI-WR1-3 and WT. The scale is 3 μm with images taken at ×5,000. P: cuticular papillae

(GC–MS) to analyze the wax components in WT, OxWR1-1 and RI-WR1-3. The GC–MS data indicated that wax production increased about 26% in Ox-WR1-1 leaves and decreased 21% in RI-WR1-3 seedlings, compared to this in WT leaves. And the alterations were mainly due to changes in fatty acids, alkanes and esters, whereas the changes in primary alcohols in WT and RI-WR1-3 leaves were not significant and even reduced in Ox-WR1-1 leaves (Fig. 7a). Further analyses showed that the range in chain lengths of these compounds was C18–C52 with strong predominance of even carbon numbers. The most prominent compounds were the C30 primary alcohol and C30 fatty acid, accounting for >60% of the total wax coverage (Fig. 7b). The knockdown of *OsWR1* in RI-WR1-3 mainly declined wax compound classes fatty acids and alkanes (Fig. 7a); and there were different declines in the contents of C16, C18, C20, C22, C26, C28, C30 and C32, excluding the C24 fatty acid (Fig. 7b). The change of content of the C30 fatty acid was most notable in RI-WR1-3 plants, which declined about 57% relative to WT. In contrast to RI-WR1-3, Ox-WR1-1 plants increased 36% of the C30 fatty acid, compared to this of WT, one of the most significant changes in wax composition. In addition, C25 alkanes and C48 esters were notably increased, whereas the content of primary alcohols somewhat decreased in C22, C24, C28 and C30 in Ox-WR1-1 leaves (Fig. 7b).

OsWR1 modulates the expression of wax biosynthesis related genes in rice

To further demonstrate that the changes of wax synthesis in transgenic seedlings were associated with wax synthesis, we determined the effects of OsWR1 on the expression of wax synthesis related genes. A set of genes that were potentially implicated in epidermal wax biosynthesis were obtained by genome annotation of *O. sativa* ssp. *japonica* and comparative genome analysis with *Arabidopsis thaliana* (Table S2). Among the selected putative wax synthesis related and possible regulatory genes, 12

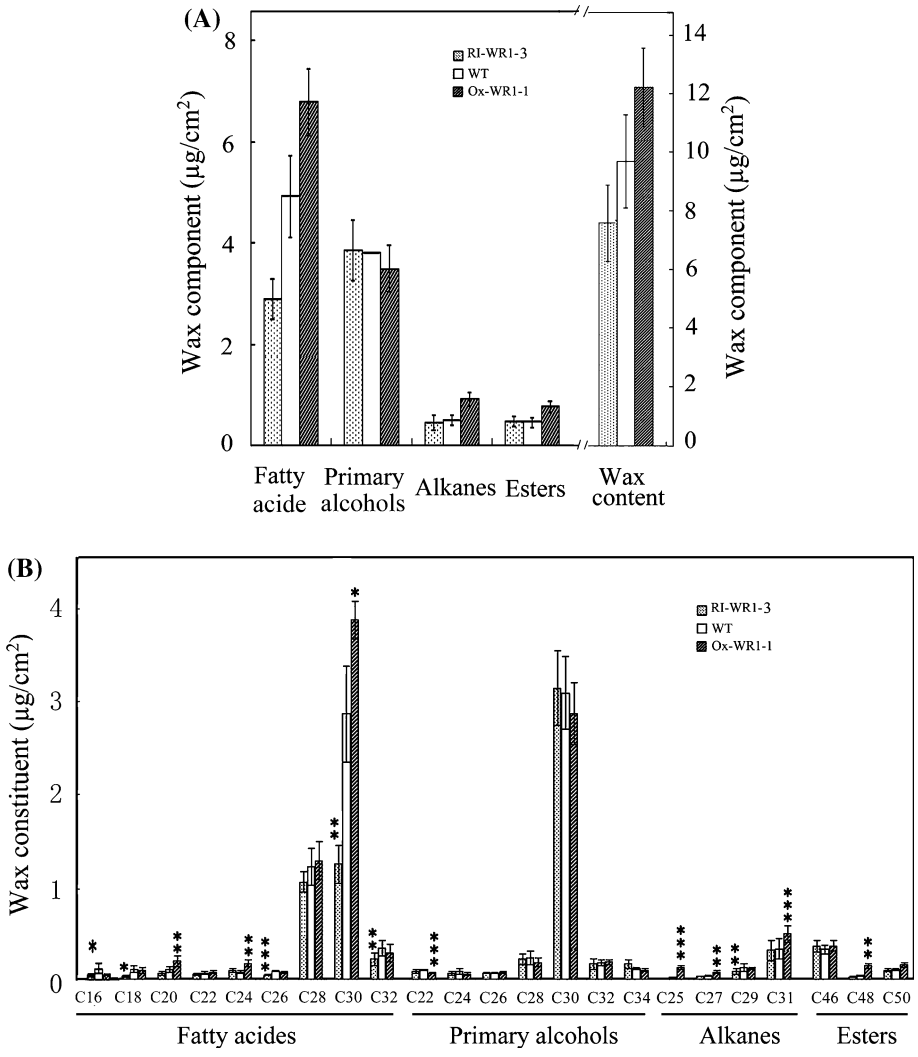

Fig. 7 Analyses of wax contents, components and constituents in rice leaves. **a** Quantitative analysis of wax components and contents in rice leaves. **b** Wax constituents of fatty acids, primary alcohols, alkanes and esters in leaves. The results show averages of three replicates, and error bars indicate ± SD. Levels of significance between WT, Ox-WR1-1 and RI-WR1-5 were determined using Student's *t* test for unequal variance and marked by the following: $*P < 0.1$; $**P < 0.05$ and $***P < 0.01$

were found to be regulated by OsWR1. Our results showed that *OsLACS1*, *OsLACS1-2*, *OsFDH1/2*, *OsCER1/2*, *OsCUT1*, *OsKCS1*, *OsFAE1-L* and *OsFAE1-L'* were >twofold upregulated in Ox-WR1 plants but obviously downregulated in RI-WR1 seedlings. In addition, *OsCER3* and *OsKCS2* were significantly increased in Ox-WR1, but not obviously decreased in RI-WR1 plants (Fig. 8a), indicating that OsWR1 modulates the expression of wax synthesis related genes.

Since shorter chain fatty acids (C16–C18), which might be precursors of cutin, decreased in RI-WR1-3, though did not obviously increase in Ox-WR1-1 (Fig. 7b), we then determined the effects of OsWR1 on the expression of cutin synthesis related genes (Table S1). Among the 11 selected putative cutin synthesis related genes, 7 (*OsCYP86A7-1–3*, *OsHTH-like*, *OsPDHc1-1*, *OsGPAT4* and *OsGDSL-2*) were regulated by Ox-WR1, but only 4 (*OsCYP86A7-1–3* and *OsGDSL-2*) were consistently downregulaed by RI-WR1 (Fig. 8b), revealing that the possible activation of OsWR1 in cutin synthesis as well.

OsWR1 binds to fragments containing GCC box and DRE in the promoters of the *OsFAE1-L'* and *OsLACS1* genes

To determine which genes were most likely targeted by OsWR1, we screened sequences upstream of the coding regions for the presence of GCC box and DRE, which have been described to interact with the ERF proteins (Nakano et al. 2006, Zhang et al. 2009; Wan et al. 2011). For this analysis, promoter sequences up to 2 kb in length were examined using an online program (PLACE) designed to recognize plant transcription factor-specific motifs (Higo et al. 1999). Among the above detected genes possibly activated by OsWR1, seven wax synthesis related genes (e.g. *OsFDH1*, *OsCER2*, *OsKCS1*/2, *OsLACS1*, *OsFAE1-L* and *OsFAE1-L'*), and 9 cutin synthesis related genes (e.g. *OsCYP86A7-1–3*, *OsGPDHc1-1–2, OsHTH-like, OsGDSL-1, OsGPAT4* and *OsLACS2-2*) contain GCC box or/and DRE in their promoters (Fig. S3).

Because of the character of ERF proteins that identify the GCC box and DRE, it is greatly possible that OsWR1 at

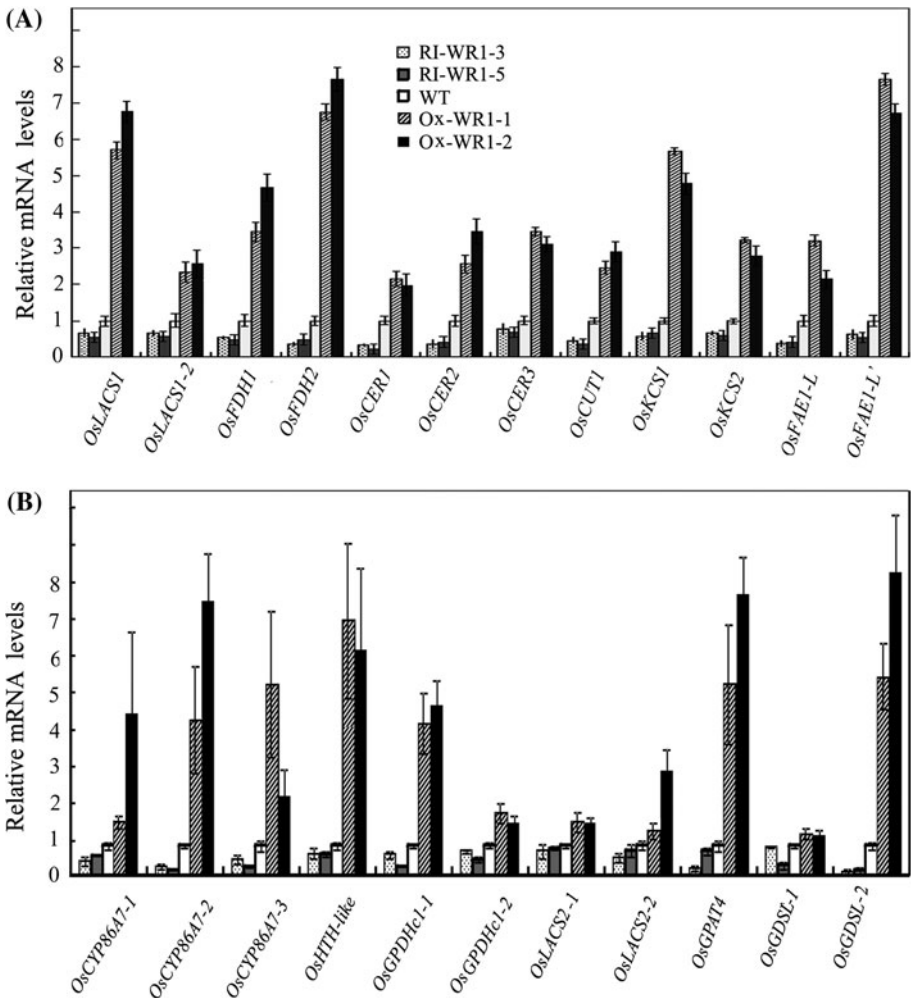

Fig. 8 The expression of wax and cutin synthesis related in rice. Expression of wax (**a**) and cutin synthesis related genes (**b**). *OsActin1* was used as an internal control. The expression of wax/cutin synthesis related genes was assessed by the relative expression level of those genes in WT rice (transcripts of each gene in WT were taken as 1). The result shows the average of three independent experiments, and *error bars* indicate ± SD

least partially directly activates some wax and/or cutin synthesis related genes. Then we were questioned whether OsWR1 directly interacts with the promoters of wax/cutin synthesis related genes. To address this query, we performed chromatin immunoprecipitation (ChIP) experiments using a specific anti-HA antibody (Orlando and Paro 1993). In our assays, we used Ox-WR1-1 transgenic line that contained HA tag. Input DNAs before immunoprecipitation were used as positive controls. After PCR screening, only two of the wax synthesis related genes were found to be directly targeted by OsWR1 in plants. The P3 region that contained one GCC box in *OsLAE1-L'* promoter was immunoprecipitated by OsWR1, but P1 and P2 regions were not (Fig. 9a); whereas the P1 region in *OsLACS1* promoter containing one DRE interacted with OsWR1 as well, although P2 and P3 fragments containing GCC box and DRE did not show any interaction with OsWR1 (Fig. 9b).

Next we applied an EMSA to further delimit the OsWR1 target site in P3 of *OsFAE1-L'* and P1 of *OsLACS1*. The GCC box from P3 of *OsFAE1-L'* and the DRE from P1 of *OsLACS1* were first made by annealing the synthesized oligonucleotides (Table S1). Using these fragments as probes, we revealed that OsWR1–GST, but not GST-only (as negative control), retarded the movement of DRE and GCC box probes (Fig. 9c, d). To further examine the specific binding, competition assays were performed with an excess of unlabeled probes. Our assays showed that unlabeled GCC box or DRE (at 500 times excess) almost completely inhibited the binding activity (Fig. 9c, d). Thus our data evidence that OsWR1 physically interacts with the GCC box and DRE in the wax synthesis related gene promoters.

Discussion

Accumulating research has shown the major genes that encode the enzymes catalyzing some of the steps in the wax pathway in Arabidopsis (Kunst and Samuels 2009;

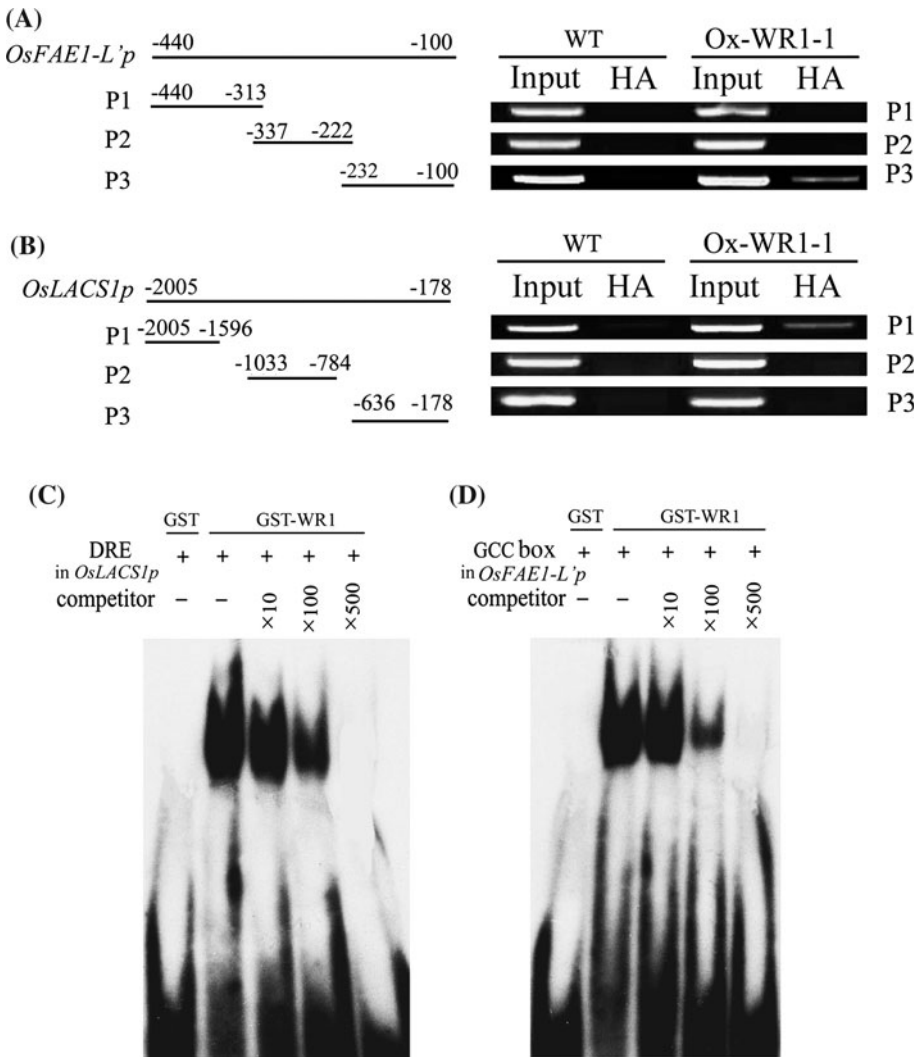

Fig. 9 Interaction of OsWR1 with the promoters of wax synthesis related genes in vivo and in vitro. The interaction of *OsFAE1-L'* promoter (**a**) and *OsLACS1* promoter (**b**) with OsWR1 in plants. *Left panels* in **a** and **b** show that the fragments of promoters of wax synthesis related genes were used for PCR amplifications. *Numbers* indicate the positions of the nucleotides at the 5′ or 3′ ends of each fragment relative to the translation start site. *Right panels* present PCR samples before input and after immunoprecipitation with antibody HA detected using specific primers. Detection of the interaction of OsWR1 with DRE (**c**) and GCC box (**d**) using the electrophoretic mobility-shift assay. Probe sequences of DRE from *OsLACS1* and GCC box from *FAE1-L'* are given in Table S1. Competition assays were processed using unlabeled probes

Schreiber 2010). Some ERF proteins and other proteins have been reported in wax biosynthesis; however, few of the mechanisms concerning how these genes are associated with this process have been elucidated in rice. In the present research, we reported a homolog ERF protein of Arabidopsis wax synthesis regulator WIN1/SHN1, OsWR1 that at least partially directly activates the expression of wax synthesis related genes to enhance wax production, through the regulation of the components of wax biosynthesis. And this regulation results in the changes of cuticular permeability and water loss, subsequently improving the drought tolerance in rice. Thus our data indicate that OsWR1 is a positive regulator of wax synthesis in rice.

The Arabidopsis genes, such as *CER1* (McNevin et al. 1993), *CUT1*/*CER6* (Millar et al. 1999), *KCS1* (Todd et al. 1999), *FDH* (Pruitt et al. 2000), and the maize genes *GL1* (Sturaro et al. 2005) and *GLOSSY2* (Tacke et al. 1995) have been proposed or shown to encode enzymes or components of the secretory pathway (Millar et al. 1999; Todd et al. 1999; Chen et al. 2003; Islam et al. 2009; Qin et al. 2011). Other genes, such as *CER2* and *CER3*, encode regulatory proteins that might be involved in wax biosynthesis (Xia et al. 1996; Rowland et al. 2007). In Arabidopsis, ERF protein WIN1/SHN1 at least directly activates the expression of cutin synthesis gene and controls compositional changes and cutin production. This transcriptional regulation is followed after a delay by the induction of wax biosynthesis genes, which in turn causes the wax production (Aharoni et al. 2004; Broun et al. 2004; Kannangara et al. 2007). Distinct to the regulation of WIN1/SHN1, alteration of *OsWR1* gene in rice directly affected the expression of wax synthesis related genes, subsequently causing wax accumulation. Among these genes, several exhibited expression patterns associated with expression of *OsWR1*, which is mainly expressed in leaves. In addition, Arabidopsis WIN1/SHN1 downregulated the expression of *FAE* gene (Aharoni et al. 2004; Broun et al. 2004; Kannangara et al. 2007), while OsWR1 upregulated two *FAE* genes that encode ketoacyl-CoA synthase. It is known that acyl chain extensions are carried out by several distinct elongases with unique substrate chain specificities (Kunst and Samuels 2003). Thus OsWR1 may positively impact on the fatty acid elongation process for the production of VLCFA used for producing aliphatic wax components. Although OsWR1 modulates the expression of cutin related genes, however, contents of C16 fatty acid, which might be cutin monomers, were not increased in the OsWR1-overexpressing plant, especially we did not detect the direct interaction of OsWR1 with these gene's promoters (Data not shown), suggesting that the regulation of OsWR1 on cutin synthesis genes is mediated by other cofactors. Thus, our data reveal that OsWR1 at least partially directly modulates the wax synthesis, different from the regulation of the homolog of WIN1/SHN1 in cutin synthesis.

Wax composition in mature leaves including rice (Jung et al. 2006), Arabidopsis (Suh et al. 2005; Seo et al. 2011), maize (Blaker and Greyson 1988), tree tobacco (Cameron et al. 2006) and tomato (Leide et al. 2007) was predominantly odd numbered alkanes, while recent report indicates that the cuticular wax in rice consists mainly of fatty acids, primary alcohols and aldehydes (Mao et al. 2011). Interestingly, in the present research, we found that VLCFAs and primary alcohols are shown as the major constituents. Actually, increasing evidence reveals that different wax biosynthesis genes, even the homologous genes from different plants, confer different wax compositions (Aarts et al. 1995; Chen et al. 2003; Sturaro et al. 2005; Zhang et al. 2005). For instance, Arabidopsis WIN1/SHN1 increases cuticular wax levels, mainly because of an increase in alkanes that comprised approximately half of the total waxes (Aharoni et al. 2004; Broun et al. 2004); Medicago WXP1 confers cuticular wax accumulation and the increase was mainly contributed by C30 primary alcohol (Zhang et al. 2005); whereas rice OsGL1-2 has a critical role in fatty acid precursors C18 and C20 biosynthesis of cuticular wax (Islam et al. 2009). Distinctive to the regulation of WIN1/SHN1 and OsGL1-2, our results in this report show that OxWR1 regulates wax production mainly through affecting the constituent of C30 fatty acid.

Many factors including water potential, osmotic adjustment, cell membrane stability, cuticular wax characteristics and epidermal conductance affect plant water status and drought tolerance. Due to the multiple importance of wax as a waterproof barrier and for primary protection against environmental stresses, increasing evidence reveals that the key function of wax is to prevent uncontrolled loss of water (Post-Beittenmiller 1996; Millar et al. 1999; Raffaele et al. 2008). To elucidate the molecular basis underlying wax biosynthesis related drought response, we firstly found that the changes of the water loss were associated with the alteration of *OsWR1* transcripts in transgenic lines. Then, with chlorophyll leaching assays, we further evidenced that Ox-WR1 lines had much less chlorophyll leaching, whereas the RI-WR1 lines lost chlorophyll significantly faster than the wild type did. Furthermore, *OsWR1*, showing high similarity to Arabidopsis wax/cutin controlled stress regulator WIN1/SHN1, was induced by drought, ABA and salt, suggesting that the OsWR1 might be associated with wax/cutin controlled stress regulator. To test the hypothesis, we checked the wax status using Ox-WR1 and RI-WR1 plants, showing significant increase and decrease, respectively. These observations suggest that the regulation of OsWR1 in wax synthesis related genes and wax production possibly affects the water loss and drought tolerance in plants, consistent with the wax function in

drought response (Aharoni et al. 2004; Broun et al. 2004; Islam et al. 2009, Qin et al. 2011; Seo et al. 2011). Therefore, data in the present report show that OsWR1 is, to our knowledge, the first example of an ERF transcriptional activator affecting the expression of wax synthesis related genes in rice. Manipulation of OsWR1 levels will lead to a better understanding of wax biosynthesis and the associated mechanisms.

It was reported that the increase in wax load in maize and Arabidopsis led to the retarded phenotype (Aharoni et al. 2004; Broun et al. 2004). Similarly, though Ox-WR1 lines did not show any obvious development disadvantages in the seedling stage, however, the height of Ox-WR1 lines was obviously shorter than the wild type during flowering stage (data not shown), demonstrating that the OsWR1 affected the development of rice but not so obvious as that in Arabidopsis. In addition, the Arabidopsis *lacs1* and *lacs2* just resulted in 5–35% decrease in cuticular wax (Lu et al. 2009). Because of the prevention of water loss caused by cuticular wax in Ox-WR1 lines, it would be possible that plants need a compensation system to keep a balance in stress tolerance. To address this possibility, we propose that ROS-scavenging enzymes would have contribution to the wax-associated drought response. In the present report we reveal that OsWR1 participates in the regulation of oxidative stress response and membrane stability, and this regulation might be a compensation for the modulation of OsWR1 in wax synthesis-associated drought response.

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