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GENETIC TRANSFORMATION AND HYBRIDIZATION

Enhanced heat and drought tolerance in transgenic rice seedlings overexpressing *OsWRKY11* under the control of *HSP101* promoter

Xiaolan Wu · Yoko Shiroto · Sachie Kishitani · Yukihiro Ito · Kinya Toriyama

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Abstract An *OsWRKY11* gene, which encodes a transcription factor with the WRKY domain, was identified as one of the genes that was induced by both heat shock and drought stresses in seedlings of rice (*Oryza sativa* L.). To determine if overexpression of *OsWRKY11* confers heat and drought tolerance, *OsWRKY11* cDNA was fused to the promoter of *HSP101* of rice and introduced into a rice cultivar Sasanishiki. Overexpression of *OsWRKY11* was induced by heat treatment. After heat pretreatment, the transgenic lines showed significant heat and drought tolerance, as indicated by the slower leaf-wilting and less-impaired survival rate of green parts of plants. They also showed significant desiccation tolerance, as indicated by the slower water loss in detached leaves. Our results indicate that the *OsWRKY11* gene plays a role in heat and drought stress response and tolerance, and might be useful for improvement of stress tolerance.

Keywords Drought tolerance · Heat tolerance · Transgenic rice · WRKY

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X. Wu · Y. Shiroto · Y. Ito · K. Toriyama (✉)
Laboratory of Environmental Biotechnology,
Graduate School of Agricultural Science, Tohoku University,
1-1 Tsutumidori-Amamiyamachi, Aoba-ku,
Sendai 981-8555, Japan
e-mail: torikin@bios.tohoku.ac.jp

S. Kishitani
Laboratory of Plant Breeding and Genetics,
Graduate School of Agricultural Science,
Tohoku University, 1-1 Tsutumidori-Amamiyamachi,
Aoba-ku, Sendai 981-8555, Japan

Introduction

Drought stress has been found to be one of the major causes of reduced crop yield (Ozturk et al. 2002), and great efforts have been made to breed drought-tolerant crop varieties. As the most important world food crop, cultivated rice (*Oryza sativa* L.) demands tremendous amounts of water during growth, which results in a number of production challenges. Rice is also a popular model plant for studies of monocots. Improvements in the tolerance of cereal plants to abiotic stress are important when the efficiency of food production is to be increased. Breeding of transgenic rice cultivar with drought tolerance can help increase and stabilize crop yield under stress environments.

Many transgenic approaches have been carried out to increase biotic and abiotic stress tolerance (see Bajaj and Mohanty 2005 for a review). One successful approach to increase abiotic stress tolerance is overexpression of certain stress-inducible transcription factors, such as the following: CBF1/DREB1B, CBF2/DREB1C, CBF3/DREB1A (Thomashow 1999); DREB1A (Kasuga et al. 1999); DREB2A (Sakuma et al. 2006); EmBP1, OSBZ8, TAF1 (Zhu 2002); SNAC1 (Hu et al. 2006). However, employment of a constitutive promoter such as CaMV35S to drive genes for transcription factors may present some risk of deleterious effect on plant growth (Romero et al. 1997; Kasuga et al. 1999). Overexpression of the OsDREB1A proteins has been reported to cause growth retardation under unstressed control conditions in transgenic rice plants (Ito et al. 2006). Similar phenomena concerning improvement of stress tolerance and growth retardation have been observed in transgenic *Arabidopsis* overexpressing CBF1/DREB1B, CBF2/DREB1C, or CBF3/DREB1A (Thomashow 1999; Kasuga et al. 1999) and in tobacco (Kasuga et al. 2004). It has been reported

that the stress-inducible rd29A promoter instead of the constitutive promoter minimizes this negative effect on plant growth (Kasuga et al. 1999, 2004). It is, therefore, desirable to use stress-inducible promoters to produce stress tolerant plants.

To search for drought and heat-inducible transcription factors in rice, we previously focused on rice orthologs of *Arabidopsis* genes for transcription factors known to be induced by drought or heat (Seki et al. 2002; Oono et al. 2003; Rizhsky et al. 2004). Using hydroponically grown seedlings, *OsWRKY11* (DDBJ accession no. AK108745; previous name was *WRKY16* in accession no. AY341856) was identified to show enhanced expression by placing the plants at 38°C for 1 h (heat treatment), by leaving the plants out of water for 10 h (drought treatment) and by combined heat/drought treatment for 0.5 h (Shiroto et al. 2004).

The *WRKY* genes encode a large group of transcription factors. There are over 70 *WRKY* genes in *Arabidopsis* and over 100 members in rice (Wu et al. 2005; Zhang and Wang 2005; Liu et al. 2007; Ramamoorthy et al 2008). This family is defined by a domain of 60 amino acids containing the amino acid sequence WRKY at its amino-terminal end and a putative zinc finger motif at its carboxy-terminal end. It binds specifically to the DNA sequence motif (T)(T)TGAC(C/T), which is known as the W box. *WRKY* genes are known to participate in various developmental and physiological programs, including disease resistance (Chen and Chen 2000; Dellagi et al. 2000; Du and Chen 2000; Eulgem et al. 2000; Kim et al. 2000; Asai et al. 2002), senescence (Hinderhofer and Zentgraf 2001; Robatzek and Somssich 2001), biotic and abiotic stress responses (Yoda et al. 2002; Dong et al. 2003; Huang and Duman 2002; Hara et al. 2000; Rizhsky et al. 2002, 2004; Pnueli et al. 2002; Seki et al. 2002), and growth and developmental processes (Lagacé and Matton 2004; Johnson et al. 2002; Sun et al. 2003; Xu et al. 2004). To the best of our knowledge, however, no information is available regarding the relationship between overexpression of WRKY genes and heat and drought tolerance.

To determine if overexpression of *OsWRKY11* confers heat and drought tolerance, *OsWRKY11* cDNA was fused to the promoter of *HSP101* of rice and introduced into rice. The promoter of *HSP101* was employed in this study, because the *HSP101* gene has been reported to be induced by heat shock (Agarwal et al. 2003). Although the promoter activity of *HSP101* has not been reported, it was expected that the promoter was stress-inducible and could be used to minimize possible deleterious effects of *OsWRKY11* expression under unstressed conditions. In this study, we first examined activation of *HSP101* promoter by heat and drought stress, and the phenotype of transgenic plants overexpressing *OsWRKY11*. Then, we evaluated survival of green parts of plants after heat and drought treatment, and water loss of detached aerial parts during desiccation. Enhanced heat and drought tolerance was observed.

Materials and methods

Construction of *HSP101* promoter::*GUS*

Genomic DNA was extracted from leaves of *O. sativa* L. cv. Nipponbare using DNeasy (Qiagen, Tokyo, Japan). An approximately 1 kb-fragment containing the promoter region of *HSP101* (DDBJ accession no. AJ316025) was amplified from the DNA using Ex Taq (TaKaRa-Bio, Ohtsu, Japan), the PCR enhancer system (Invitrogen, Carlsbud, USA), and the following primers: OsHSP101p-F2 primer (5′-GGCCAACATGATACAAGAAACCG-3′) and OsHSP101p-R1 primer (5′-CGTTGGTCTTGTGCGT GAAGTTG-3′). Cycling conditions were as follows: 35 cycles at 94°C for 30 s, at 58°C for 30 s, and at 68°C for 30 s. The amplified fragments were gel-purified using the MinElute Gel Extraction Kit (Qiagen, USA). A *Hind* III site and a *Nhe* I site (italicized) were added using Ex Taq (Takara, Kyoto, Japan), the PCR enhancer system (Invitrogen, Carlsbud, USA), and OsHSP101p-F primer (5′-*AA GCTT*CCTTCCGGCGATCTTGCAG-3′) and OsHSP101p-R primer (5′-*GCTAGC*TCCTCCTCTCCTCACACAATC-3′). The amplified fragment was ligated to pGEM-T Easy Vector Systems (Promega, Madison, USA).

The *HSP101* promoter-*GUS* was taken as a *Hind* III-*Sac* I fragment and inserted in the same sites of pBI101-Ubi-Hyg, which contains a maize ubiquitin promoter (Christensen et al. 1992) and a hygromycin phosphotransferase gene as a selection marker.

Construction of *HSP101* promoter::*OsWRKY11*

A full length cDNA of *OsWRKY11* in pCMVFL3 (DDBJ accession no. AK108745) was obtained from the Rice Genome Resource Center at the National Institute of Agrobiological Sciences, Japan (accession no. AK108745 in the rice full-length cDNA database, KOME, http://cdna01.dna.affrc.go.jp/cDNA/). The *HSP101* promoter was taken as a *Eco* RI fragment in pGEM-T Easy Vector and inserted into the same site located in front of the *OsWRKY11* cDNA. The *HSP101* promoter-*OsWRKY11* contains two *Spe* I sites, one derived from pGEM-T easy vector and the other from the 3′ end of the cDNA. The *Spe* I fragment was inserted into the *Xba* I site of pBI101H, which contained CaMV35S promoter–hygromycin phosphotransferase gene as a selection marker (Yokoi et al. 1998).

Production of transgenic plants

The final construct, *HSP101* promoter::*OsWRKY11 and HSP101* promoter::*GUS*, was transferred into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) and used for *Agrobacterium*-mediated transformation (Yokoi et al. 1997). Primary tranformants, To plants, were grown in pots (4.5 cm in diameter and 5.7 cm in depth) in a greenhouse at 28/23°C (12/12 h) under natural day length conditions, as described by Shirasawa et al. (2006).

Southern blot analysis

Genomic DNA (2 μg) was extracted from leaves using DNeasy (QIAGEN K.K., Japan) and digested with *Hin*dIII and electrophoresed on a 0.8% agarose gel. DNA fragments were blotted onto nylon membrane (GE Healthcare, UK) and hybridized with a 708-bp *HPT* probe labeled with digoxigenin (Roche, Switzerland) using primers HPT-F (5′-AATGAGTTGGACCAGCAGAAG-3′) and HPT-R (5′-CATTCAGGTCAAACATAGGCC-3′), or with a GUS probe using primers G_{787}-F (5′-TGTGAATTCGATATCTACCCGCTTCGCGTC-3′) and G_{1809}-R (5′-GATGAATTCTCATTGTTTGCCTCCCTGCTG-3′). After hybridization, blots were washed at 65°C in 0.1× SSC and 0.1% SDS.

GUS assay

Eight independent transgenic plants with *HSP101* promoter::*GUS* were grown in soil in pots at 22/18°C (12/12 h) for 2 weeks and were subjected to heat shock, cold, NaCl, or desiccation treatment. For heat shock treatment, the excised fully expanded leaf blades (0.5–1 cm in length), leaf sheaths, and roots were placed in water in microcentrifuge tubes and incubated in a water bath at 37, 42, or 45°C for 1 h. The control was simultaneously placed at room temperature for 1 h. For cold treatment, the leaves in the microcentrifuge tubes were placed in water at 4°C for 1 h. Desiccation treatment was carried out by leaving the leaf segments in the tubes with neither water nor a lid at 25°C for 1 h. For NaCl treatment, the excised leaves were placed in 250 mM NaCl solution in the microcentrifuge tubes and incubated at 25°C for 1 h. Histochemical staining for GUS activity was performed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) as a chromogenic substrate (Jefferson et al. 1987). Tissues were vacuum infiltrated with X-Gluc reaction buffer (100 mM sodium phosphate buffer pH 7.2, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% Triton X-100 and 1 mg/ml X-gluc) and incubated at 37°C overnight. After incubation, chlorophyll was removed from green tissues by immersion in 70% ethanol, and tissue samples were observed on a Nikon stereomicroscope.

RT-PCR

Two-week-old seedlings grown in soil at 22/18°C were exposed to heat pretreatment at 37/22°C (12/12 h) for 2 weeks with water everyday. For no-heat pretreatment, plants were grown at 22/18°C. Induction of *OsWRKY11* expression was determined by RT-PCR. Total RNA was isolated from leaves using RNeasy (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Poly A RNA was isolated using the Dynabeads mRNA Purification Kit (Invitrogen, Carlsbud, USA). cDNA synthesis was accomplished using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbud, USA) and then used as a template for PCR amplification with the following primers: 5′-AAATCGTCACGCCGGTGCAAG-3′ and 5′-TCAACCTCGCTCTTGGTGAGGAA-3′ for OsWRKY11; 5′-TACAACGGTTGGCGTCGCAC-3′ and 5′-AACTTGCGCACACGGTCCAG-3′ for *tubulin alpha-1* chain (accession no. AK069140 in DDBJ database). PCR was carried out with Ex Taq polymerase (TaKaRa-Bio, Ohtsu, Japan) for 30 cycles of denaturation for 0.5 min at 94°C, annealing for 0.5 min at 66°C, and extension for 0.5 min at 72°C, followed by final extension for 5 min at 72°C. For quantitative RT-PCR analysis, PCR was performed using SYBR *Premix Ex Taq* polymerase (TaKaRa-Bio) and Thermal Cycler Dice Real Time System TP800 (TaKaRa-Bio) for 55 cycles of denaturation for 5 s at 95°C, annealing for 10 s at 55°C, and extension for 30 s at 72°C.

Evaluation of heat and drought tolerance of seedlings

Evaluation of tolerance against heat and drought stress was performed by determining the extent of recovery of whole plants from exposure to 37/22°C (12/12 h) without a water supply for 2 days. Prior to combined heat/drought treatment, 2-week-old seedlings were grown for 2 weeks at 37/22°C with a water supply. Then the plants were exposed to 37/22°C for 2 days without a water supply. The plants were returned to 22/18°C and grown with a water supply for 2 weeks. Arial parts were separated into green living parts and nongreen dead parts. Each dry weight (DW) was determined after drying at 80°C for 24 h. The percentage of surviving green parts was calculated from the following formula: (DW of green parts/DW of green parts plus dead parts) × 100. Heat and drought treatment was also carried out at 37/22°C for 2.5 days without a water supply. Survival of plants was observed after 2 weeks of recovery.

Evaluation of water loss in detached aerial parts

For heat pretreatment, 2-week-old seedlings grown in soil at 22/18°C (12/12 h) were exposed to heat pretreatment at 37/22°C for 2 weeks with water everyday. For the control

condition with no-heat pretreatment, plants were grown at 22/18°C. After the pretreatment for 2 weeks, the aerial parts of transgenic plants and wild type (WT) were detached and weighed immediately and then placed on a laboratory bench at 25°C and weighed at designated times. Detached aerial parts were then dried at 80°C for 24 h to determine final DW. Water content was standardized as a percentage relative to the initial water content of aerial parts of the plant; it was calculated as follows: $[(FW_i - DW)/(FW_0 - DW)] \times 100$, where FW_i and FW_0 are fresh weight for any given interval and original fresh weight, respectively. Four T_2 plants per line were used for each test. All tests were simultaneously repeated three times.

Results

Activation of *HSP101* promoter in transgenic rice plants with *HSP101* promoter::*GUS*

An approximately 1 kb-fragment containing the promoter region of *HSP101* (DDBJ accession no. AJ316025) was amplified from rice genomic DNA and inserted in pBI101-UbiHyg, which contains a hygromycin phosphotransferase gene as a selection marker (Fig. 1). The *GUS* gene was fused to the promoter of *HSP101* and introduced into an elite cultivar Sasanishiki. Excised leaf blades of the eight independent transgenic plants grown at 22/18°C (12/12 h) were subjected to histochemical GUS assay. Incubation of the leaf segments at 37, 42, or 45°C for 1 h induced a high level of *GUS* expression, while no *GUS* expression was detected at room temperature (Fig. 2a). Blue staining was stronger at 42 and 45°C. GUS expression was not observed in WT leaf blades. Strong GUS expression was also

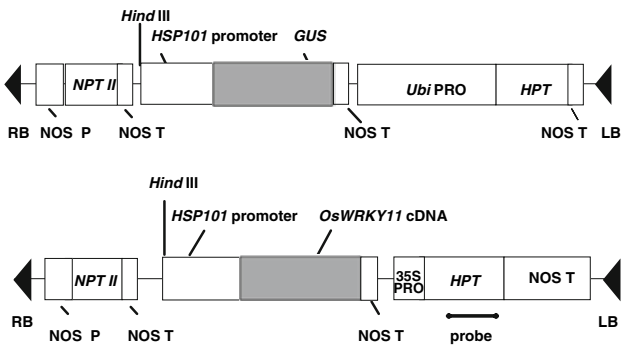

Fig. 1 The T-DNA region of *HSP101* promoter::*GUS and HSP101* promoter::*OsWRKY11* used for transformation. *HSP101* promoter was amplified from rice genome based on the sequence of DDBJ accession no. AJ316025. *OsWRKY11* cDNA was a full length cDNA (accession no. AK108745). *LB* left border, *NOS P* nopaline synthase promoter, *NPTII* neomycin phosphotransferase, *NOS T* 3′ signal of nopaline synthase terminator, *35S PRO* cauliflower mosaic virus 35S promoter, *HPT* hygromycin phosphotransferase

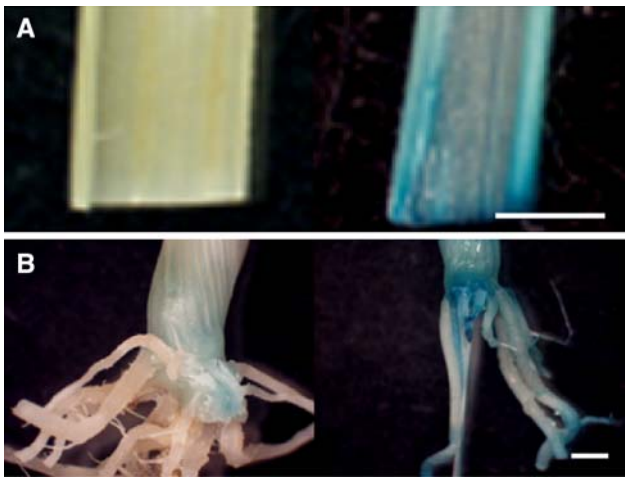

Fig. 2 GUS assay of leaf blades (**a**), leaf sheaths and roots (**b**) in transgenic plants with *HSP101* promoter::*GUS* with or without heat shock. Strong GUS activity, blue staining, was observed after heat shock at 45°C for 1 h (*right*) but not evident at control condition without heat shock (*left*). *Bar* 2 mm

observed in leaf sheaths and roots, whereas weak blue staining was observed in the base of stems at room temperature. The GUS activity under other stresses, i.e., cold (4°C), 250 mM NaCl or drought for 1 h was not detected. Thus, the heat shock activation of the *HSP101* promoter was confirmed.

A transgenic line carrying a single copy of the *GUS* gene, which was revealed by Southern blot analysis (data not shown), was selected (line #g8). T_2 plants homozygous for the introduced *GUS* gene were used for further analysis.

Production of transgenic rice plants carrying *HSP101* promoter::*OsWRKY11*

OsWRKY11 cDNA (DDBJ accession no. AK108745) was fused to the promoter of *HSP101* of rice (Fig. 1) and introduced into rice. Four independent transgenic lines (#ox2, #ox3, #ox4, and #ox5) were obtained. The number of copies of the T-DNA was determined by Southern blot analysis. We used *Hind* III, which cuts only once within the introduced T-DNA, and probed with the *HPT* gene (data not shown) so that the number of bands represented the copy number. The transgenic lines, #ox2 and #ox3, were identified to have a single copy of T-DNA, and #ox4 contained two copies and #ox5 carried four copies (Table 1).

T_1 plants segregated for the introduced T-DNA were grown to maturity under normal growth conditions. Three types of phenotypes were observed: dwarf with bent leaves, normal plant length with bent leaves and normal plant length with normal leaves. In case of the bent leaves, the edges of leaf blades were bent toward the abaxial side, i.e. toward the opposite direction observed in the typical wilted

Table 1 Phenotype of mature transgenic plants (T1) segregating for the introduced *OsWRKY11*

Transgenic line	#ox2	#ox3	#ox4	#ox5	WT
Copy number of the transgene	1	1	2	4	0
Total number of plants examined	5	24	6	4	4
Number of plants without the transgene	1	5	2	1	4
Number of dwarf plants (plant length < 85 cm)	0	0	0	0	0
Number of normal plant length (>94 cm) with bent leaves	0	0	0	0	0
Number of normal plant length (>94 cm) with normal leaves	1	5	2	1	4
Number of plants with the transgene	4	19	4	3	0
Number of dwarf plants (plant length < 85 cm)	0	0	2	1	0
Number of normal plant length (>94 cm) with bent leaves	4	16	0	0	0
Number of normal plant length (>94 cm) with normal leaves	0	3	2	2	0

leaf-rolling response. The number of plants with each phenotype is shown in Table 1. Most of the *OsWRKY11* transgenic plants show the phenotype of normal plant length with bent leaves; plant length was more than 94 cm. In contrast, #ox4 showed dwarf phenotype; the plant length was less than 85 cm, and the WT plant had a plant length of 110–128 cm. Nontransgenic plants segregating from each primary transformant showed a phenotype of normal plant length with normal leaves. These results suggested that introduction of *HSP101* promoter::*OsWRKY11* might have caused some deleterious effects on plant growth.

Transgenic lines with a single copy of the T-DNA (i.e., #ox2 and #ox3) were selected, and T_2 plants homozygous for the T-DNA were used for further study. A transgenic line, #g8, which contained *HSP101* promoter::*GUS*, was used as a negative control. In 2-week-old seedlings of T_2 plants, plant length was identical among #ox2, #ox3, #g8, and WT. However, the plant length of #ox3 was slightly shorter when compared to that of #ox2, #g8, and WT in 4-week-old seedlings grown at 22/18°C ($n > 26$, $P < 0.0001$). The dwarf phenotype and bent leaves were manifested when the plants were subjected to heat treatment at 37/22°C (12/12 h) for 2 weeks (Fig. 3). Plant length of

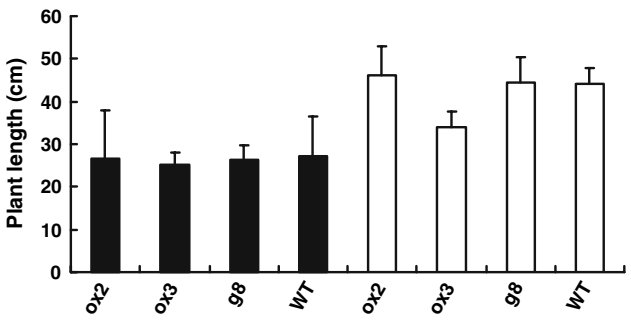

Fig. 3 Plant lengths of 2-week-old seedlings before or after heat treatment for 2 weeks (**a**). Transgenic line #ox2 showed a dwarf phenotype after heat shock. #ox2 and #ox3, T2 plants with *HSP101* promoter::*OsWRKY11*; *#g8*, *HSP101* promoter::*GUS*

#ox3 was significantly shorter than that of #ox2, #g8, and WT ($n > 28$, $P < 0.0001$). Under normal growth conditions, the seed set percentage was 36.7 ± 5.8 for #ox2 and 98.0 ± 0.8 for #ox3.

Induction of *OsWRKY* expression by heat treatment

Two-week-old seedlings grown in soil at 22/18°C (12/12 h) were exposed to heat pretreatment at 37/22°C (12/12 h) for 2 weeks with water everyday. Plants were maintained at 37°C during the day and at 22°C at night. For the control condition of no-heat pretreatment, plants were grown at 22/18°C. Induction of *OsWRKY11* expression was investigated in leaf blades using RT-PCR. A 164-bp band of *OsWRKY11* was detected in #ox2 and #ox3 after heat treatment, but was not detected in #g8 or WT (data not shown). In the case of no-heat pretreatment, a band of *OsWRKY11* was hardly detected in these PCR conditions.

The relative Os*WRKY11* expression level was also investigated using real-time quantitative RT-PCR (Fig. 4). The value in Fig. 4 indicates the relative expression level compared to that of *tubulin*. The relative Os*WRKY11* expression level of #ox2 and #ox3 with no-heat treatment (control condition) was the same as that of WT. In contrast, the values were 3.7 times higher in #ox2 and 3.0 times higher in #ox3 than in WT after the heat pretreatment. These results demonstrated that overexpression of *WRKY11* was induced by heat treatment, but it was not detectable under unstressed normal conditions.

Heat and drought tolerance of seedlings

Evaluation of tolerance against combined heat/drought stress was performed by determining the extent of recovery of whole plants from exposure to 37/22°C (12/12 h) without a water supply for 2 days. Prior to the combined heat/drought treatment, 2-week-old seedlings were grown for 2 weeks at 37/22°C with a water supply for the

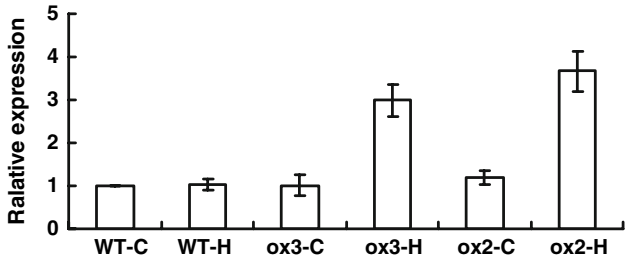

Fig. 4 Relative *OsWRKY11* expression level compared to that of *tubulin alpha-1* chain detected by real-time quantitative RT-PCR in plants with no-heat treatment (C) at 22/18°C (12/12 h) or plants with heat treatment (H) at 37/22°C (12/12 h) for 2 weeks. *Values* indicate relative expression level against that of WT-C in three biological replicates from cDNA prepared from leaf blades. Overexpression of Os*WRKY11* was induced by heat treatment. The expression level after no-heat treatment was the same as that in WT. #ox2 and #ox3, T_2 plants with *HSP101* promoter::*OsWRKY11*; *#g8*, *HSP101* promoter::*GUS*

induction of *OsWRKY11* expression. Figure 5a shows the photographs of #ox2, #ox3, and WT after the combined heat/drought treatment for 2 days without a water supply. The *OsWRKY11* transgenic lines #ox2 and #ox3 show a weaker leaf-wilting phenotype.

After the plants were exposed to 37/22°C for 2 days without water supply, they were returned to 22/18°C and grown with water supply for 2 weeks. Then, the percentage of surviving green parts was investigated (Fig. 6). The value (%) was 81.7 ± 1.9 for #ox2, 94.1 ± 1.6 for #ox3, while it was 39.8 ± 11 for #g8 and 46.6 ± 7.8 for WT. The difference was significant based on a *t* test ($P < 0.001$).

Figure 5b shows the photographs of #ox2, #ox3, and WT at 2 weeks of recovery after combined heat/drought treatment at 37/22°C for 2.5 days without a water supply. Prolonged heat and drought treatment caused all the WT plants to die, while the *OsWRKY11* transgenic lines #ox2 and #ox3 survived. These results demonstrated that the *OsWRKY11* transgenic lines #ox2 and #ox3 gained significant heat and drought tolerance.

Evaluation of water loss in detached aerial parts

We determined whether water loss was affected in Os*WRKY11* transgenic plants #ox2 and #ox3 by comparing the rates of change of the fresh weight of the detached aerial parts of plants during dehydration. For heat pretreatment, 2-week-old seedlings grown in soil at 22/18°C were exposed to heat pretreatment at 37/22°C for 2 weeks with water everyday. For the control condition with no-heat pretreatment, plants were grown at 22/18°C. After pretreatment for 2 weeks, the aerial parts of the transgenic plants and WT were detached and placed on a laboratory bench at 25°C and weighed at designated times.

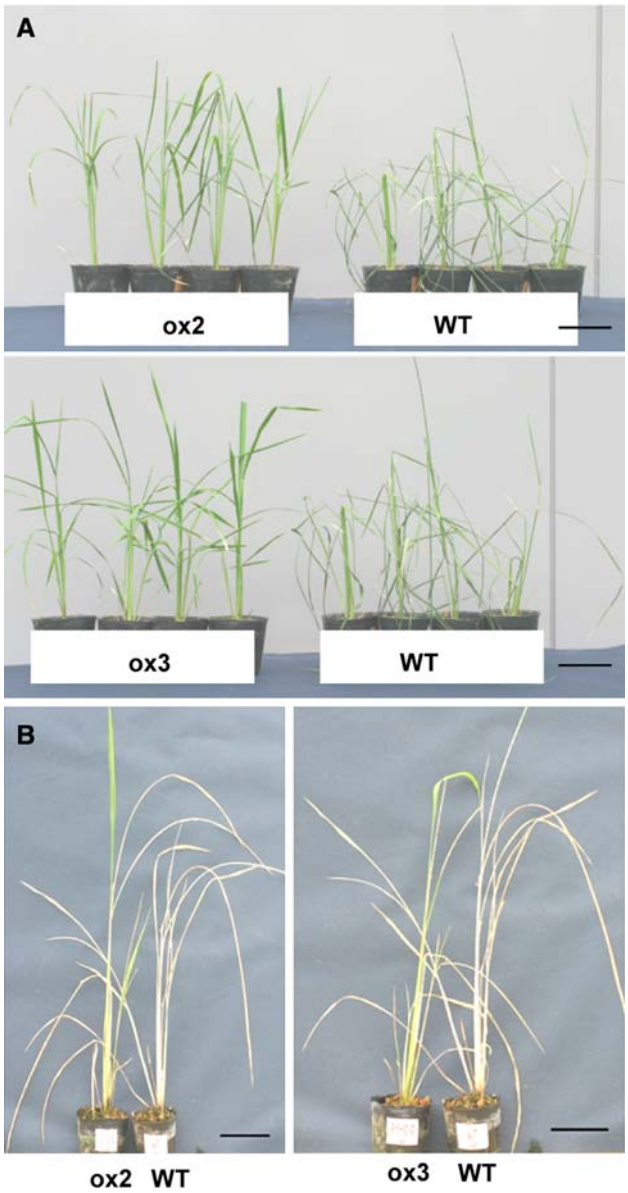

Fig. 5 Photographs of *OsWRKY11* transgenic lines #ox2 and #ox3, and WT after combined heat/drought treatment at 37/22°C (12/12 h) without water supply. Two-week-old seedlings were grown for 2 weeks at 37/22°C with water supply, and then the plants were exposed to combined heat and drought treatment. **a** The photographs were taken after combined heat/drought treatment for 2 days. The *OsWRKY11* transgenic lines #ox2 and #ox3 show weaker leaf-wilting phenotype. **b** The photographs were taken after recovery at 22/18°C for 2 weeks following heat/drought treatment for 2.5 days. The *OsWRKY11* transgenic lines #ox2 and #ox3 survived, whereas WT plants died after recovery at 22/18°C for 2 weeks. *Bar* 5 cm

The time course of water loss of plants with the heat pretreatment is shown in Fig. 7a and that of plants with the no-heat pretreatment is shown in Fig. 7b. In plants of both cases, water loss in #ox2 and #ox3 was slower than that in WT or #g8. In the case of plants with the heat pretreatment, the time (min) at which 20% of water loss was achieved

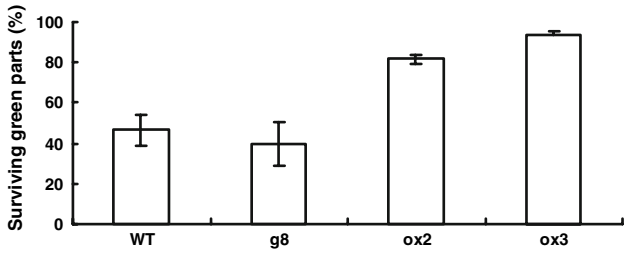

Fig. 6 Survival of green parts in seedlings after recovery for 2 weeks from exposure to combined heat and drought treatment. Two-week-old seedlings were grown for 2 weeks at 37/22°C with water supply, and then the plants were exposed to combined heat and drought treatment at 37/22°C (12/12 h) for 2 days without water supply. After recovery for 2 weeks, areal parts were separated to green living parts and nongreen dead parts, and each DW was determined. *Values* (mean ± SD) indicate the percentage of surviving green parts calculated using the following formula: (DW of green parts/DW of green parts plus dead parts) × 100 ($n = 4$). The *OsWRKY11* transgenic lines #ox2 and #ox3 conferred significant heat and drought tolerance compared to WT and #g8, plants with *HSP101* promoter::*GUS*

was 141 ± 13 (mean ± SD) for #ox2 and 121 ± 31 for #ox3, whereas it was 51 ± 13 for WT and 56 ± 32 for #g8. The difference was significant based on a *t* test ($P < 0.01$). In the case of plants with the no-heat pretreatment, the time (min) at which 20% of water loss was achieved was 69 ± 8 for #ox2 and 68 ± 13 for #ox3, whereas it was 34 ± 12 for WT and 35 ± 4 for #g8. The difference was significant based on a *t* test ($P < 0.01$). When we compared the ratio between the time of 20% water loss of plants with the heat treatment and those with the no-heat pretreatment, the ratio was 1.5 for WT, 1.6 for #g8, 2.1 for #ox2, and 1.8 for #ox3, indicating that heat pretreatment enhanced drought tolerance especially in the #ox2 and #ox3 plants. It is considered that the heat pretreatment induced the expression of the introduced *OsWRKY11* in addition to induction of endogenous *OsWRKY11*. Crosstolerance of heat and drought stresses was implicated even in WT plants. This experiment was repeated two more times and reproducibility was confirmed (data not shown).

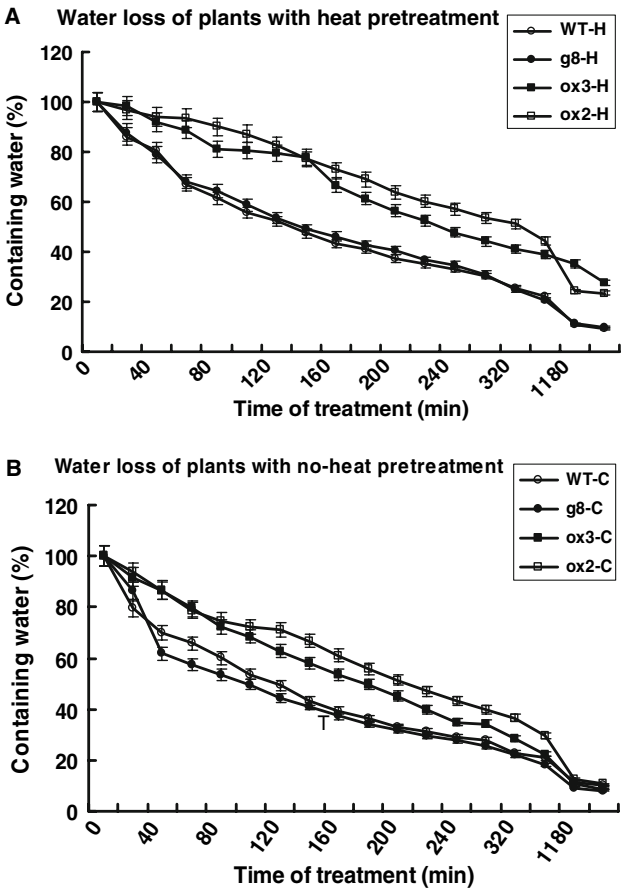

Fig. 7 The time courses of water loss in detached aerial parts. The water loss in #ox2 and #ox3 was slower than in WT and #g8. The water loss of plants with heat pretreatment (H) was slower than that with no-heat pretreatment (C). #ox2 and #ox3, T2 plants with *HSP101* promoter::*OsWRKY11*; *#g8, HSP101* promoter::*GUS*. *Values* (mean ± SD) indicate relative water content calculated using the following formula: $[(FW_i - DW)/(FW_0 - DW)] \times 100$, where FW_i and FW_0 are fresh weight for any given interval and original fresh weight, respectively, and DW is the dry weight ($n = 15$ for #ox2, #ox3, and WT; $n = 14$ for #g8)

Discussion

Advantage of using of *HSP101* promoter

Overexpression of transcription factor genes has sometimes been reported to accompany growth defects resulting in reduced productivity. This is presumably due to constitutive activation of defense-response genes under the regulation of introduced transcription factors. Such growth defects have been reported in transgenic rice plants overexpressing *OsDREB1A* (Ito et al. 2006) and *WRKY45* (Shimono et al. 2007). The *WRKY45* gene is one of the benzothiadiazole (BTH)- and salicylic acid (SA)-inducible *WRKY* genes. Growth retardation has been observed in transgenic rice plants with *WRKY45* driven by maize ubiquitin promoter (Shimono et al. 2007). It has been reported that growth conditions have a profound influence on the expression of pathogen-related genes during concurrent *WRKY45* expression, which may in turn affect the growth of *WRKY45*-overexpressing plants. This is quite similar to our results of the appearance of dwarf phenotype in the transgenic *OsWRKY11* lines. Dehydration-responsive element-binding protein 1 (DREB1)/C-repeat-binding factors (CBFs) are known to specifically interact with the DRE/CRT *cis*-acting element and to control the expression of many stress-inducible genes in *Arabidopsis*. Overexpression of the *OsDREB1A* under the control of CaMV35S

promoter or maize ubiquitin promoter has also been reported to cause growth retardation under unstressed control conditions in transgenic rice (Ito et al. 2006). Some transgenic lines showed a dwarf phenotype even at the reproductive stage. Kasuga et al. (1999, 2004) previously showed that the stress-inducible *rd29A* promoter instead of the constitutive promoter minimizes this negative effect on plant growth. Ito et al. (2006) demonstrated that the *Arabidopsis rd29A* gene promoter did not function in rice leaves; therefore, strong stress-inducible promoters of rice were necessary.

Our results indicated that the *HSP101* promoter of rice is heat-inducible (Figs. 2, 4) and can be successfully used for the induction of *OsWRKY11* expression to enhance heat and drought tolerance. Some leaky expression of *OsWRKY11*, although it was undetectable in leaf blades by RT-PCR, was likely to have occurred, because some plants, such as #ox3 and #ox4, showed a dwarf phenotype under unstressed normal conditions (Table 1). The *HSP101* promoter might drive *OsWRKY11* expression in the base of shoots, because weak GUS expression was observed in the base of stems without heat treatment (Fig. 2b). It is also possible that the CaMV 35S promoter in front of hygromycin phosphotransferase gene affected the *HSP101* promoter activity. Leaky expressions of *OsWRKY11* could also explain the fact that less impaired water loss of detached leaves was observed in plants without heat pretreatment (Fig. 7).

Involvement of *OsWRKY11* in stress tolerance

Successful enhancement of drought tolerance by overexpression of transcription factors has been reported in rice. Ito et al. (2006) reported that overexpression of *Arabidopsis DREB1* and rice ortholog *OsDREB1* showed improved tolerance to drought, high-salt, and low-temperature stresses in transgenic rice. Hu et al. (2006) reported that overexpression of stress-responsive gene SNAC (STRESS-RESPONSIVE NAC 1) significantly enhances drought resistance in transgenic rice. Improvement of water use efficiency and drought tolerance in rice has been reported by expression of *HARDY*, an *Arabidopsis* drought and salt tolerance gene encoding an AP2/ERF-like transcription factor (Karaba et al. 2007). Xu et al. (2008) reported that overexpression of *ZFP252*, which encodes a TFIIIA-type zinc finger protein, in rice increased the amount of free proline and soluble sugar, elevated the expression of stress defense genes, and enhanced tolerance to salt and drought stresses.

WRKY proteins constitute a large family of transcription factors implicated in many different processes. *WRKY* genes play a variety of developmental and physiological roles in plants. The most reported studies for this superfamily of genes address their involvement in disease responses. Overexpression of *AtWRKY18* in transgenic *Arabidopsis* plants results in enhanced expression of pathogenesis-related genes (Chen and Chen 2002). Enhancement of biotic stress by overexpressing *WRKY* genes has been reported. Shimono et al. (2007) reported that overexpression of BTH- and SA-inducible *WRKY45* gene in rice markedly enhanced resistance to rice blast fungus. Overexpression of *OsWRKY71* gene in rice resulted in enhanced resistance to virulent bacterial pathogens *Xanthomonas oryzae pv. oryzae* (*Xoo*) 13751 (Liu et al. 2007). However, employment of *WRKY* genes to enhance abiotic stresses has not yet been reported.

The relationship between *WRKY* and abiotic stress responses has been reported for some plant species. A barley gene coding for WRKY protein, Hv-WRKY38, has been reported to be involved in cold- and drought-response in barley (Mare et al. 2004). Involvement of an ABA-inducible *WRKY* gene in abiotic stresses has recently been reported in creosote bush (*Larrea tridentate*) (Zou et al. 2007). Qiu et al. (2004) reported that 10 of 13 *OsWRKY* genes (*OsW8-9*, *12–14*, *16*, *17*, *21–24*, *26*, *30*, and *45*) were differentially regulated in plants treated by the four following abiotic stress factors: NaCl, PEG, cold (4°C), and heat (42°C). The resulting expression profiles exhibited significant differences in both the manner and timing of their response to the four different abiotic treatments. The difference of gene expression profiles suggested the different physiological functions among the *WRKY* genes.

Our results in the current study have demonstrated that the transgenic lines #ox2 and #ox3 showed significant desiccation tolerance, as indicated by the slower water loss in detached leaves (Fig. 7). After heat pretreatment, the transgenic lines #ox2 and #ox3 showed significant heat and drought tolerance, as indicated by the slower leaf-wilting and less-impaired survival rate of green parts (Figs. 5, 6). *OsWRKY11* gene products may function in heat and drought stress response and tolerance. This is the first report of enhancement of abiotic stress by overexpression of *WRKY* genes.

Xie et al. (2005) undertook a comprehensive computational analysis of rice genomic sequences and predicted the structures of 81 *OsWRKY* genes, 48 of which are supported by full-length cDNA sequences. Recently, Ramamoorthy et al. (2008) have predicted 103 genes encoding WRKY transcription factors and reported comprehensive transcriptional profiles of the WRKY gene family in rice under various abiotic and phytohormone treatments. *OsWRKY11* (LOC_Os01g43650) was shown to be relatively highly expressed in young and mature panicles and weakly expressed in young and mature leaves and roots under normal growth conditions. They reported that *OsWRKY11* was not induced under abiotic (cold, drought, and salinity)

or various hormone (ABA, IAA, gibberellic acid, methyl jasmonate, and salicylic acid) treatments. Ryu et al. (2006) carried out a systematic expression analysis of *OsWRKY* genes and identified that, among 45 tested genes, the expression of 15 genes, including *OsWRKY11*, was increased remarkably in an incompatible interaction between rice and rice blast fungus (*Magnaporthe grisea*). They also suggested that these genes are also involved in defense response to abiotic stress, such as humidity, touch stress induced by spraying, and altered diurnal rhythms due to prolonged darkness during the pathogen infection procedure. The induction of some *OsWRKY* expressions, including *OsWRKY11*, has been shown to be induced by wounding in clipped leaves. It is considered that OsWRKY11 is involved in biotic stress response to disease, as well in as heat and drought response. The transgenic plants #ox2 and #ox3 are expected to gain tolerance to such abiotic stresses.

The most similar protein to *OsWRKY11* in *Arabidopsis* is *AtWRKY28* encoded by AT4G18170 with a similarity of 88% in the WRKY domain and 48% in the whole proteins. OsWRKY11 contains a single WRKY domain with C_2H_2-type (C-X_{4-5}-C-X_{22-24}-H-X_{1-2}-H) zinc finger motif and belongs to Group II of the WRKY family (Xie et al. 2005). OsWRKY11 contains the consensus coactivator motif, LXXLL, where L is Leu and X is any amino acid. OsWRKY11, therefore, is expected to bind specifically to the DNA sequence motif (T)(T)TGAC(C/T), which is known as the W box, in promoter regions and to activate certain gene expression. We are now investigating what kinds of genes are upregulated by *OsWRKY11*.

Conclusion

Drought and heat are major abiotic stresses on crop production associated with global warming. The *HSP101* promoter was shown to be activated by heat but not by drought stress. Heat stress, however, often precedes drought stress. The *HSP101* promoter, therefore, will be useful to confer heat and drought tolerance in field conditions, minimizing growth defects in unstressed conditions. Our data suggest that the *OsWRKY11* gene, together with the *HSP101* promoter, holds promising utility in improving drought and heat tolerance in rice.

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