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OsWRKY42 Represses *OsMT1d* and Induces Reactive Oxygen Species and Leaf Senescence in Rice

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We isolated a rice (*Oryza sativa* L.) *WRKY* gene which is highly upregulated in senescent leaves, denoted *OsWRKY42*. Analysis of *OsWRKY42-GFP* expression and its effects on transcriptional activation in maize protoplasts suggested that the OsWRKY42 protein functions as a nuclear transcriptional repressor. *OsWRKY42*-overexpressing (*OsWRKY42OX*) transgenic rice plants exhibited an early leaf senescence phenotype with accumulation of the reactive oxygen species (ROS) hydrogen peroxide and a reduced chlorophyll content. Expression analysis of ROS producing and scavenging genes revealed that the metallothionein genes clustered on chromosome 12, especially *OsMT1d*, were strongly repressed in *OsWRKY42OX* plants. An *OsMT1d* promoter:*LUC* construct was found to be repressed by *OsWRKY42* overexpression in rice protoplasts. Finally, chromatin immunoprecipitation analysis demonstrated that OsWRKY42 binds to the W-box of the *OsMT1d* promoter. Our results thus suggest that OsWRKY42 represses *OsMT1d*-mediated ROS scavenging and thereby promotes leaf senescence in rice.

INTRODUCTION

Senescence is a final developmental process in the life cycle of plants and a type of programmed cell death. Senescence is controlled by multiple developmental factors and also external biotic and abiotic stress signals (Buchanan-Wollaston et al., 2005; Lim et al., 2007; Munne-Bosch and Alegre, 2004). Comprehensive analyses of altered gene expression patterns in genetically controlled senescent leaves, as a study model for plant senescence, has identified important regulatory factors. To date, over 100 transcription factors, most notably belonging to the WRKY, NAC, C2H2 zinc finger, AP2/EREBP, and MYB families, have been identified to be highly upregulated in senescent leaves in *Arabidopsis* (Balazadeh et al., 2008; Buchanan-Wollaston et al., 2005; Guo et al., 2004). These results suggest that the transition from mature to senescent leaves is achieved by a massive reprogramming of gene expression.

The WRKY transcription factors constitute one of the largest transcription factor families in plants. WRKY proteins have the highly conserved DNA-binding domain of one or two 60 amino acid regions harboring WRKYGQK and a zinc finger structure. WRKYs have been shown to play important roles in the senescence process. For instance, the *Arabidopsis atwrky53* mutant shows a delayed leaf senescence whereas *AtWRKY53* overexpression resulted in precocious senescence, suggesting that *AtWRKY53* functions as a positive regulator of leaf senescence (Miao et al., 2004; 2007). The *Arabidopsis* double mutant *atwrky54*/*atwrky70* displays a significantly enhanced senescence phenotype, suggesting that AtWRKY70 and AtWRKY54 act as negative regulators of leaf senescence (Besseau et al., 2012).

The production of reactive oxygen species (ROS) is one of the earliest components of the leaf senescence pathway (Jing et al., 2008; Mittler et al., 2004; Zentgraf and Hemleben, 2008). For instance, AtWRKY53 and its regulators are controlled by hydrogen peroxide (H_2O_2) (Miao et al., 2007; 2008). Cellular levels of ROS have been positively correlated with the severity of leaf senescence in *Arabidopsis* and oilseed rape (Bieker et al., 2012). The ROS status has been found to be controlled by a fine-tuned network of enzymatic and antioxidative components consisting of ROS-producing and ROS-scavenging proteins (Mittler et al., 2004; Kim et al., 2012). ROS are produced under stress conditions primarily as byproducts of normal metabolic processes, such as respiration and photosynthesis, in chloroplasts, mitochondria and peroxisomes and also at the cell surface and exterior by the activity of multiple enzymes including NADPH oxidases (Apel and Hirt, 2004; Mittler et al., 2004; Noctor et al., 2014). ROS are scavenged by enzymes such as superoxide dismutase, catalase, and peroxidase (Apel and Hirt, 2004; Jang et al., 2012) and also nonenzyme components that include low molecular antioxidants, such as ascorbate, glutathione, carotenoids, and metallothioneins (MTs) (Gechev et al., 2006). MTs harbor conserved cysteine-rich domains (Hassinen et al., 2011; Yang et al., 2009), and the OsMT1d and OsMT2b proteins in rice have also been shown to be ROS scavengers

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(Hu et al., 2011; Steffens and Sauter, 2009; Wong et al., 2004).

Few studies to date have focused on leaf senescence in rice, a vital commercial crop plant that feeds more than half of the world's population. In particular, the molecular regulatory mechanism of transcription factors underlying leaf senescence remains largely unknown in rice. The OsWRKY family comprises over 100 members in the rice genome (Rice WRKY Working Group, 2012). To date, most of the functionally characterized OsWRKYs have been reported to play roles in defense responses to biotic pathogens and also abiotic stress responses to environmental stimuli and hormones (De Vleesschauwer et al., 2013; Jang et al., 2010; Ryu et al., 2006). In our present study, we describe the isolation and characterization of a leaf senescence-inducible *OsWRKY* gene in rice, *OsWRKY42*. We provide evidence that the OsWRKY42 protein functions as a positive regulator of leaf senescence in rice by increasing the ROS level *via* the repression of the *OsMT1d* gene.

MATERIALS AND METHODS

Plant materials and growth conditions

Rice [japonica cultivar (cv.) Dongjin] plants were grown in a greenhouse under a 14/10 h light and dark period, at 24-28°C temperature and 70-80% humidity.

RNA isolation and RT-PCR analysis

Total RNA was prepared from various tissues of rice plants using Trizol reagent (Invitrogen, USA) with DNase treatment (TURBO DNA-free kit; Ambion-Life technologies, USA). The extracted RNA was reverse-transcribed using AMV reverse transcriptase XL (2620A; Takara, Japan) with RNase inhibitor (2312A; Takara) and an oligo-dT primer. The synthesized first strand cDNA was used in subsequent PCR reactions with gene-specific primers and control primers for *OsUBQ5*. The primers were designed in the region encompassing at least one intron for each gene to exclude any influence by genomic DNA contamination (Supplementary Table 1; Jain et al., 2006).

Transient expression assay in mesophyll protoplasts

To examine the transcriptional activation ability of OsWRKY42, the effector vector was constructed by fusing the *OsWRKY42* cDNA amplified by PCR using FL-F/R primers containing *Sma*I and *Sal*I restriction sites, respectively (Supplementary Table 1). The resulting vector contained the 35S promoter, the tobacco mosaic virus (TMV) translation enhancer (Ω) sequence, and the *OsWRKY42* cDNA insert fused to the GAL4 DNA binding domain (BD) in frame. The *OsWRKY30*-carrying effector vector was then used as a control transcriptional activator (Han et al., 2013). The GAL4-responsive reporter vector contained 5X GAL4, minimal TATA, the Ω sequence, and the *Luciferase* (*LUC*) gene (Ohta et al., 2000). The maize *Ubiquitin1* promoter:*β-glucuronidase* (*ZmUBQ1*:*GUS*) construct was used as an internal control (Cho et al., 2009). Maize mesophyll protoplasts isolated from the second leaves of dark grown plants were cotransfected with the effector, reporter and internal control vectors as described previously (Cho et al., 2009).

To analyze transcriptional repression by *OsWRKY42*, two reporter vectors, *OsMT1a*:*LUC* and *OsMT1d*:*LUC*, were constructed with promoter regions from nucleotides -1496 to -1 and -1427 to -1, respectively. These positions are relative to the +1 A translational initiation codons of *OsMT1a* and *OsMT1d* which were amplified by PCR using *OsMT1a* pro F/R and *OsMT1d* pro F/R primers containing *Bam*HI and *Sal*I restriction sites (Supplementary Table 1). The amplified fragments were inserted into the JJ803 vector carrying *LUC* derived from pGL2 (Promega, USA). The *35S*:*OsWRKY42-cMyc* fusion effector construct was generated by insertion of an *OsWRKY42* cDNA fragment without the stop codon that had been amplified using GFP-F/R primers, between the Ω and c-Myc of the pJJ1754 vector (Supplementary Table 1). *ZmUBQ1*:*GUS* was used as an internal control (Cho et al., 2009). The constructed vectors were transformed into rice protoplasts isolated from the second leaves of young plants (Zhang et al., 2011) using the polyethylene glycol-calcium-mediated method (Cho et al., 2009). Transfected protoplasts were incubated for 12 h in W5 solution (Zhang et al., 2011) and harvested in lysis buffer (Promega). LUC activity was analyzed using a luciferase assay system (E1500; Promega) and a VICTOR2 multilabel counter (PerkinElmer, USA). GUS assays were performed using a previously described method (Jefferson et al., 1987). All transient expression experiments were repeated three times and gave similar results.

Subcellular localization analysis of *OsWRKY42*

The *OsWRKY42* cDNA insert without its stop codon was amplified using GFP-F/R primers containing *Xba*I and *Xho*I restriction sites, respectively (Supplementary Table 1). The amplified fragments were digested with the respective restriction enzymes and then subcloned between the 35S promoter and *sGFP* of the pJJ461 vector derived from the binary vector pC1300intC (Ouwerkerk et al., 2001). The resulting GFP fusion construct was transiently expressed in maize protoplasts (Cho et al., 2009), which were subsequently examined by laser-scanning confocal microscopy (LSM 510 META, Carl Zeiss, Germany). Chlorophyll autofluorescence (Han et al., 2012) and OsABF1-RFP (Han et al., 2013; Hossain et al., 2010; Truernit and Haseloff, 2008) were used as chloroplast and nuclear markers, respectively.

Rice transformation

OsWRKY42 cDNA was amplified by RT-PCR using the cDNA F/R primers containing *Sma*I and *Kpa*I restriction sites, respectively (Supplementary Table 1), and inserted between the *ZmUBQ1* promoter and *Nopaline synthase* (*Nos*) terminator of pGA1611 (Kim et al., 2003). The resulting construct, *ZmUBQ1*:*OsWRKY42*, was used for rice (cv. Dongjin) transformation *via* *Agrobacterium tumefaciens* (LBA4404) as described previously (Jeon et al., 2000).

Transient expression assay in tobacco leaves

The *35S*:*OsWRKY42-cMyc*, *35S*:*GFP* (pJJ461) and the corresponding empty vector (pJJ1754) were introduced into *A. tumefaciens* strain GV3101. Overnight cultures were harvested and resuspended in 10 mM $MgCl_2$ to an OD_{600} of 0.5. After incubation at room temperature for 3 h, the cells were used to infiltrate six-week-old *Nicotiana benthamiana* leaves using a needleless syringe (1 ml) as described previously (Romeis et al., 2001; Seo et al., 2012). Plants were grown and maintained throughout the experiments in a growth chamber at a temperature of 25°C and under 12 h of light.

Hydrogen peroxide assay

For the detection of H_2O_2, fully expanded second leaves from the top of six-week-old wild type and transgenic plants were placed in solution of 1 mg ml^{-1} 3,3-diaminobenzidine (DAB) and vacuum infiltrated at 650 mmHg for 10 min. The leaves were then immersed in DAB solution for 3 h in darkness. Subsequently, the treated leaves were decolorized in 80% ethanol,

placed onto glass slides and observed under a stereomicroscope (Olympus, USA). The H_2O_2 content was also measured colorimetrically in the same fully expanded second leaves from the tops of six-week-old wild type and transgenic plants using 50 mM phosphate buffer (pH 6.5) containing 1 mM hydroxylamine (Jana and Choudhuri, 1982). After centrifugation at 6,000 × *g* for 25 min, the extracts were mixed with 0.1% titanium sulphate in 20% (v/v) H_2SO_4. After centrifugation, the absorbance was measured at 410 nm and the H_2O_2 content was calculated using the extinction coefficient 0.28 μmol^{-1} cm^{-1}.

Chlorophyll assay

Rice leaves were prepared from the fully expanded second leaves from the tops of six-week-old wild type and transgenic plants. The total chlorophyll contents were measured spectrophotometrically after extraction in 80% acetone using the method of Porra et al. (1989).

Promoter sequence analysis of *OsMT1* genes

The sequences at approximately 1.5-kb upstream from the coding regions of *OsMT1* genes were analyzed for the W-box using the PLACE Signal Scan program (Higo et al., 1999). W-box elements harboring the consensus sequence were identified according to published criteria (Eulgem et al., 2000; Kim and Zhang, 2004).

Chromatin immunoprecipitation analysis

For chromatin immunoprecipitation (ChIP) analysis, the *35S*:*OsWRKY42-cMyc* construct was transfected into rice protoplasts as described above. ChIP analysis was then performed using the EpiQuik Plant ChIP kit (Epigentek, USA) with the cMyc antibody (G3109; Santa Cruz Biotechnology, USA). Transfected protoplasts were harvested with 1% formaldehyde solution to cross-link the proteins to the DNA. The cross-linked DNA was sheared by sonication into 200-500 bp fragments in the presence of a protease inhibitor cocktail. A dilution of the resulting supernatant was incubated with an anti-cMyc- bound strip at room temperature for 90 min with orbital shaking. Relative enrichment was measured by comparing the input and ChIP values. Normal mouse IgG was used as a negative control antibody. All of the primers used in the ChIP assays are listed in Supplementary Table 1.

RESULTS

Identification of a senescence-inducible *OsWRKY42* gene in rice leaves

Through a systematic screen of the digital expression profile of rice *WRKY* genes using the RiceXPro (Sato et al., 2011; http://ricexpro.dna.affrc.go.jp/) database, we identified the *OsWRKY42* gene (LOC_Os02g26430) to be upregulated during the ripening stage that accompanies leaf senescence. Subsequent RT-PCR confirmed that *OsWRKY42* is highly expressed only in senescent leaves, and is present at much lower levels in the young and mature leaves of rice plants (Fig. 1). The senescence-associated gene, *Staygreen* (*SGR*) (Park et al., 2007), was also found to be increased only in senescent leaves. The *Rubisco small subunit* (*RbcS*) gene was strongly downregulated in the senescent rice leaves, while the housekeeping gene *OsUBQ5* was constitutively transcribed. Our results thus indicated that *OsWRKY42* is strongly upregulated in senescent rice leaves.

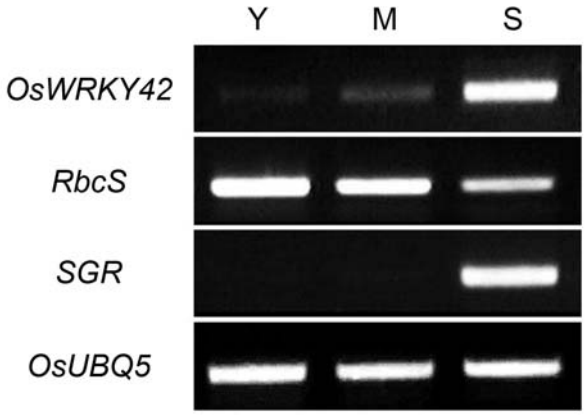

Fig. 1. Expression pattern of the *OsWRKY42* gene in rice leaves. *RbcS* was used as a senescence-downregulated marker, whilst *SGR* was used as a senescence-inducible gene. Y, young leaves of three-week-old plants; M, fully expanded mature leaves of ten-week-old plants; S, senescent flag leaves of 17-week-old plants. *OsUBQ5* was amplified as an internal RT-PCR control.

Subcellular localization and transcriptional activator properties of OsWRKY42

To next examine the subcellular localization of OsWRKY42, we generated an *OsWRKY42-GFP* fusion construct for introduction into maize mesophyll protoplasts (Fig. 2A). The green OsWRKY42-GFP signal merged very tightly with the RFP (red) signal of the OsABF1-RFP nuclear marker (Hossain et al., 2010). The auto-fluorescence of chlorophyll was used as a chloroplast marker. This indicated that the OsWRKY42 protein localizes in the nucleus.

The transcriptional activator ability of OsWRKY42 was examined using the maize protoplast transient expression system (Cho et al., 2009). The effector vectors used for this experiment contain either BD alone, or *OsWRKY30* or *OsWRKY42* fused to BD (Fig. 2B). BD- and BD-OsWRKY30 were used as negative and positive controls, respectively. In the maize protoplasts cotransfected with the effector and reporter vectors, the LUC activity of the effector BD-OsWRKY42 (OsW42) was reduced by about 0.4-fold of the empty vector (EV) control (Fig. 2C). The known transcriptional activator OsWRKY30 (OsW30; Han et al., 2013) increased LUC reporter activity (Fig. 2C). This result suggests that OsWRKY42 functions as a transcriptional repressor in the nucleus.

Phenotype of *OsWRKY42* transgenic rice plants

To further investigate the role of *OsWRKY42* in rice, we produced 12 transgenic plants expressing *ZmUBQ1*:*OsWRKY42*. Ten of the primary transgenic rice plants that highly expressed *OsWRKY42* displayed an extremely early onset of leaf senescence, eventually leading to plant death (Supplementary Fig. 1). Only two independent transgenic rice lines, *OsWRKY42OX1* and *OsWRKY42OX3*, with relatively low transgene expression could grow and set a small number of seeds. The progeny of these plants were analyzed in further experiments (Fig. 3A) and clearly showed an early leaf senescence phenotype at the three-week-old plant stage (Fig. 3B).

To examine whether *OsWRKY42* could induce early leaf senescence in other heterologous species, we expressed the gene under the control of 35S promoter in tobacco leaves by

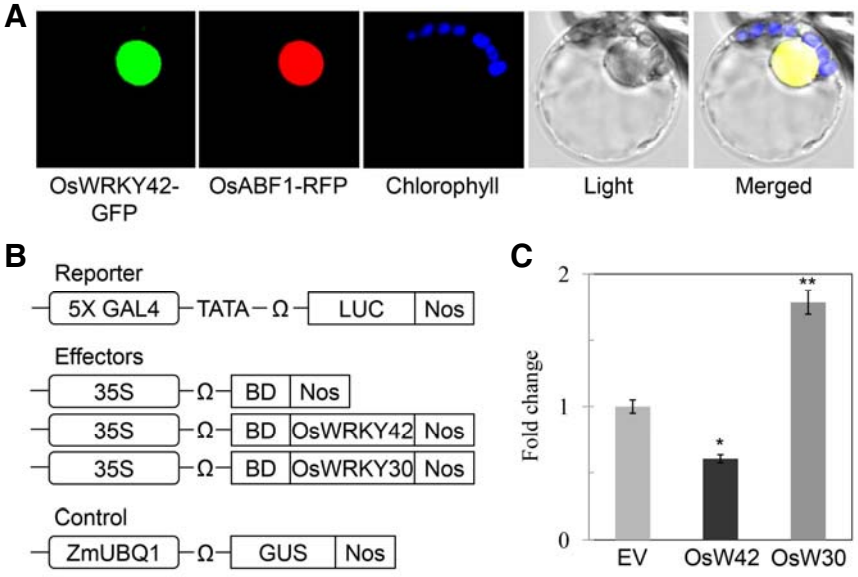

Fig. 2. Subcellular localization and transcriptional activation ability analysis of OsWRKY42. (A) Nuclear localization of the OsWRKY42-GFP fusion protein in maize protoplasts. Photographs were taken in the dark field for green fluorescence and in the bright field for cell morphology. OsABF1-RFP was used as a nuclear marker. (B) Schematic representation of constructs used for analysis of GAL4-responsive transcription by OsWRKY42 fused to the GAL4 BD in maize protoplasts. *ZmUBQ1*:*GUS* was used as an internal control. (C) Relative luciferase activities in maize protoplasts after co-transfection with the reporter, effector and control plasmids. LUC activities were normalized after transfection with the empty vector control (arbitrarily set at 1). EV, empty vector; OsW42, BD-OsWRKY42; W30, BD-OsWRKY30. **P* < 0.05, ***P* < 0.01.

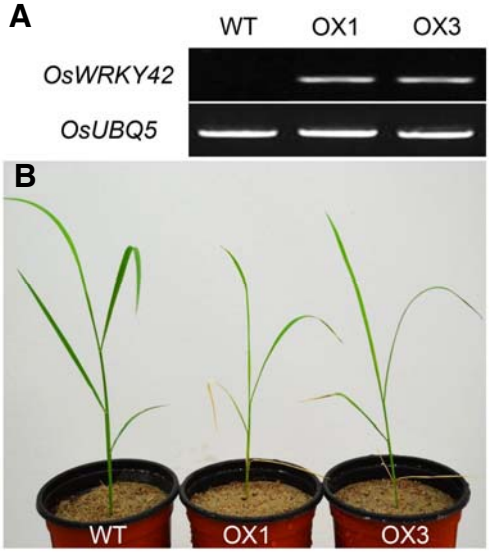

Fig. 3. Characterization of *OsWRKY42*-overexpressing (*OsWRKY42OX*) transgenic rice plants. (A) RT-PCR analysis of *OsWRKY42* expression in *OsWRKY42OX1* (*OX1*) and *OsWRKY42OX3* (*OX3*) transgenic lines. *OsUBQ5* was amplified as an internal RT-PCR control. (B) Phenotype of three-week-old *OsWRKY42OX* transgenic lines (OX1 and OX3) in comparison with wild type (WT).

Agrobacterium-mediation (Supplementary Fig. 2) (Romeis et al., 2001; Seo et al., 2012). At 10 days after inoculation, only *OsWRKY42-cMyc* expression (OsW42) caused early leaf senescence compared to the controls, *Agrobacterium* background GV3101 strain and *Agrobacterium* carrying empty vector (EV) or *35S:GFP* (GFP). This result suggests that *OsWRKY42* can also function as a positive regulator of leaf senescence in plant species other than rice.

Hydrogen peroxide measurements in *OsWRKY42* transgenic rice plants

A disruption of the critical and tightly regulated balance between the production and scavenging of ROS has been well established as a promoter of leaf senescence (Jing et al., 2008; Mittler et al., 2004; Zentgraf and Hemleben, 2008). The early leaf senescence phenotype we observed in our current analysis prompted us examine whether the ROS level was increased in *OsWRKY42OX* transgenic rice lines. We thus examined the level of H_2O_2, the most significant ROS with a relatively long half-life, in the fully expanded second leaves from top of six-week-old *OsWRKY42OX1* and *OsWRKY42OX3* lines. DAB staining exhibited strong brown colors in the leaves of both transgenic lines compared to wild type (Fig. 4A). Consistently, we found increased levels of H_2O_2 in the leaves of *OsWRKY42OX1* and *OsWRKY42OX3* lines (Fig. 4B), which also showed lower total chlorophyll contents than wild type plants (Fig. 4C). These results suggest that *OsWRKY42* overexpression induces ROS accumulation and promotes leaf senescence.

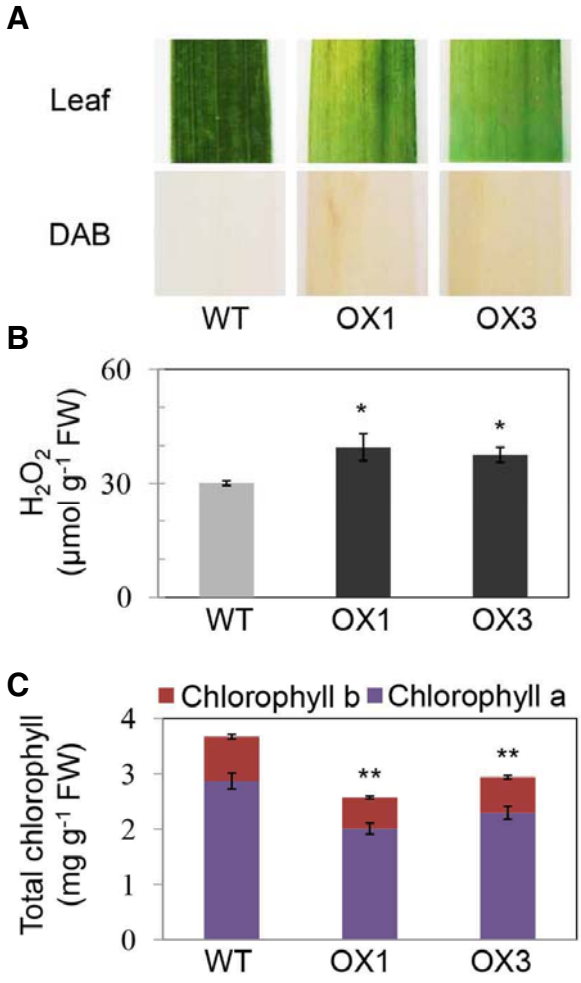

Fig. 4. DAB staining for measurement of the relative H_2O_2 levels and total chlorophyll contents in the fully expanded second leaves from the top of six-week-old *OsWRKY42OX1* (*OX1*) and *OsWRKY42OX3* (*OX3*) transgenic plants and wild type rice plants. (A) Representative leaves visualized by DAB staining of *OsWRKY42OX* lines and wild type (WT). (B) H_2O_2 levels of the *OsWRKY42OX* lines and wild type (WT). (C) Histogram of total chlorophyll contents of *OsWRKY42OX* lines and wild type (WT). Each datapoint represents the mean (± SD) from three separate experiments. $^*P < 0.05$, $^{**}P < 0.01$.

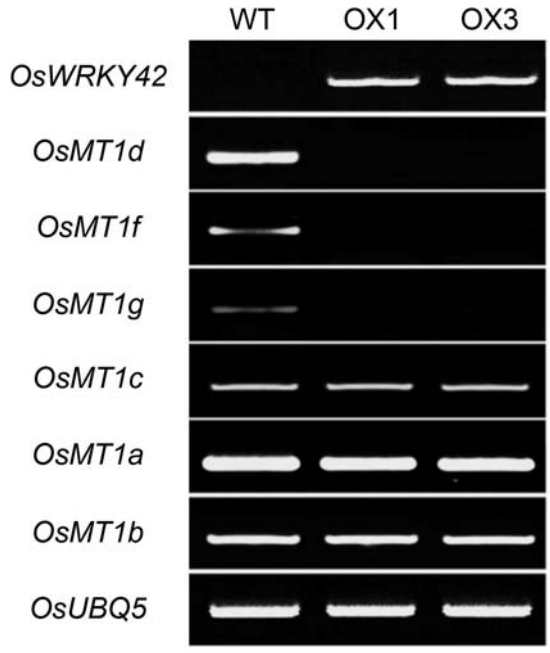

Fig. 5. Expression analysis of rice metallothionein I (*OsMT1*) genes in the leaves of *OsWRKY42OX1* (*OX1*) and *OsWRKY42OX3* (*OX3*) transgenic rice lines. Locus numbers of *OsMT1* genes are: *OsMT1a*, LOC_Os12g38270; *OsMT1b*, LOC_Os03g17870; *OsMT1c*, LOC_Os12g38051; *OsMT1d*, LOC_Os12g38300; *OsMT1f*, LOC_Os12g38010; *OsMT1g*, LOC_Os12g38290. Os*UBQ5* transcripts were amplified as an internal RT-PCR control.

Expression of ROS producing and scavenging genes in *OsWRKY42* transgenic rice plants

To examine whether any ROS producing and scavenging genes were disrupted in *OsWRKY42OX1* and *OsWRKY42OX3* lines, we compared the expression levels of a number of previously described ROS-related genes (Hu et al., 2011) in transgenic lines and wild type plants. Interestingly, we found that the highly expressed gene *OsMT1d* was strongly downregulated in *OsWRKY42OX* lines compared to wild type. The weakly expressed genes, *OsMT1f* and *OsMT1g*, were also found to be repressed in *OsWRKY42OX* lines (Fig. 5). In contrast, none of the other tested ROS producing and scavenging genes were significantly altered in this comparison (Supplementary Fig. 3). This indicates that the overexpression of *OsWRKY42* represses ROS non-enzyme scavenger *MT1* genes, in particular *OsMT1d* clustered in a short interval of chromosome 12, and thereby induces ROS accumulation and precocious leaf senescence.

Analysis of *OsMT1d* transcriptional repression by *OsWRKY42* in rice protoplasts

Analysis of about 1.5 kb upstream regulatory sequences of *OsMT1d*, *OsMT1f* and *OsMT1g*, revealed that these three genes each harbor three potential W-boxes, which are known as WRKY binding elements (Supplementary Fig. 4; Eulgem et al., 2000; Kim and Zhang, 2004). To examine whether OsWRKY42 represses *OsMT1* genes by binding to these W-boxes, we performed a transient expression assay using *LUC* reporter system (Fig. 6). For this experiment, we isolated promoters of *OsMT1d* (the nucleotides -1427~-1) and *OsMT1a* (-1496~-1) as positive (the most strongly repressed *OsMT1* gene in *OsWRKY42OX* lines) and negative (constantly expressed *OsMT1* gene) downstream targets, respectively (Fig. 6A). After coexpression of reporter and effector vectors in rice protoplasts, we found that the expression of *OsWRKY42-cMyc* strongly repressed *OsMT1d*:*LUC* expression but not *OsMT1a*:*LUC* (Fig. 6B). This indicates that OsWRKY42 represses *OsMT1d* expression.

Analysis of promoter binding by OsWRKY42 *in vivo*

The *OsMT1d* promoter contains three potential W-boxes, two copies (-1099~-1094 and -945~-940 within P2) that harbor a consensus sequence variant A/CTGACC and one copy (-729~-

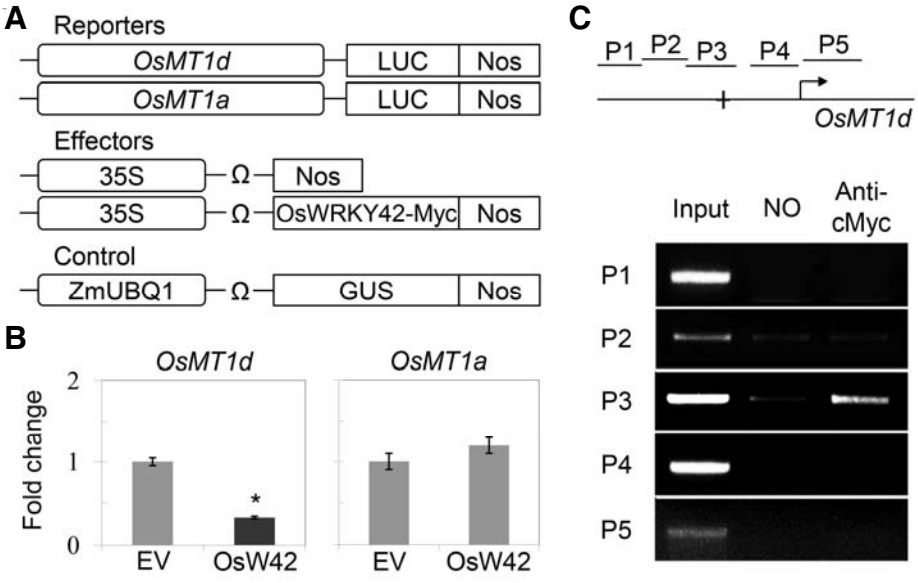

Fig. 6. Transient expression analysis of promoters of *OsMT1a* and *OsMT1d* and ChIP analysis of the *OsMT1d* promoter in rice protoplasts. (A) Schematic representation of reporters and effectors. *OsMT1a* and *OsMT1d* promoters contain the nucleotides -1496 to -1 and -1427 to -1, respectively. *ZmUBQ1*:*GUS* was used an internal control. (B) Fold changes calculated using a luciferase assay for the changed expression level of target promoter:*LUC* constructs by expression of *35S*:*OsWRKY42-cMyc* (OsW42) in rice protoplasts. LUC activities were normalized after transfection with the empty vector (EV) control that was arbitrarily assigned a value of 1. **P* < 0.01. (C) Selected promoter fragments of *OsMT1d* (top). ChIP analysis was performed of the *OsMT1d* promoter using chromatin prepared from *35S*:*OsWRKY42-cMyc* transfected rice protoplasts (bottom). ┼, W-box with the consensus sequence TTGACT.

724 within P3) with the consensus sequence TTGACT (Fig. 6C, top; Supplementary Fig. 4). To verify the *in vivo* association of OsWRKY42 and the W-box present in the *OsMT1d* promoter, we performed chromatin immunoprecipitation (ChIP) analysis (Fig. 6C, bottom). We analyzed DNA before immunoprecipitation (Input) and immunoprecipitated DNA by PCR using primers that amplify the P1 to P5 regions of the *OsMT1d* promoter. The ChIP assay revealed that the OsWRKY42-cMyc protein specifically binds to the P3 region on the *OsMT1d* promoter (Fig. 6C, bottom). This result indicates that the OsWRKY42 transcriptional repressor binds to the W-box region of the *OsMT1d* promoter and represses its expression. This in turn disrupts the ROS balance which results in the precocious leaf senescence seen in *OsWRKY42OX* transgenic rice plants.

DISCUSSION

The processes underlying senescence influence many important agricultural traits including the number and quality of seeds and timing of seed setting. However, our knowledge concerning the regulatory mechanisms that control senescence is still limited in rice, one of the world's most agronomically important crops. It has been evident that ROS trigger leaf senescence (Zentgraf and Hemleben, 2008). Plants have developed a fine-tuned network of enzymatic and low-molecular-weight antioxidative components to regulate their ROS status. Oxidative stress and senescence occur when this critical balance is disrupted because of a depletion of antioxidants or excess accumulation of ROS.

The association between ROS and the WRKY transcription factors was revealed by the finding that the *Arabidopsis AtWRKY53* gene is induced by H_2O_2 treatment (Miao et al., 2004). H_2O_2 was found to induce reporter gene expression driven by the *AtWRKY53* promoter in protoplasts prepared from wild type plants. In contrast, this induction was abolished in protoplasts prepared from *AtWRKY53* knockout lines, indicating that the AtWRKY53 protein is directly involved in the positive regulation of genes that respond to H_2O_2 (Miao et al., 2007). *AtWRKY70* was also found previously to be constitutively expressed in an ROS scavenging enzyme gene mutant, *atapx1*, revealing its responsiveness to ROS (Ciftci-Yilmaz et al., 2007). However, it remains to be determined how these WRKY factors can participate in ROS homeostasis in the senescent leaf. In our current study, we found that the rice OsWRKY42 transcription factor functions as a key repressor of antioxidant *OsMT1* isoforms, in particular *MT1d*. Our present findings thus provide direct evidence that WRKY can directly regulate ROS scavenging genes during the onset of leaf senescence.

ROS participate in a diverse range of plant processes including pollen development (Hiscock and Allen, 2008; McInnis et al., 2006a; 2006b). In this regard, it is noteworthy that the *OsMT1d* (former name *MT-1-4b*) gene has been previously shown to be a downstream target of the *OsMADS3* gene that regulates late anther development and pollen formation in rice (Hu et al., 2011). Microarray analysis of an Os*MADS3* mutant allele, *mads3-4*, which displays defective anther walls, aborted microspores, and complete male sterility, revealed the altered expression of a number of ROS producing and scavenging genes in this mutant. OsMADS3 protein was further found to bind to the consensus CArG- core element [CC(A/T)4NNGG] of promoter of *OsMT1d*. In addition, a recombinant OsMT1d revealed ROS scavenging activity in a previous study (Hu et al., 2011). Thus, the modulation of the ROS level through OsMT1d *via* OsMADS3 appears to be essential for rice male reproductive development. Taken together with previous findings, our current study results suggest that at least two transcription factors, OsMADS3 and OsWRKY42 regulate *OsMT1d* positively and negatively, respectively, to balance the ROS levels. It would be interesting in the future to examine whether *OsWRKY42* has any function in rice male development.

Histochemical GUS analysis of a serial deletion of the rice *OsMT1d* promoter in transgenic *Arabidopsis* revealed an important promoter region demarcated by nucleotides -1052~-583, a region which contains metal-responsive cis-elements such as a putative animal metal-regulatory motif and a potential copper responsive element which responds to treatments with different

heavy metals, such as Cu^{2+}, Pb^{2+} and Al^{3+} (Dong et al., 2010). It would be therefore interesting to determine whether the W box element at -729~-724, defined as the OsWRKY42 binding site in our present study and located in the interval of the important *OsMT1d* promoter region, can also participate in the regulatory response to heavy metals.

The *OsMT1a*, *OsMT1c*, *OsMT1d*, *OsMT1f* and *OsMT1g* genes are tightly clustered on chromosome 12. In RT-PCR analysis of the shoots of rice seedlings, three of these genes, *OsMT1a*, *OsMT1c* and *OsMT1d*, with high transcript levels were downregulated under salinity stress and ABA treatment, whilst they were previously found to be upregulated after drought stress (Kumar et al., 2012). This result suggests that these clustered *OsMT1* genes may be coregulated under certain stress conditions. Consistently, we found in our current experiments that *OsMT1d*, *OsMT1f*, and *OsMT1g* are downregulated in *OsWRKY42* transgenic rice plants, although *OsMT1a*, *OsMT1b*, and *OsMT1c* showed unaltered expression (Fig. 5). In our current study also, we confirmed that OsWRKY42 can bind to the *OsMT1d* promoter (Fig. 6). Given that the promoters of *OsMT1f* and *OsMT1g*, as well as of *OsMT1d*, contain conserved W-boxes (Supplementary Fig. S4) and both are similarly downregulated in *OsWRKY42OX* lines, it is likely that OsWRKY42 also associates with the regulatory elements of *OsMT1f* and *OsMT1g* genes and represses both genes.

In summary, we have here isolated and characterized a rice leaf senescence-inducible factor, *OsWRKY42*. Analysis of transgenic rice plants overexpressing *OsWRKY42* has revealed a novel regulatory mechanism by which WRKY can participate in ROS balancing and in the modulation of leaf senescence. To achieve a more in-depth understanding of *OsWRKY42* function during leaf senescence, it would be valuable in the future to identify additional targets of this gene through genome wide expression analysis of *OsWRKY42OX* transgenic rice.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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