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ORIGINAL ARTICLE

Functional analysis of a cold-responsive rice WRKY gene, *OsWRKY71*

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Abstract Publicly available microarray data and RNA gel blot analysis identified a rice WRKY transcription factor, *OsWRKY71*, that is highly upregulated in response to cold stress. Experiments with *OsWRKY71* promoter:*GFP* transgenic rice confirmed its cold-inducible expression. Transient expression of *OsWRKY71-GFP* in maize mesophyll protoplasts indicated that it is localized predominantly in the nucleus and to a lesser extent in the cytosol. Transcriptional activation assays revealed that OsWRKY71 suppresses luciferase reporter activity in maize protoplasts, suggesting that it functions as a transcriptional repressor in rice. To characterize the function of *OsWRKY71* in rice, we generated transgenic rice plants carrying *CaMV35S* promoter:*OsWRKY71*. Upon cold (4 °C) treatment, two selected *OsWRKY71* transgenic lines, *OX12* and *OX21,* recovered much better with respect to survival rate, photosynthetic ability, fresh weight, and dry weight than the control lines. RT-PCR analysis of known cold-responsive genes found that expression of *OsTGFR* and *WSI76* was increased in *OsWRKY71* transgenic lines in response to cold stress. Our results suggest that *OsWRKY71* has a positive function in cold tolerance by regulating downstream target genes.

Keywords Cold · OsWRKY71 · Rice · Transgenic · Tolerance

Introduction

Unlike animals, plants cannot move away from disadvantageous environmental conditions such as cold, drought, and high salinity. Cold exposure (low temperature or chilling) represents one of the major environmental stresses that adversely affect rice productivity through poor germination, yellowing leaves, growth retardation, and improper microspore development (Andaya and Mackill 2003; Baruah et al. 2009; Shimono et al. 2011a; de Los Reyes et al. 2013). Therefore, screening for cold tolerance genes is particularly important in light of the current increase in occasional extreme weather events.

To cope with diverse environmental stresses plants regulate the expression of a large number of stress-associated genes. The gene products include water channel proteins, the enzymes required for the biosynthesis of osmoprotectants, lipid desaturases for membrane modification, protective proteins, such as antifreeze proteins and chaperones, detoxification proteins, and transcription factors (Shinozaki and Yamaguchi-Shinozaki 1996; Singh et al. 2002). In particular, transcription factors play an essential role in the regulation of the plant response to abiotic stresses through the binding of transcription factors

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to cognate *cis*-acting elements present in the promoter region of their target genes (Casaretto and Ho 2003; Fujita et al. 2005; Yamaguchi-Shinozaki and Shinozaki 2006; Hu et al. 2008; Zou et al. 2010; Yang et al. 2012; Peng et al. 2013). One class of proteins that is unique to plants and plays a vital role in abiotic stress response is the WRKY family of proteins that bind to the consensus *cis*-element W box (TTGACT/C) (Eulgem et al. 2000; Cai et al. 2008; Ciolkowski et al. 2008; Rushton et al. 2010). Previous studies have identified a large number of WRKYs, including 74 WRKY-encoding genes in *Arabidopsis* (Ulker and Somssich 2004) and 125 in rice (Rice WRKY working group 2012).

Until now, the functions of WRKY proteins have been mainly analyzed with a focus on defense responses upon pathogen infection. For instance, the *Arabidopsis* WRKYs *AtWRKY18*, *AtWRKY40*, and *AtWRKY60* are negative regulators of resistance to the hemibiotrophic bacterial pathogen *Pseudomonas syringae* (Xu et al. 2006) and the barley WRKYs *HvWRKY1* and *HvWRKY2* are negative regulators of the basal defense response against the fungal pathogen *Blumeria graminis* (Shen et al. 2007). The rice WRKY *OsWRKY45* enhances resistance to *Magnaporthe oryzae* and *Xanthomonas oryzae* pathovar *oryzae* (*Xoo*) (Shimono et al. 2007; Jiang et al. 2010; Shimono et al. 2011b; Matsushita et al. 2012). Overexpression of *OsWRKY62* and *OsWRKY76* compromises XA21-mediated resistance to *Xoo* (Peng et al. 2008; Seo et al. 2011) and overexpression of *OsWRKY28* enhances rice susceptibility to *M. oryzae* (Chujo et al. 2013). Consistent with these findings, reduced expression of these rice WRKYs enhances resistance to *Xoo* and *M. oryzae* (Peng et al. 2010; Delteil et al. 2012).

Notably, a number of WRKY genes are involved in the coordination of multiple biological processes. For instance, *AtWRKY33* regulates disease resistance, NaCl tolerance and thermotolerance (Birkenbihl et al. 2012; Jiang and Deyholos 2009; Li et al. 2011), and a pepper WRKY, *CaWRKY40*, modulates resistance to *Ralstonia solanacearum* and tolerance to heat stress (Dang et al. 2012). These findings suggest that WRKYs can serve as nodes in the crosstalk between different physiological processes; however, the functions of the majority of WRKY family members and their possible roles in signaling crosstalk remain poorly understood.

Rice *OsWRKY71* is upregulated by several defense-signaling molecules, such as methyl jasmonate, salicylic acid and 1-aminocyclo-propane-1-carboxylic acid, as well as by biotic elicitors, wounding and pathogen infection. Accordingly, *OsWRKY71* overexpression enhances resistance to *Xoo* (Liu et al. 2007; Chujo et al. 2008). On the other hand, OsWRKY71 acts as a transcriptional repressor of gibberellin-responsive genes (Zhang et al. 2004). In the present study, we show that *OsWRKY71* expression is induced by cold stress. In an attempt to investigate the in vivo function of *OsWRKY71*, we analyzed the responses of *OsWRKY71* overexpression lines upon cold treatment. Our data indicate that *OsWRKY71* may have an important role in cold tolerance in rice.

Materials and methods

Plant materials and exposure of rice seedlings to chilling stress

Rice (*Oryza sativa* japonica cultivar [cv.] Dongjin) plants were grown in a greenhouse under a 14/10-h light/dark period, at a temperature of 24–28 °C and 70–80 % humidity. To investigate the expression of *OsWRKY71* in rice, seedlings and mature leaves and panicles of flowering plants were subjected to cold (4 °C), abscisic acid (ABA, 100 μM), salt (300 mM NaCl), and drought (20 % polyethylene glycol and air-drying) treatments. To analyze the response to cold stress, homozygous transgenic plants of the T_2 generation were used. Five-week-old seedlings were treated at 4 °C in a low-temperature environment room, and thereafter moved to the greenhouse for 6 days. Surviving seedlings were photographed and characterized to investigate the response of *OsWRKY71* overexpressing transgenic plants (*OsWRKY71-OX*) to chilling stress. To analyze the expression of cold-responsive genes in seedlings in response to cold stress, 5-week-old seedlings of transgenic plants of T_2 generation were exposed to 4 °C in the low-temperature environment room. At 0, 12, and 24 h of chilling treatment, leaf blades of cold-stressed seedlings were sampled for analysis.

RNA gel blot and RT-PCR analysis

Total RNA was prepared from various rice tissues after different abiotic stress treatments using Trizol reagent (Invitrogen, Carlsbad, CA, USA). For RNA gel blot analysis, 20 μg of isolated total RNA was fractionated on a 1.3 % agarose gel and transferred onto Hybond-N^+ nylon membranes (Amersham Biosciences, Pittsburgh, PA, USA). Hybridization was then carried out with [α-^{32}P] dCTP-labeled gene-specific probe according to standard procedures under high-stringency hybridization conditions (Cho et al. 2006). Gene-specific probes for *OsWRKY71* (LOC_Os02g08440) and *SalT* (LOC_Os01g24710) were amplified using the primers listed in Supplementary Table S1. PCR amplification was performed in a final volume of 40 μL (100 pmol of each primer, 20 μM each of dNTPs, 10 mM Tris–HCl pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100, and 0.5 U of Taq polymerase) using 50 ng of genomic DNA as template.

For RT-PCR analysis, total RNA isolated from wild type (WT) and transgenic rice plants was reverse transcribed with oligo-dT primer and a first-strand cDNA synthesis kit (Roche, Mannheim, Germany). The synthesized first-strand cDNA was used in subsequent PCR reactions as described above with gene-specific primers and control primers for *OsUBQ5* (Jain et al. 2006; Supplementary Table S1). The cold-responsive genes analyzed included *OsDREB1A* (LOC_Os09g35030), *Glutamate decarboxylase* (LOC_Os03g13300), *TPP1* (*Trehalose-6-phosphate phosphatase1*; LOC_Os02g44230), *OsTGFR* (LOC_Os01g68510), *WSI76* (LOC_Os07g48830), *OsCORTM1* (*Cold-regulated 413 thylakoid membrane1*; LOC_Os05g49170) and *OsMAT1* (*Methionine adenosyltransferase1*; LOC_Os05g04510).

Analysis of *OsWRKY71* promoter:GFP expression

The 1943-bp promoter region was isolated from rice genomic DNA by PCR using pOsWRKY71-F/R primers (Supplementary Table S1). After adapter-conjugating PCR with *att*B1 and *att*B2 adaptors, the isolated promoter fragment was linked to the *Green fluorescent protein* (*GFP*) gene by the Gateway LR clonase (Invitrogen). The transformation vector contained the *bar* gene between the *CaMV35S* promoter and the terminator of nopaline synthase gene (Park et al. 2010). Through *Agrobacterium*-mediated rice transformation and propagation of the transgenic lines, several independent lines of the T_2 generation were obtained. Among them, one representative transgenic line was selected for further study.

Total RNA was extracted from 1-month-old leaves that were subjected to air-drying (drought), low temperature (4 °C) or high salinity (300 mM NaCl) for 0 to 6 h, and reverse transcribed with an oligo-dT primer and a first-strand cDNA synthesis kit (Roche). Real-time qRT-PCR was carried out with 50 ng of cDNA template using the *GFP*-specific primers GFP-F/R. Fluorescence from the amplicon was detected by a Stratagene Mx3000p Real-Time PCR machine and Mx3005P software v2.02 (Stratagene, USA). All results were measured from triplicate reactions for each sample. To normalize the real-time qRT-PCR data, the *OsUBI1* (LOC_Os06g46770) gene primers OsUbi1-F/R were used as a reference.

Subcellular localization of OsWRKY71-GFP

Full-length cDNA of *OsWRKY71* was amplified by PCR using full-length F/R primers (Supplementary Table S1) and the PCR product was cloned into the pENTR^{TM}/D-TOPO vector (Invitrogen). The insert was confirmed by sequencing and then subcloned into the destination vector p2FGW7 for N-terminal GFP fusion (Karimi et al. 2002) using the Gateway LR clonase (Invitrogen). The resulting construct was introduced into maize mesophyll protoplasts using PEG-calcium mediated transformation method (Cho et al. 2009). After incubation for 24 h, expression of the GFP fusion protein was monitored using a confocal microscope (LSM510 META, Carl Zeiss, Germany). GFP fluorescence was excited at 488 nm and detected in the emission range of 500–525 nm. OsABF1-RFP was used as a nuclear marker (Amir Hossain et al. 2010).

Assay for transcriptional activity of OsWRKY71

For the transient expression assay, an effector vector was constructed by fusing the full-length cDNA of *OsWRKY71*, amplified by PCR using primers containing *Nde*I and *Eco*RI sites (Supplementary Table S1), to the GAL4 binding domain (BD) sequence under the control of the *CaMV35S* promoter and the Ω sequence. The previously described GAL4-responsive reporter construct (Ohta et al. 2000) was used as a reporter vector, and contains 5X GAL4, a minimal TATA, the Ω sequence and the *Luciferase* (*LUC*) gene. The maize ubiquitin promoter:*β-Glucuronidase* (*GUS*) construct (*ZmUBQ:GUS*) was used as an internal control (Han et al. 2013). Maize mesophyll protoplasts isolated from the second leaves of dark grown plants were transfected with the effector, reporter and internal control vectors as described previously (Han et al. 2013). Transfected protoplasts were incubated in W5 solution for 12 h (Zhang et al. 2011). LUC and GUS activity assays were performed as previously described (Han et al. 2013). All experiments were repeated three times with similar results.

Production of *OsWRKY71* overexpressing transgenic plants

Full-length cDNA of *OsWRKY71* was amplified by PCR using full-length F/R primers (Supplementary Table S1), cloned into pENTR^{TM}/D-TOPO vectors (Invitrogen), and then subcloned into the binary plant vector pH2GW7 (Karimi et al. 2002) using the gateway LR clonase (Invitrogen). To produce transgenic rice plants expressing *CaMV35S*:*OsWRKY71*, the *Agrobacterium tumefaciens* LBA4404 strain harboring this vector was grown on AB media supplemented with 10 mg L^{-1} streptomycin and 50 mg L^{-1} hygromycin for 3 days at 28 °C, and transgenic calli were obtained via the *Agrobacterium*-mediated co-cultivation method as described previously (Jeon et al. 2000). *OsWRKY71-OX* transgenic plants were selected on medium containing 50 mg L^{-1} hygromycin and 250 mg L^{-1} cefotaxime. T_2 homozygous lines were selected by testing for hygromycin resistance of progeny plants and analyzed for further characterization.

Phenotype analysis of *OsWRKY71-OX* lines in response to cold stress

To examine the cold tolerance of *OsWRKY71-OX* lines, we analyzed both the chlorophyll fluorescence and survival ratio of transgenic plants (Lee et al. 2004). In brief, the youngest leaves of 10-day-old seedlings were cut and floated on distilled water at 4 °C in a cold chamber under continuous light of 110 μmol m^{-2} s^{-1} for up to 40 h and the chlorophyll fluorescence was measured using the Plant Efficiency Analyzer (Hansatech, Kings Lynn, UK). The ratio *Fv*/*Fm* was calculated to examine damage in the photosystem (Genty et al. 1989). For survival analysis, 10-day-old seedlings were treated in the cold chamber for up to 50 h. After recovery for 6 days under normal growth conditions, the third-leaf survived plants were counted and their fresh weights were measured. Dry weights were measured after completely drying at 80 °C for 2 days.

Results

OsWRKY71 expression in response to cold treatment

Analysis of publicly available microarray data revealed that expression of *OsWRKY71* is greatly increased when rice plants are challenged by cold stress (Supplementary Fig. S1). This prompted us to further investigate the expression of *OsWRKY71* in rice in response to cold and other environmental stresses by RNA blot analysis (Fig. 1). *OsWRKY71* expression was examined by subjecting rice seedlings and panicles of flowering plants to cold (4 °C), ABA (100 μM), salt (300 mM NaCl), and drought (20 % PEG and air-drying) treatments. *OsWRKY71* expression was rapidly increased in cold-treated seedlings as early as 2 h, but was barely detectable in untreated samples (Fig. 1a). Notably, none of the ABA, salt, and drought treatments increased *OsWRKY71* expression relative to that of untreated leaves, even 8 h after each treatment (Fig. 1b), indicating its unique responsiveness to cold treatment. The cold-responsive expression of *OsWRKY71* was consistently observed in the mature leaf and panicle after 3 days (I) and 7 days (II) exposure to cold stress (Fig. 1c). In contrast, the gene expression of *SalT* was upregulated in response to salt and drought, as previously reported (Claes et al. 1990).

To further confirm that *OsWRKY71* is regulated by cold at the transcriptional level, we constructed a fusion vector containing the approximately 2-kb promoter sequence of *OsWRKY71* and the reporter gene *GFP* (*pOsWRKY71:GFP*) and introduced the vector into rice. *pOsWRKY71:GFP* transgenic rice plants were treated with cold, drought and salt. *GFP* expression analysis indicated that *OsWRKY71* promoter activity was dramatically increased after only 2 h of cold treatment (Fig. 1d).

Determination of subcellular localization of OsWRKY71

To determine the subcellular localization of OsWRKY71, an *OsWRKY71-GFP* fusion construct under the control of the *CaMV35S* promoter was generated. In a transient expression assay with maize mesophyll protoplasts, OsWRKY71-GFP was detected mostly in the nucleus and to a lesser extent in the cytosol, as confirmed by the nuclear marker construct, OsABF1-RFP (Fig. 2a). Therefore, we concluded that the OsWRKY71 protein might be targeted to the nucleus.

Transcriptional repression ability of OsWRKY71

Next, we examined the transcriptional activation ability of OsWRKY71 using the maize protoplast transient expression system (Han et al. 2013). The effector constructs for this system contain the *CaMV35S* promoter, the TMV translation enhancer Ω sequence, and either the GAL4 DNA BD or the *OsWRKY71* cDNA insert fused to the BD (Fig. 2b). As a reporter, we used a GAL4-responsive reporter construct (Ohta et al. 2000) that contains five copies of the GAL4 binding site in tandem and a minimal TATA region of the *CaMV35S* promoter, the Ω sequence, and the firefly *LUC* gene (Fig. 2b). In the maize protoplasts, the reporter LUC activity was considerably reduced when co-infected with the effector *BD-OsWRKY71* fusion vector compared with the empty vector control (Fig. 2c). This result suggests that OsWRKY71 may function as a transcriptional repressor to regulate downstream target genes.

Characterization of transgenic rice plants overexpressing *OsWRKY71*

To characterize the function of *OsWRKY71* in rice, transgenic rice plants constitutively overexpressing *OsWRKY71* under the control of the *CaMV35S* promoter were generated using *Agrobacterium*-mediated transformation. The transcript level of *OsWRKY71* was much higher in two independent transgenic T_2 homozygous lines, *OsWRKY71-OX12* (*OX12*) and *OsWRKY71-OX21* (*OX21*), than in the transformation background WT (cv. Dongjin) and the segregated non-transgenic line (NT) (Fig. 3a). There was no apparent difference in agricultural phenotypic characteristics such as plant height and yield potential among these transgenic lines (data not shown).

To investigate whether *OsWRKY71* overexpression correlated with cold tolerance in rice, two 5-week-old

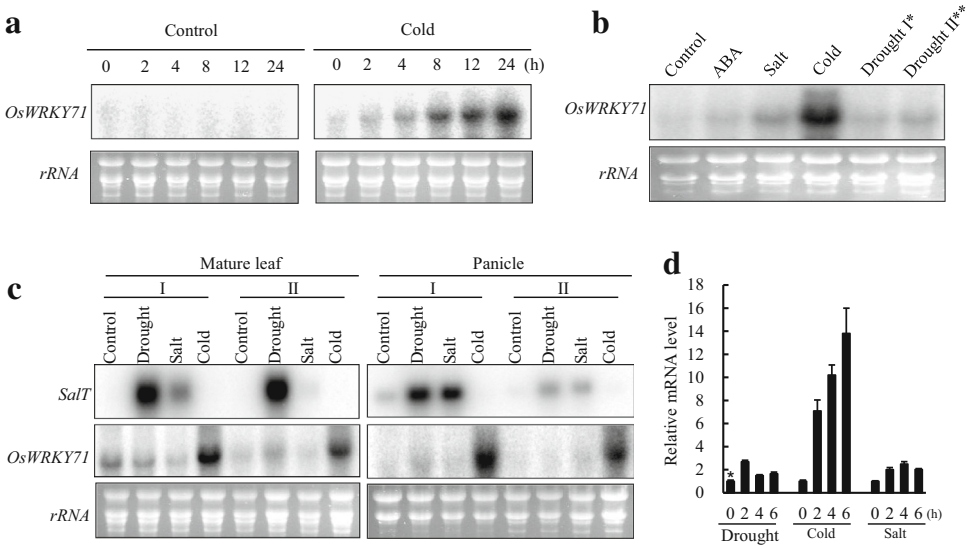

Fig. 1 Analysis of cold-responsive expression of *OsWRKY71*. **a** RNA blot analysis of the time course of *OsWRKY71* expression under control or cold-treated conditions. Two-week-old seedlings were transferred to the 4 °C growth chamber for 0, 2, 4, 8, 12, and 24 h of cold treatment and then sampled for RNA blot analysis. Untreated seedlings were sampled at the same time as controls. rRNAs are shown as a loading control. **b** RNA blot analysis of *OsWRKY71* under control, ABA (100 μM), salt (300 mM NaCl), cold (4 °C), and drought (air dry* and 20 % PEG**) conditions. All stresses were applied to 2-week-old seedlings for 8 h. **c** RNA blot analysis of *OsWRKY71* in mature leaves and panicles under control, drought, salt, and cold conditions. Mature leaves and panicles a week before anthesis were sampled after 3 (I) or 7 days (II) of various stress treatments. *SalT* expression was included as a drought- and salt-responsive marker gene. **d** Promoter activity analysis of *pOsWRKY71:GFP* in transgenic rice. A strong *GFP* signal was detected only after cold (4 °C) treatment, in contrast to drought (air dry) and salt (300 mM NaCl) treatments. The fold values were represented as a graph relative to the level of *GFP* mRNA in the lowest-expressing transgenic plants, as indicated by an *asterisk*

control lines, WT and NT, and two *OsWRKY71-OX* lines, *OX12* and *OX21*, were exposed to cold stress (4 °C) for 50 h and then allowed to recover their growth for 6 days under normal growth conditions. We found that *OX12* and *OX21* transgenic plants recovered much better, maintaining a greater number of green leaves, than WT and NT control plants, which displayed severely dried leaves (Fig. 3b).

Cold stress tolerance was examined by measuring the third-leaf survival ratio of 10-day-old seedlings. Most plants of the *OsWRKY71-OX* lines *OX12* and *OX21*, 94 and 97 %, respectively, survived after cold stress treatment followed by recovery. In contrast, only 54 and 75 % of the control WT and NT plants, respectively, survived (Table 1). Our results clearly indicate that *OsWRKY71* overexpression in rice plants increases tolerance to low-temperature stress.

The elevated tolerance of *OsWRKY71-OX* transgenic rice plants to cold stress was further evaluated by measuring chlorophyll fluorescence in two control and two transgenic lines that were subjected to low temperature (4 °C) for 20 or 40 h. The ratio F_v/F_m between the variable fluorescence (F_v) and the maximum fluorescence (F_m) is used to estimate the quantum yield of PSII photochemistry (Krause and Weis 1991). Under normal growth conditions (0 h), all tested plants showed an F_v/F_m of approximately 0.85, which is close to the value of 0.8 observed in all plant species studied (Fig. 4a). When exposed to cold stress, the control plants showed a significant reduction in the F_v/F_m values: 0.57 and 0.58 after 20 h and 0.2 and 0.18 after 40 h in WT and NT, respectively. In contrast, *OsWRKY71-OX* lines maintained relatively high F_v/F_m values of 0.7 and 0.71 after 20 h and 0.3 and 0.37 after 40 h for *OX12* and *OX21*, respectively (Fig. 4a). Concordant with these findings, *OX12* and *OX21* plants retained higher levels of both fresh weight and dry weight than control plants (Fig. 4b, c). These results further suggest that overexpression of *OsWRKY71* enhances cold tolerance in transgenic rice.

Expression of cold-responsive genes in *OsWRKY71-OX* lines

To obtain clues regarding the molecular mechanism of *OsWRKY71*-mediated cold tolerance, we monitored the expression of previously known cold-responsive genes in *OsWRKY71-OX* lines by RT-PCR analysis. Transcription levels of the selected cold marker genes were examined in both control and transgenic lines under cold (4 °C) treatment for 12 or 24 h. We found remarkable upregulation of the rice DREB gene *OsDREB1A* (Dubouzet et al. 2003; Ito et al. 2006) in the control, WT, NT, and transgenic *OX12* and *OX21* plants. These plants showed no difference in the pattern of *OsDREB1A* expression (Fig. 5), which peaked

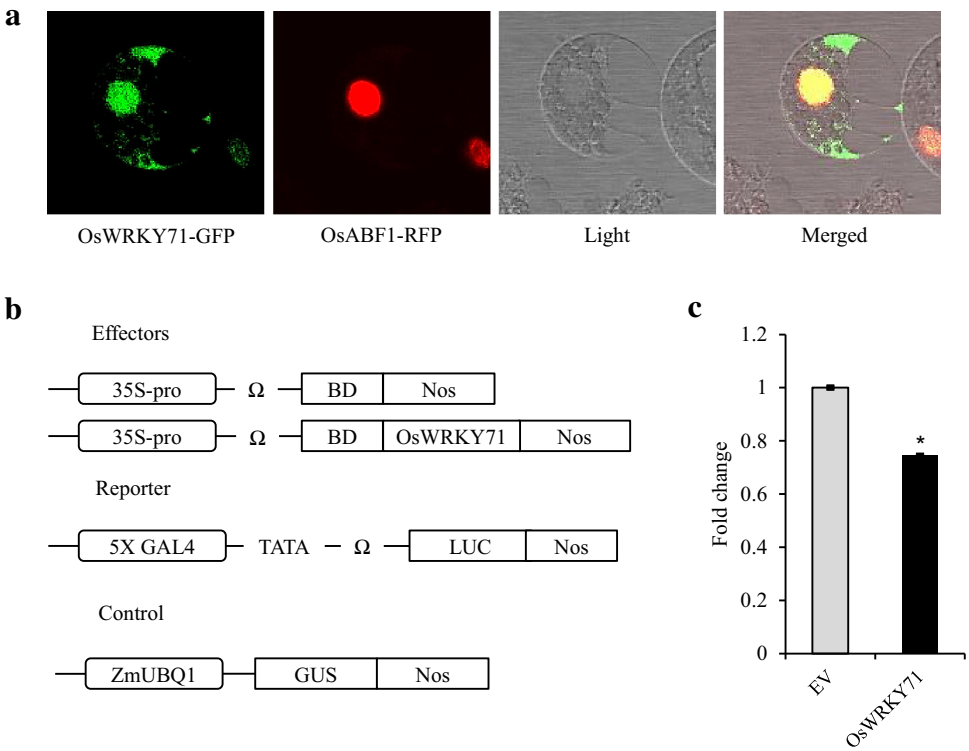

Fig. 2 Subcellular localization of OsWRKY71 and transcriptional repression assay. **a** Expression of OsWRKY71-GFP fusion protein in maize protoplast cells. The OsWRKY71-GFP signal is green; OsABF1-RFP is used as a nuclear marker. Bright light field and merged images are also shown. **b** Structures of the reporter and effector vectors used for the transcriptional activity assay of OsWRKY71. *ZmUBQ:GUS* was used as an internal control. *Nos*, *nopaline synthase* terminator. **c** Luciferase activity assay. The effector vector, either empty vector (EV) or *BD-OsWRKY71* (OsWRKY71), was co-infected with the reporter *LUC* vector and the internal control vector *ZmUBQ:GUS*. The fold change was calculated as the LUC/GUS activity ratio. The values are the averages with standard deviation of the results from three independent experiments. Normalized LUC activity recorded after transfection with the empty vector control alone was arbitrarily set at 1. $*P < 0.05$

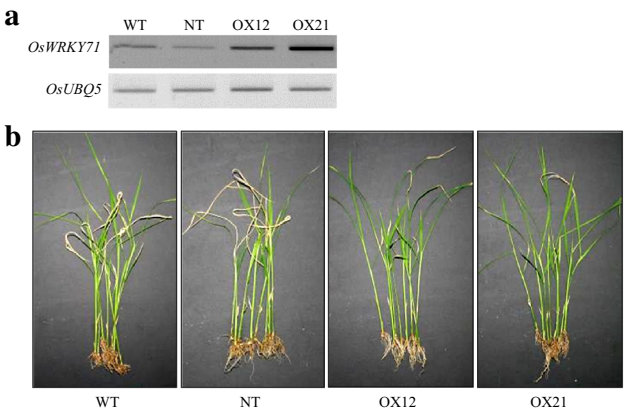

Fig. 3 Production and characterization of *OsWRKY71-OX* transgenic rice plants. **a** RT-PCR analysis of *OsWRKY71* expression in *OsWRKY71-OX12* (*OX12*) and *OsWRKY71-OX21* (*OX21*) transgenic lines. *OsUBQ5* was amplified as an internal RT-PCR control. **b***OsWRKY71* overexpression confers cold tolerance in rice. Five-week-old rice seedlings of wild type (WT), segregant non-transgenic (NT), and *OsWRKY71-OX* transgenic lines (*OX12* and *OX21*) were exposed to cold stress (4 °C) for 50 h and then allowed to recover for 6 days under normal conditions

Table 1 Cold tolerance of *OsWRKY71-OX* transgenic rice judged by the third-leaf survival ratio

Line	No. of plants tested	No. of plants surviving	Survival ratio
WT	63	34	54.0
NT	63	47	74.6
OX12	62	58	93.5*
OX21	63	61	97.0*

Cold-stressed 10-day-old seedlings were allowed to recover for 6 days and the third-leaf survival ratios were measured. Experiments were repeated three times

WT wild type, *NT* segregant non-transgenic line, *OX12* and *OX21OsWRKY71-OX* transgenic rice lines

* $P < 0.05$

12 h after cold treatment. Likewise, expression of two marker genes, *Glutamate decarboxylase* (Su et al. 2010) and *TPP1* (Pramanik and Imai 2005), reached a similar peak 12 h after cold treatment in control and transgenic lines and thereafter decreased. *OsCORTM1* (*OsCOR413-*

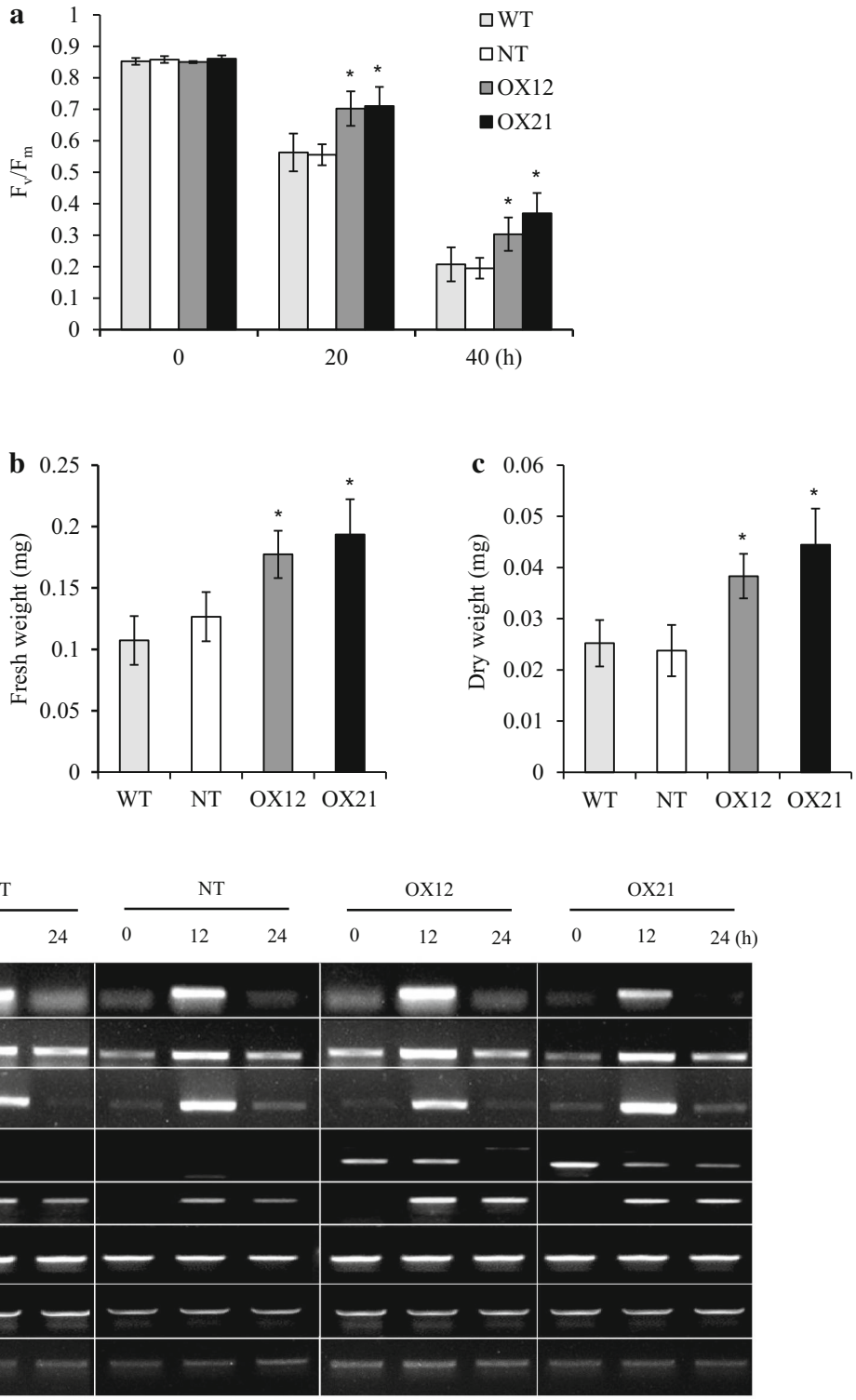

Fig. 4 Phenotype analysis of *OsWRKY71-OX* transgenic rice seedlings in response to cold treatment. **a** Changes in chlorophyll fluorescence of wild type (WT), segregant non-transgenic (NT), and *OsWRKY71-OX* transgenic lines (*OX12* and *OX21*) at 20 h and 40 h of cold stress treatment. Fresh weight (**b**) and dry weight (**c**) after 6 days of recovery. $^*P < 0.05$

Fig. 5 Expression analysis of cold-responsive marker genes in *OsWRKY71-OX* lines in response to cold stress. Leaves of 5-week-old seedlings exposed to 4 °C were harvested at 0, 12, and 24 h. *OsUBQ5* was used as an internal control. *WT* wild type, *NT* segregant non-transgenic line, *OX12* and *OX21OsWRKY71-OX* transgenic rice lines

TM1; Ma et al. 2009) and *OsMAT1* (Ma et al. 2009), as well as the PCR control gene *OsUBQ5,* did not respond to cold treatment in control and transgenic lines.

Interestingly, two cold-responsive genes, *OsTGFR* and *WSI76*, which are the rice homologs of target genes of *Arabidopsis* DREBs (Dubouzet et al. 2003; Chen et al.

2008; Saito and Yoshida 2011), showed differential induction in *OsWRKY71-OX* plants under normal and cold stress conditions (Fig. 5). *OsTGFR* expression was detected in *OsWRKY71-OX* plants before and after cold treatment. *WSI76* expression was more highly induced after cold treatment in *OX12* and *OX21* than in control lines. This result suggests that OsWRKY71 may have a regulatory function in the expression of *OsTGFR* and *WSI76* in response to cold treatment.

Discussion

Among several families of transcription factors that are involved in the transcriptional regulation of plant stress-responsive genes, increasing attention has recently been paid to the role of WRKY transcription factors in regulating the response to abiotic and biotic stresses. However, relatively little progress has been made in understanding the function of WRKY proteins under abiotic stresses (Chen et al. 2012).

Here, we provide evidence that *OsWRKY71* positively regulates cold tolerance in rice plants. First, we showed that *OsWRKY71* was induced specifically by cold (Fig. 1). Second, we found that it functioned as a nuclear transcriptional repressor (Fig. 2). Third, *OsWRKY71* overexpression enhanced cold tolerance in transgenic rice, and this elevated tolerance was further confirmed by measurement of chlorophyll fluorescence, F_v/F_m (Figs. 3, 4; Table 1). Finally, we identified two potential downstream targets of OsWRKY71, *OsTGFR* and *WSI76,* in response to cold treatment (Fig. 5). Together, these data suggest that *OsWRKY71* may have an important transcriptional regulatory role in response to cold stress, and that the *OsWRKY71*-mediated pathway is likely essential for cold tolerance in rice. It is noteworthy that *OsWRKY71* was not upregulated by ABA and other abiotic stresses such as salt and drought in rice seedlings (Fig. 1), implying that *OsWRKY71* may function in an ABA-independent cold response pathway.

Numerous transcriptional expression data have revealed cold-responsive genes in common with those under observation in the present study that are associated with cold stress in different plant species (Fowler and Thomashow 2002; Maruyama et al. 2004; Seki et al. 2002; Dubouzet et al. 2003; Wang et al. 2004; Fowler et al. 2005; Skinner et al. 2005; Ito et al. 2006; Badawi et al. 2007; Cheng et al. 2007; Zhou et al. 2008; Mittal et al. 2012; Zhang et al. 2012). For instance, *DREB1s* act as upstream regulators during cold stress and are rapidly induced several fold after cold treatment. Our experimental observation of expression patterns of cold-responsive genes other than *OsCORTM1* and *OsMAT1,* such as *OsDREB1A*, *Glc decarboxylase*, and *TPP1*, indicated that many of the rice homologs of cold-responsive genes are also rapidly regulated in cold-exposed rice (Fig. 5), supporting conserved regulatory mechanism(s) in a range of plant species.

In *Arabidopsis* there are seven members belonging to the *GolS* gene family, of which *GolS3* is controlled by DREB1A and induced by cold stress (Taji et al. 2002). Expression of the rice homolog of *GolS3*, *WSI76*, was found to be increased under chilling stress (Saito and Yoshida 2011). In this context, it is noteworthy that we found a remarkable increase in the transcripts of *WSI76* in *OsWRKY71-OX* transgenic rice compared with the controls WT and NT after cold treatment (Fig. 5). In *Boea hygrometrica*, a WRKY is involved in the regulation of *GolS* expression (Wang et al. 2009). *OsTGFR* is a homolog of target genes of *Arabidopsis* DREBs (Dubouzet et al. 2003; Chen et al. 2008; Ma et al. 2009; Saito and Yoshida 2011). We found an alteration in *OsTGFR* expression in *OsWRKY71-OX* transgenic rice in response to cold compared with expression in the controls. The enhancement of stress tolerance in *OsWRKY71* transgenic plants likely results from changes in expression of these cold-responsive genes. In previous studies, *OsWRKY28* (Chujo et al. 2013) and *OsWRKY76* (Yokotani et al. 2013), closely related paralogues of *OsWRKY71*, exhibited transcriptional repressor activity. Consistent with these findings, we found that *OsWRKY71* functioned as a transcriptional repressor (Fig. 2c). This result suggests that OsWRKY71 exerts its function through transcriptional suppression of target genes that may negatively regulate the downstream genes *OsTGFR* and *WSI76*. How the transcriptional repressor *OsWRKY71* regulates downstream target genes in response to cold stress remains to be determined.

Strong constitutive overexpression of transcription factors often results in negative growth phenotypes in transgenic plants. For example, growth retardation has been observed in transgenic rice plants overexpressing stress-responsive transcription factors such as *OsDREB1A*, *OsDREB1B*, *OsWRKY89* and *OsNAC6* (Ito et al. 2006; Nakashima et al. 2007; Wang et al. 2007). Although constitutive expression of these transgenes produced the best result in respect to cold tolerance, the overall advantage was reduced by significant differences in agronomical traits such as flowering time and yield potentials of transgenic lines. In the present study, *OsWRKY71* overexpression resulted in no obvious phenotypic alteration, and plant architecture was indistinguishable from that of WT under normal growth conditions. Given that *OsWRKY71* overexpression improved cold tolerance with an unaltered growth phenotype under normal conditions, our data imply the potential application of *OsWRKY71* for genetic improvement of cold tolerance in rice. To further understand the molecular mechanism of enhanced cold tolerance

responses mediated by *OsWRKY71*, it would be useful to analyze global gene expression changes in *OsWRKY71-OX* in response to cold stress and to identify and characterize downstream targets in future studies.

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