

RESEARCH PAPER

OsWRKY45 alleles play different roles in abscisic acid signalling and salt stress tolerance but similar roles in drought and cold tolerance in rice

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Received 2 December 2010; Revised 11 April 2011; Accepted 12 April 2011

Abstract

Although allelic diversity of genes has been shown to contribute to many phenotypic variations associated with different physiological processes in plants, information on allelic diversity of abiotic stress-responsive genes is limited. Here it is shown that the alleles *OsWRKY45-1* and *OsWRKY45-2* play different roles in abscisic acid (ABA) signalling and salt stress adaptation in rice. The two alleles had different transcriptional responses to ABA and salt stresses. *OsWRKY45-1*-overexpressing lines showed reduced ABA sensitivity, whereas *OsWRKY45-1*-knockout lines showed increased ABA sensitivity. *OsWRKY45-1* transgenic plants showed no obvious difference from negative controls in response to salt stress. In contrast, *OsWRKY45-2*-overexpressing lines showed increased ABA sensitivity and reduced salt stress tolerance, and *OsWRKY45-2*-suppressing lines showed reduced ABA sensitivity and increased salt stress tolerance. *OsWRKY45-1* and *OsWRKY45-2* transgenic plants showed differential expression of a set of ABA- and abiotic stress-responsive genes, but they showed similar responses to cold and drought stresses. These results suggest that *OsWRKY45-1* negatively and *OsWRKY45-2* positively regulates ABA signalling and, in addition, *OsWRKY45-2* but not *OsWRKY45-1* negatively regulates rice response to salt stress. The different roles of the two alleles in ABA signalling and salt stress may be due to their transcriptional mediation of different signalling pathways.

Key words: Abiotic stress, biotic stress, *Oryza sativa*, transcription factor.

Introduction

In plants, naturally occurring intraspecific allelic diversity results in phenotypic variations. Different combinations of alleles generate numerous varieties in a species. Allelic diversity has been reported to contribute to variation in development, flower morphology, fruit and seed morphology and colour, grain weight and quality, plant architecture, disease resistance specificity, and pathogen-induced defence signalling (Jones, 2001; Alonso-Blanco *et al.*, 2005; Fan *et al.*, 2006; Bhave and Morris, 2008; Tao *et al.*, 2009). Allelic diversity may also provide clues to understanding heterosis (Springer and Stupar, 2007).

Genomic analyses of the transcriptomes of plant genomes have revealed that many genes are involved in plant responses

and adaptation to diverse abiotic stresses, such as drought, low temperature, and high salinity (Seki *et al.*, 2002; Mahajan *et al.*, 2005). Although relatively few genes have been characterized for their functions in plant tolerance to abiotic stresses, these genes function either positively or negatively in the pathways leading to stress adaptation. As regulatory proteins, transcription factors play important roles in almost every stress response. Much progress has been made in the characterization of rice transcription factors involved in response to abiotic stresses. At least four NAC-type transcription factors positively regulate rice tolerance to drought, salt, or cold stresses (Hu *et al.*, 2006, 2008; Zheng *et al.*, 2009; Jeong *et al.*, 2010). Several bZIP-type transcription factors

positively or negatively mediate rice adaptation to abiotic stresses (Xiang *et al.*, 2008; Zou *et al.*, 2008; Lu *et al.*, 2009; Amir Hossain *et al.*, 2010), and the transcription factors AP2/EREBP, MYB, and zinc-finger also play important roles in rice responses to various abiotic stresses (Xu *et al.*, 2006; Chen *et al.*, 2008; Huang *et al.*, 2009; Ma *et al.*, 2009).

The WRKY transcription factor family consists of 98–102 members in different rice varieties (Ross *et al.*, 2007). The expression of ~40% of the members was in response to abiotic stresses (Ramamoorthy *et al.*, 2008), suggesting that WRKY-type regulators may play important roles in rice adaptation to abiotic stresses. However, only three rice WRKY genes have been functionally characterized for their roles in abiotic stress responses in rice. OsWRKY13 negatively regulates rice tolerance to salt and cold stresses, and its role in tolerance may be at least partly due to suppressing the expression of *SNAC1*, which encodes a transcription factor that regulates rice response to abiotic stresses (Hu *et al.*, 2006; Qiu *et al.*, 2008). Overexpression of *OsWRKY89* enhanced tolerance to ultraviolet B irradiation (Wang *et al.*, 2007). Overexpression of *OsWRKY11* under the control of the heat shock protein 101 (HSP101) promoter enhanced heat and drought tolerance in rice (Wu *et al.*, 2009). Although a number of genes have been shown to function in pathways leading to the adaptation of various abiotic stresses in plants, the allelic diversity of abiotic stress-responsive genes is poorly understood.

The phytohormone abscisic acid (ABA) is involved in abiotic stress responses (Verslues and Zhu, 2005), in addition functioning in various aspects of plant growth throughout development (Himmelbach *et al.*, 2003; Finkelstein *et al.*, 2008). ABA regulates stomatal opening and ion channel activity in plant guard cells when a plant adapts to water deficiency and salt stress (Verslues and Zhu, 2005). ABA is also involved in the transcriptional and post-transcriptional regulation of some stress-responsive genes (Chinnusamy *et al.*, 2004). However, there is also ABA-independent signalling in plant responses to abiotic stresses (Chinnusamy *et al.*, 2004; Yamaguchi-Shinozaki and Shinozaki, 2006). Accumulating evidence suggests that ABA is also involved in host–pathogen interactions. In most cases, ABA functions as a negative regulator in pathogen-induced defence signalling, although it sometimes acts as a positive regulator (Bari and Jones, 2009). The multiple roles of ABA in abiotic and biotic stresses suggest that it may be an important signalling molecule in the cross-talk of transduction pathways leading to abiotic stress tolerance and disease resistance. However, the molecular mechanisms of ABA in this cross-talk are poorly understood.

Asian cultivated rice (*Oryza sativa* L.) consists of two major groups, which are known by the subspecies names *indica* (*O. sativa* L. ssp. *indica*) and *japonica* (*O. sativa* L. ssp. *japonica*). A previous study revealed that *OsWRKY45-1* from *japonica* rice and *OsWRKY45-2* from *indica* rice are alleles located on rice chromosome 5 (Tao *et al.*, 2009). The two alleles, which encode proteins with a difference of 10 amino acids, play opposite roles in rice resistance against bacterial pathogens. OsWRKY45-1 negatively and OsWRKY45-2

positively regulates rice resistance against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), which causes bacterial blight disease, and *Xanthomonas oryzae* pv. *oryzicola*, which causes bacterial streak disease. However, both alleles positively regulate rice resistance against *Magnaporthe grisea*, which causes fungal blast disease. The opposite roles of the two alleles in rice–bacterium interactions appear to be due to their mediation of different defence signalling pathways (Tao *et al.*, 2009). In rice–pathogen interactions, the expression of *OsWRKY45-1* and *OsWRKY45-2* is regulated by another transcriptional regulator, OsWRKY13 (Qiu *et al.*, 2007, 2009). Furthermore, both OsWRKY45-1 and OsWRKY45-2 appear to regulate *OsWRKY13* expression in rice–*Xoo* interaction (Tao *et al.*, 2009). OsWRKY13 positively regulates rice resistance against *Xoo* and *M. grisea* (Qiu *et al.*, 2007) but negatively regulates rice tolerance to abiotic stresses (Qiu *et al.*, 2008). These results suggest that OsWRKY45-1 and OsWRKY45-2 may also mediate the cross-talk between abiotic and biotic stresses, as does OsWRKY13. Here a series of experiments are reported whose results support this hypothesis. The two alleles are involved in rice response or adaptation to abiotic stresses. They play different roles, however, either negatively or positively regulating rice responses to ABA signalling and salt stress, but both negatively regulate rice responses to cold and drought stresses.

Materials and methods

Rice materials and stress treatment

Independent homozygous T₂ or T₃ generations of the *OsWRKY45-1*-overexpressing (oe) lines (D113UM3, D113UM10, and D113UM11), *OsWRKY45-2*-oe lines (D114UM4, D114UM6, and D114UM11), the *OsWRKY45-1*-knockout (KO) line (2C-50229), and *OsWRKY45-2*-suppressing [RNA interference (RNAi)] lines (D115RMH1 and D115RMH6) were used. The *OsWRKY45-1* gene from *japonica* rice variety Nipponbare (*O. sativa* L. ssp. *japonica*) and the *OsWRKY45-2* gene from *indica* rice variety Minghui 63 (*O. sativa* L. ssp. *indica*) were previously used for generating overexpressing transgenic lines (Tao *et al.*, 2009). The *OsWRKY45-1*-oe and *OsWRKY45-2*-oe plants were generated using the maize ubiquitin gene promoter to drive *OsWRKY45-1* or *OsWRKY45-2*, respectively, in the genetic background of rice variety Mudanjiang 8 (*O. sativa* L. ssp. *japonica*) that carried *OsWRKY45-1* (Tao *et al.*, 2009). The *OsWRKY45-1*-KO mutant, which had a T-DNA inserted into the promoter of *OsWRKY45-1*, had the background of variety Dongjin (*O. sativa* L. ssp. *japonica*) and was kindly provided by Professor Gynheung An (Jeong *et al.*, 2006; Tao *et al.*, 2009). The *OsWRKY45-1* genes and their promoter regions in Mudanjiang 8, Dongjin, and Nipponbare have identical sequences (Tao *et al.*, 2009). The *OsWRKY45-2*-RNAi plants were generated using the RNAi strategy in the genetic background of variety Minghui 63 (Tao *et al.*, 2009). The negative siblings from corresponding transgenic segregating populations were used as controls. For experiments testing cold, drought, and salt stresses, plants were grown in a greenhouse with light strength maintained at 12 000–14 000 lux and with a 14 h light/10 h dark cycle at 25 °C until the 4- to 5-leaf stage. The humidity in the greenhouse was maintained at 50–60%. The stress treatments were performed as reported previously (Hu *et al.*, 2008).

For analysing the influence of ABA on plant growth, plants germinated and grown on 1/2 strength MS medium at room temperature (25–28 °C) for 3 d were transferred to culture boxes

containing 1/2 strength MS medium plus 3 μ M ABA; each box contained two groups of plants, the transgenic and control plants. After 7–10 d of treatment (until the transgenic and control plants showed visible differences in growth rate), the phenotype was recorded.

For cold stress, plants were grown in soil in pots. Each pot contained two groups of plants, the transgenic and control plants. Seedlings were transferred to a growth chamber under a 14 h light/10 h dark cycle at 4 °C for 4–6 d (until almost all the leaves of one group in the pot became completely rolled and some leaves died); the plants were then recovered by maintaining them at room temperature for 3 d or 7 d and the phenotype was recorded.

For drought stress, water was withheld from seedlings of transgenic and control plants growing in the same pot filled with a mixture of sand and soil (1:1) for 3–5 d (until almost all the leaves of one group in the pot became completely rolled); water was then provided for 3–7 d and the survival rates were recorded.

For salt stress, seedlings of transgenic and control plants growing in the same pot filled with a mixture of sand and soil (1:1) were irrigated with a solution containing 200 mM NaCl for 4–6 d (until almost all the leaves of one group in the pot lost their green colour and some leaves died). During the treatment, phenotypes were recorded.

DNA sequencing and promoter analyses

The genomic fragment of *OsWRKY45-1* was amplified from rice cultivar IRAT109 (*O. sativa* L. ssp. *japonica*) using primers w45F4 and w45R4, and sequenced using primers w45F4, w45R4, w45F6, and w45R6 (Supplementary Table S1 available at *JXB* online). To analyse the putative conserved *cis*-elements, which are strongly related to various abiotic and biotic stresses, in the promoters of *OsWRKY45-1* and *OsWRKY45-2*, the ~1500 nucleotide sequences upstream of the transcription initiation sites of the two genes were used to search the PLACE (Plant Cis-acting Regulatory DNA Elements, <http://www.dna.affrc.go.jp/PLACE/index.html>) database.

Gene expression analyses

To analyse the expression patterns of *OsWRKY45-1* and *OsWRKY45-2* under various abiotic stresses, rice cultivars IRAT109 and Zhenshan 97 (*O. sativa* L. ssp. *indica*) were grown in a greenhouse until the 4- to 5-leaf stage with a 14 h light/10 h dark cycle at 25 °C. For ABA treatments, seedlings were sprayed with a solution containing 0.1 mM ABA and 0.02% Tween-20. For cold stress, seedlings were transferred to a growth chamber under 14 h light/10 h darkness at 4 °C for 3–24 h. For drought stress, seedlings grown on sandy soil (Hu *et al.*, 2008) were not watered for 3–6 d. For salt stress, seedlings were irrigated with 200 mM NaCl solution for 3–24 h. The quantitative reverse transcription-PCR (qRT-PCR) analysis was conducted as described previously (Qiu *et al.*, 2007) using gene-specific primers (Supplementary Table S1 at *JXB* online). The expression level of the rice actin gene was used to standardize the RNA sample for each qRT-PCR. The expression level relative to the control was presented.

Statistical analysis

The significant differences between control and treatment of the samples or between control and transgenic plants were analysed by the pair-wise *t*-test installed in the Microsoft Office Excel program.

Results

Both *OsWRKY45-1* and *OsWRKY45-2* a transcriptional response to ABA and abiotic stresses

The upland *japonica* rice variety IRAT109 (*O. sativa* L. ssp. *japonica*) is more drought resistant than the lowland *indica* rice variety Zhenshan 97 (*O. sativa* L. ssp. *indica*; Yue *et al.*,

2006). Zhenshan 97 carries *OsWRKY45-2* (Tao *et al.*, 2009). Sequencing the DNA fragment (~3.7 kb) harbouring the *OsWRKY45* gene in IRAT109, including the 1.5 kb (upstream of the transcription initiation site) promoter region, coding region, and 5'- and 3'-untranslated regions, showed that it carried *OsWRKY45-1*, with its sequence identical to that in the *japonica* variety Mudanjiang 8 (Tao *et al.*, 2009). To ascertain whether the two alleles play roles in rice responses to abiotic stresses, the expression patterns of the two genes were examined under drought, high salinity (250 mM NaCl), cold (4 °C), and ABA (100 μ M) stresses by qRT-PCR.

OsWRKY45-1 and *OsWRKY45-2* showed a similar response to cold and drought stresses (Fig. 1). The expression of the two alleles was markedly induced after cold stress and suppressed after drought treatment. However, the two alleles showed different expression patterns after ABA and salt stresses (Fig. 1). In ABA treatment, *OsWRKY45-1* expression was first slightly suppressed, then induced, and later suppressed again; *OsWRKY45-2* expression was first rapidly suppressed, then recovered to a normal level, and later suppressed again. In salt stress, *OsWRKY45-1* expression was first induced and then suppressed, whereas *OsWRKY45-2* expression was first suppressed, then induced, and later suppressed again. These results suggest that the two alleles may be involved in rice responses to ABA signalling and abiotic stresses, but they may function differently in ABA and salt stresses.

The differential expression patterns of *OsWRKY45-1* and *OsWRKY45-2* in response to ABA and salt stresses prompted the examination of whether there are different ABA- and stress-responsive *cis*-acting elements in their promoters. Sequence analysis showed that both promoters contained ABRE, DRE, GCC, LTRE, MYB, and MYC *cis*-elements that have been reported to be responsive to ABA, cold, drought, or salt stresses (Supplementary Table S2 and Fig. S1 at *JXB* online). However, the *OsWRKY45-2* promoter had two additional ABREs and one additional MYC element compared with the *OsWRKY45-1* promoter. The different structures of the two promoters may explain the differential expression patterns of the two alleles in response to ABA and salt treatments.

OsWRKY45-1 and *OsWRKY45-2* play different roles in response to ABA treatment

Xiang *et al.* (2008) reported that exogenous ABA suppresses the growth of rice seedlings. To ascertain whether this pair of alleles plays different roles in ABA signalling, the response of rice to ABA stress was analysed using different transgenic lines. The seedling growth of *OsWRKY45-1-oe*, *OsWRKY45-1-KO*, *OsWRKY45-2-oe*, and *OsWRKY45-2-RNAi* plants showed no obvious difference from that of corresponding control plants, when grown on medium without ABA (Fig. 2A; Supplementary Fig. S2A at *JXB* online). After treatment with 3 μ M ABA, *OsWRKY45-1-oe* lines had significantly longer shoots than the control, whereas *OsWRKY45-1-KO* lines had significantly shorter shoots than the control (Fig. 2B; Supplementary Fig. S2B).

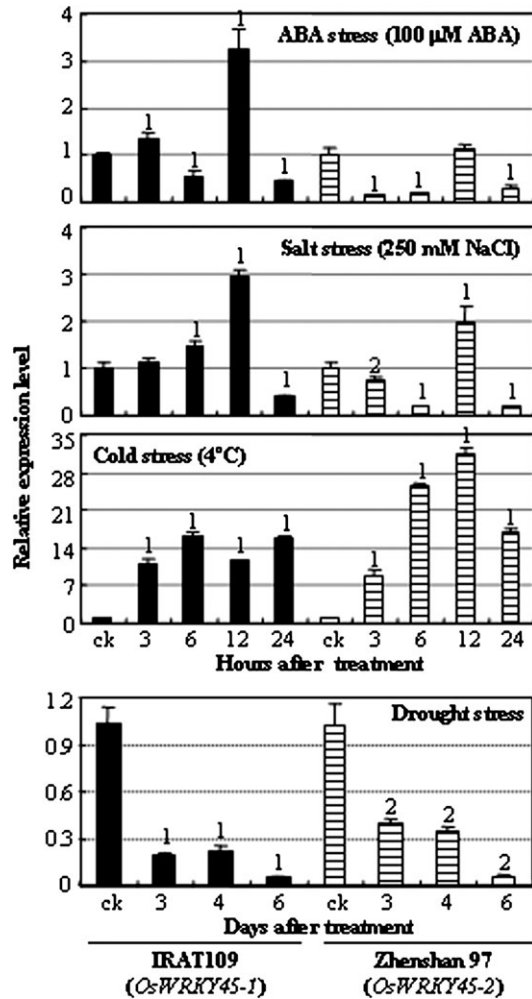


Fig. 1. Expression patterns of *OsWRKY45-1* and *OsWRKY45-2* in response to ABA and abiotic stresses in rice cultivars IRAT109 and Zhenshan 97. Bars represent the mean (three technical replicates) \pm standard deviation. The '1' or '2' indicates that a significant difference was detected between treated plants and untreated control (ck) at $P < 0.01$ or $P < 0.05$, respectively.

Modulating *OsWRKY45-1* expression appeared to have no obvious influence on the growth of root after ABA treatment compared with controls. In contrast, *OsWRKY45-2-oe* lines had significantly shorter roots and shoots than those of the control, whereas *OsWRKY45-2-RNAi* lines had significantly longer roots and somewhat longer shoots than those of the control. These results further suggest that the two alleles respond differently to ABA-mediated signalling, and *OsWRKY45-1* negatively and *OsWRKY45-2* positively regulates rice response to ABA signalling.

OsWRKY45-1 and *OsWRKY45-2* function differently in regulating the salt stress response

Transgenic lines with modified expression of either *OsWRKY45-1* or *OsWRKY45-2* showed no obvious difference from corresponding controls before salt stress. After treating the plants with 200 mM NaCl for 5 d, the survival rates of *OsWRKY45-1-oe* and *OsWRKY45-1-KO* lines were

similar to those of corresponding controls, whereas the survival rates of *OsWRKY45-2-oe* and *OsWRKY45-2-RNAi* lines were significantly lower and higher than corresponding controls, respectively (Fig. 3; Supplementary Fig. S3 at *JXB* online). When *OsWRKY45-1-oe* and *OsWRKY45-2-oe* plants, which had the same genetic background, were grown in the same pot, the former was significantly more tolerant to salt stress than the latter; the survival rate of the *OsWRKY45-1-oe* plants was 3.6-fold higher than that of the *OsWRKY45-2-oe* plants (Fig. 3). These results suggest that *OsWRKY45-2* negatively regulates the salt stress response and *OsWRKY45-1* is insensitive to salt stress, although it was transcriptionally induced by salt stress (Fig. 1).

OsWRKY45-1 and *OsWRKY45-2* play similar roles in regulating the cold stress response

Both *OsWRKY45-1-oe* and *OsWRKY45-2-oe* plants showed a similar level of sensitivity to cold. The leaves of *OsWRKY45-oe* plants rolled extensively in a cold environment. After recovery, these plants died quickly compared with the controls (Fig. 4A; Supplementary Fig. S4 at *JXB* online). After 7 d of recovery, the average survival rate of different *OsWRKY45-oe* lines was 6–18% compared with 76% for the control (Fig. 4A). In contrast, *OsWRKY45-1-KO* and *OsWRKY45-2-RNAi* plants were more tolerant to cold than the control (Fig. 4A; Supplementary Fig. S4). After 7 d of recovery, the average survival rate of *OsWRKY45-1-KO* plants was 48–55% compared with 16% for the control; the average survival rates of the two *OsWRKY45-2-RNAi* lines were 36% and 51% compared with 13% and 36% for corresponding negative controls, respectively (Fig. 4B). When *OsWRKY45-1-oe* and *OsWRKY45-2-oe* lines were grown in the same pot, the two lines showed a similar level of sensitivity to cold stress (Fig. 4). The marked difference of the survival rates among different control plants could be due to the difference in genetic background or due to the varying durations of cold stress (4–6 d) for different groups of plants. These results suggest that both *OsWRKY45-1* and *OsWRKY45-2* negatively regulate rice response to cold stress.

OsWRKY45-1 and *OsWRKY45-2* play similar roles in regulating the drought stress response

Both *OsWRKY45-1-oe* and *OsWRKY45-2-oe* plants showed a similar level of sensitivity to drought stress. After drought stress, these plants have a significantly lower survival rate than corresponding controls (Fig. 5; Supplementary Fig. S5 at *JXB* online). After 3 d of recovery, the survive rates of the two *OsWRKY45-1-oe* lines were 27% and 22% compared with 57% and 47% for the controls, respectively; the survival rates of the two *OsWRKY45-2-oe* lines were 33% and 31% compared with 86% and 72% for the controls, respectively. When the *OsWRKY45-1-oe* and *OsWRKY45-2-oe* plants were grown in the same pot, they showed no obvious difference in the sensitivity to drought stress

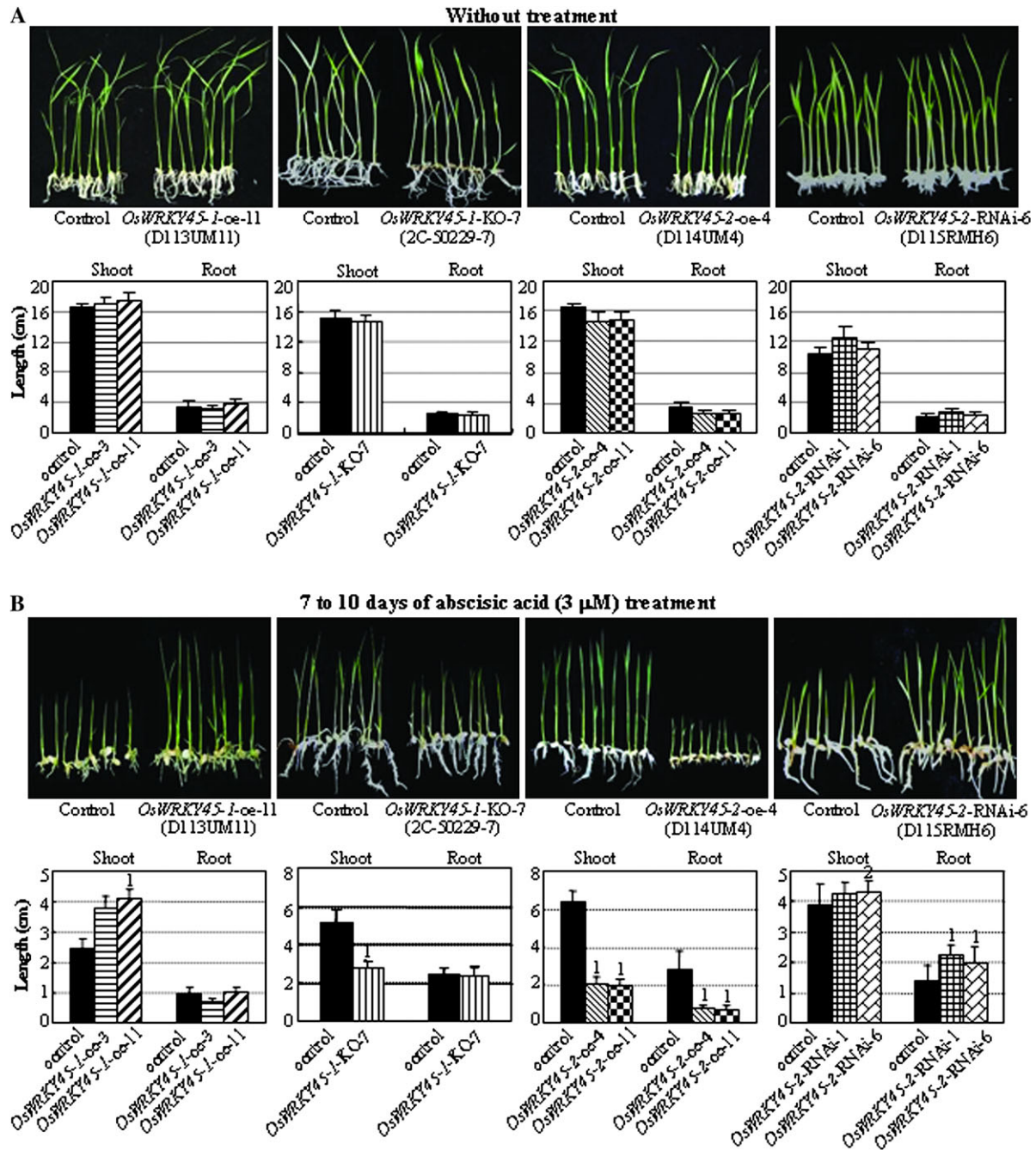


Fig. 2. Modulating *OsWRKY45-1* and *OsWRKY45-2* expression influenced rice response to ABA treatment. Control, negative siblings from corresponding transgenic segregating populations. (A) Rice seedlings before ABA treatment. Plants grown on MS medium for 10 d. (B) Phenotypes of transgenic plants after ABA treatment. Rice seedlings were grown on MS medium containing ABA for 7–10 d (until the transgenic and control plants showed marked differences in growth rate) and then phenotypes were recorded. Bars represent the mean (8–10 plants) \pm standard deviation. The '1' or '2' indicates that a significant difference was detected between a positive transgenic line and its negative control at $P < 0.01$ or $P < 0.05$, respectively. Similar results were obtained in two biological repeats and only data from one repeat are presented. Because of the varying durations of ABA treatment (7–10 d) in different groups of plants, the control plants with the same genetic background in the *OsWRKY45-1-oe* and *OsWRKY45-2-oe* groups exhibited different lengths of shoot and root.

(Fig. 5). In contrast, *OsWRKY45-1-KO* and *OsWRKY45-2-RNAi* plants were significantly more tolerant to drought stress than the controls (Fig. 5; Supplementary Fig. S5). After 3 d of recovery, the survival rate of *OsWRKY45-1-KO* line was 56% compared with 43% for the control; the survival rates of the two *OsWRKY45-2-RNAi* lines were

28% and 28% compared with 20% and 18% for their corresponding controls, respectively (Fig. 5; Supplementary Fig. S5). The marked difference in the survival rates among different control plants could be due to the difference in genetic background or due to the varying durations of drought stress (3–5 d) for the different groups of plants.

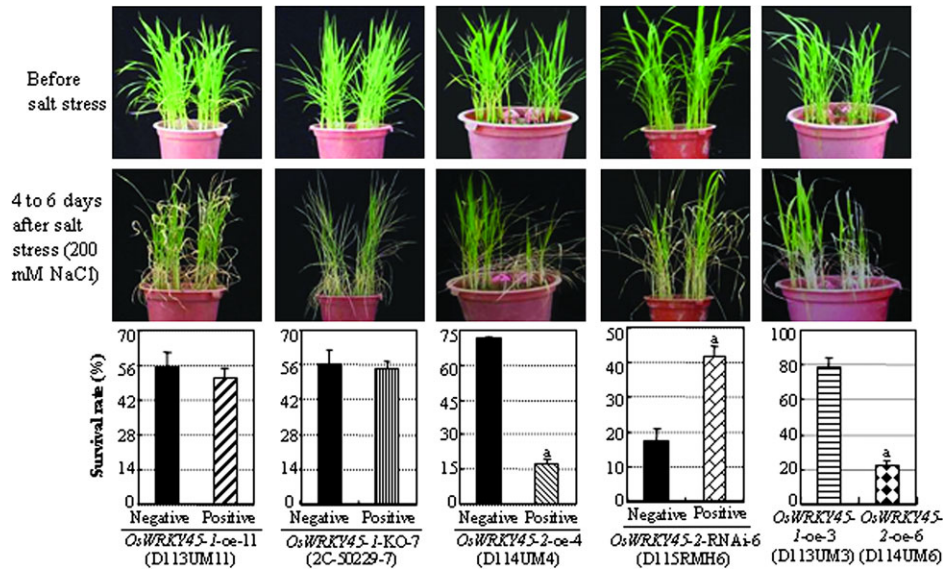


Fig. 3. Modulating the expression of *OsWRKY45-2* but not *OsWRKY45-1* influenced rice response to salt stress. Rice seedlings were irrigated with a solution containing 200 mM NaCl at the 4- to 5-leaf stage. After 4–6 d of stress (until almost all the leaves of one group in the pot lost their green colour and some leaves died), the survival rates were recorded. Negative, negative siblings from corresponding transgenic segregating populations; positive, positive transgenic plants. Bars represent the mean (three technical replicates with each replicate containing 16–20 plants) \pm standard deviation. The ‘1’ indicates that a significant difference was detected between a positive transgenic line and its negative control at $P < 0.01$. Similar results were obtained in three biological repeats and only data from one repeat are presented. Because of the varying durations of salt stress (4–6 d) in different groups of plants, the control plants with the same genetic background in the *OsWRKY45-1-oe* and *OsWRKY45-2-oe* groups exhibited different survival rates.

These results suggest that both *OsWRKY45-1* and *OsWRKY45-2* negatively regulate rice response to drought stress.

Modulating *OsWRKY45-1* and *OsWRKY45-2* expression influences expression of ABA- and abiotic stress-responsive genes

To investigate differences in the function of *OsWRKY45-1* and *OsWRKY45-2* in the regulation of ABA signalling and abiotic stress responses, the expression of 10 ABA- and putative stress-related genes in plant leaves was analysed. These genes included three ABA biosynthesis genes, *NCED2* (AK120176), *NCED4* (AK119780), and *NCED5* (AK107649) (Zhu et al., 2009); four ABA- or abiotic stress-responsive genes, *ABI5* (AK070998), *RD22* (AK065358), *Rab16D* (AK109096), and *Rab21* (AK121952) (Buchanan et al., 2004; Zou et al., 2008; Hou et al., 2009); and three known abiotic stress-resistant genes, *SNAC1* (AK067690), *OsDREB1A* (AY345233), and *OsDREB1B* (AY166833) (Dubouzet et al., 2003; Hu et al., 2006; Ito et al., 2006).

All the genes showed differential expression in at least one type of transgenic plant as compared with their expression in corresponding wild-type plants (Fig. 6). Nine of the genes (*NCED2*, *NCED4*, *NCED5*, *RD22*, *Rab16D*, *Rab21*, *SNAC1*, *OsDREB1B*, and *OsDREB1A*) showed opposite expression patterns in the overexpressing and knockout/suppressing plants for at least one *OsWRKY45* allele. Among the nine genes, *SNAC1* and *OsDREB1B* showed similar expression patterns in both the *OsWRKY45-1* and *OsWRKY45-2* transgenic plants; the two genes were significantly suppressed in

OsWRKY45-oe plants and significantly induced in *OsWRKY45-KO* and *OsWRKY45-RNAi* plants. *Rab16D* showed opposite expression patterns in *OsWRKY45-1* and *OsWRKY45-2* transgenic plants; this gene was significantly induced in *OsWRKY45-1-oe* lines and appeared to be suppressed in *OsWRKY45-1-KO* lines, but it was significantly suppressed in *OsWRKY45-2-oe* lines and significantly induced in *OsWRKY45-2-RNAi* lines. *NCED2*, *OsDREB1A*, *NCED5*, and *RD22* only showed opposite expression patterns in *OsWRKY45-1-oe* and *OsWRKY45-1-KO* plants (Fig. 6). *NCED2* and *OsDREB1A* were significantly suppressed in *OsWRKY45-1-oe* lines and significantly induced in *OsWRKY45-1-KO* lines. *NCED5* and *RD22* were significantly induced in *OsWRKY45-1-oe* lines and significantly suppressed in *OsWRKY45-1-KO* lines. *NCED4* only showed opposite expression patterns in *OsWRKY45-2-oe* and *OsWRKY45-2-RNAi* plants (Fig. 6); it was significantly suppressed in *OsWRKY45-2-oe* lines and significantly induced in *OsWRKY45-2-RNAi* lines. These results suggest that *OsWRKY45* directly or indirectly regulates the expression of at least nine of the 10 genes. The differential expression of most of the genes examined in *OsWRKY45-1* and *OsWRKY45-2* transgenic plants also suggests that this pair of alleles may regulate ABA signalling and abiotic stress responses through different pathways.

Discussion

To compare the functions of *OsWRKY45* alleles, they were separately overexpressed in the same genetic background.

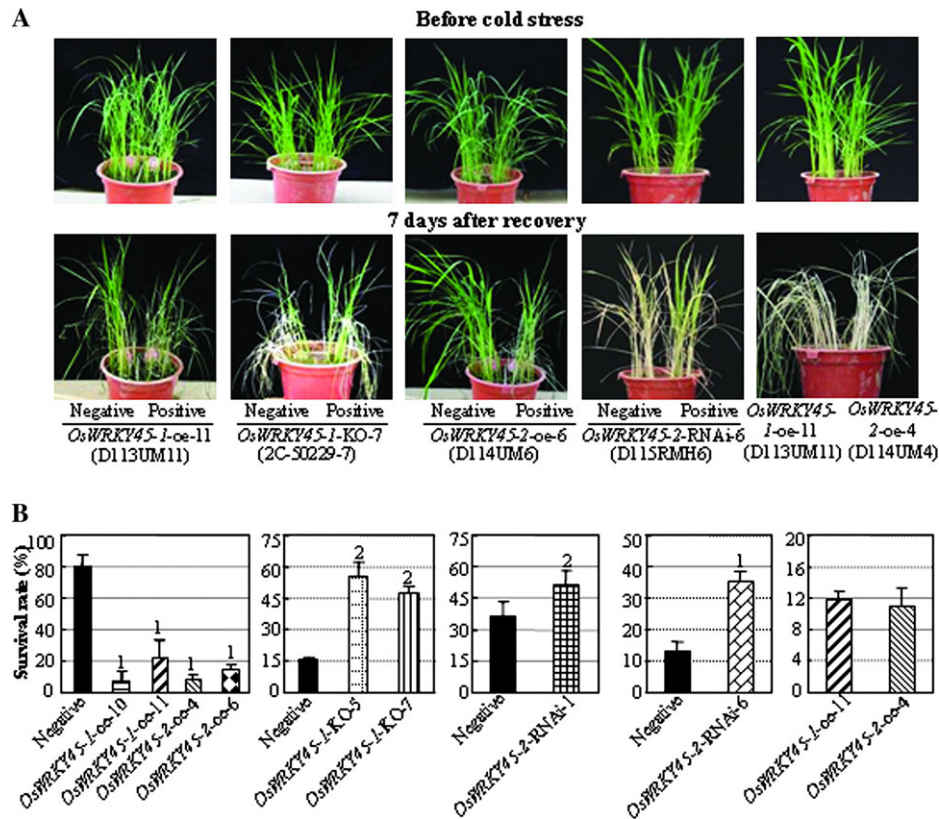


Fig. 4. Modulating *OsWRKY45-1* and *OsWRKY45-2* expression influenced rice response to cold stress. Rice seedlings were kept at 4 °C for 4–6 d (until almost all the leaves of one group in the pot became completely rolled and some leaves died) and then transferred to room temperature (25–28 °C) for recovery. Negative, negative siblings from corresponding transgenic segregating populations; positive, positive transgenic plants. (A) Phenotypes of transgenic plants before and after cold treatment. (B) Survival rate of cold-treated plants. Bars represent the mean (two or three replicates with each replicate containing 16–20 plants) \pm standard deviation. The ‘1’ or ‘2’ indicates that a significant difference was detected between a positive transgenic line and its negative control at $P < 0.01$ or $P < 0.05$, respectively. Similar results were obtained in three biological repeats and only data from one repeat are presented.

Although the *OsWRKY45-2-oe* plants expressed *OsWRKY45-2* at a high level and background *OsWRKY45-1*, the observed phenotypes should be contributed by the constitutively overexpressed *OsWRKY45-2*. This inference is supported by the following evidence. First, the *OsWRKY45-2-oe* and *OsWRKY45-2-RNAi* plants showed opposite phenotypes in response to ABA and abiotic stresses. Secondly, *OsWRKY45-1-oe* and *OsWRKY45-2-oe* plants showed different responses to ABA and salt stresses. The present findings indicate that *OsWRKY45* alleles play opposite roles in ABA signalling and salt stress responses in rice, although they have similar functions in responses to cold and drought stresses. The opposite phenotypes of *OsWRKY45-oe* and *OsWRKY45-KO* and *OsWRKY45-RNAi* plants suggest that *OsWRKY45-1* negatively and *OsWRKY45-2* positively regulates ABA signalling. Furthermore, *OsWRKY45-2* negatively regulates rice response to salt stress. *OsWRKY45-1* does not influence the response to salt stress, although its transcriptional expression was changed by this stress. However, both *OsWRKY45-1* and *OsWRKY45-2* negatively regulate rice responses to cold and drought stresses. These observations not only remind us to consider the roles of intraspecific allelic variation in plant tolerance and adaptation to abiotic stresses, but they also

provide a novel molecular-based example of how a pair of alleles functions distinctly in abiotic stress adaptation.

Alteration of the protein and promoter sequences may result in different functions of *OsWRKY45* alleles

Intraspecific allelic diversity can be caused by nucleotide sequence differences in coding regions, differential expression, and epigenetic changes (Guo *et al.*, 2004; Shiba and Takayama, 2007; Springer and Stupar, 2007). The first type of allelic diversity results in alteration of protein function, whereas the last two types cause variation in the quantity of the same protein. The difference in 10 amino acids between *OsWRKY45-1* and *OsWRKY45-2* may be a factor that results in the phenotypic variation of transgenic plants, as supported by the following evidence. First, the two alleles in *OsWRKY45-1-oe* and *OsWRKY45-2-oe* plants were regulated by the same promoter. In addition, the *OsWRKY45-1-oe* and *OsWRKY45-2-oe* plants had the same genetic background, which can eliminate the putative differential transcriptional and epigenetic regulations in the present experimental conditions.

The promoter regions (~1.5 kb upstream of the transcription initiation sites) of *OsWRKY45-1* and *OsWRKY45-2*

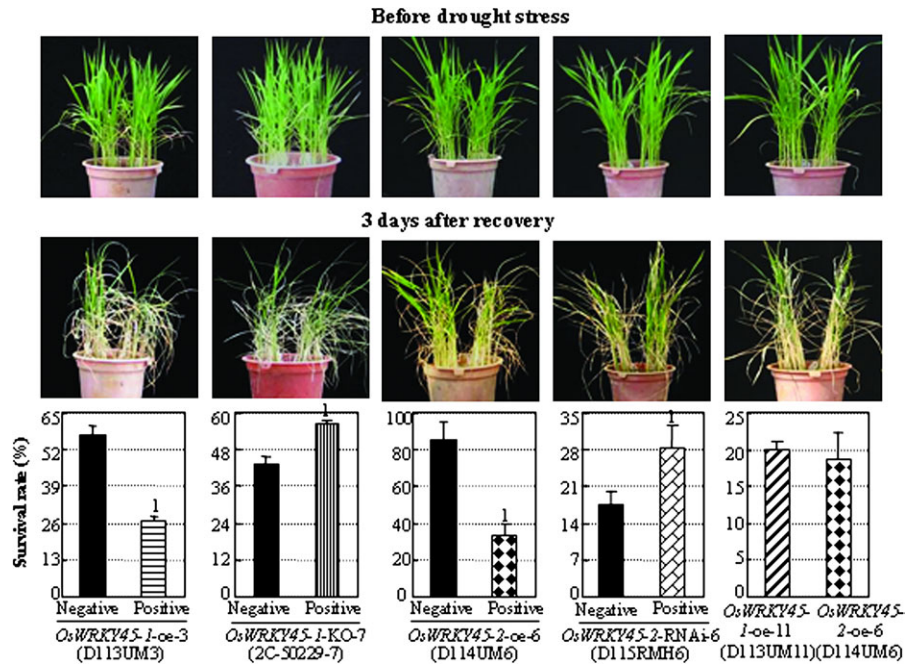


Fig. 5. Modulating *OsWRKY45-1* and *OsWRKY45-2* expression influenced rice response to drought stress. Water was withheld from rice seedlings at the 4- to 5-leaf stage for 3–5 d (until almost all the leaves of one group in the pot became completely rolled). After 3 d of recovery, the survival rates were recorded. Negative, negative siblings from corresponding transgenic segregating populations; positive, positive transgenic plants. Bars represent the mean (two or three replicates with each replicate containing 16–20 plants) \pm standard deviation. The '1' indicates that a significant difference was detected between a positive transgenic line and its negative control at $P < 0.01$. Similar results were obtained in the three biological repeats and only data from one repeat are presented. Because of the varying durations of drought stress (3–5 d) in different groups of plants, the control plants with the same genetic background in the *OsWRKY45-1-oe* and *OsWRKY45-2-oe* groups exhibited different survival rates.

differ in 19 nucleotides (Tao *et al.*, 2009). The *OsWRKY45-2* promoter has two additional ABRE-type *cis*-acting elements and one additional MYC-type *cis*-element (Supplementary Table S1 and Fig. S1 at *JXB* online). The ABRE for binding basic leucine zipper-type proteins and the MYC element for binding helix–loop–helix and leucine zipper-type proteins are important regulating elements in ABA-responsive gene expression in two ABA-dependent signalling pathways involved in drought and salt stresses (Agarwal *et al.*, 2006; Valliyodan and Nguyen, 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). Thus, the two alleles may function differently in ABA signalling and salt tolerance due to differentially regulating their expression via *trans*-acting factors under physiological conditions. This hypothesis is supported by evidence that the two alleles showed different expression patterns in response to ABA and salt stresses (Fig. 1).

The different roles of the alleles in ABA signalling and salt stress tolerance may be due to differential regulation of ABA- and abiotic stress-responsive genes

OsWRKY45-1 functions as a transcriptional regulator (Shimono *et al.*, 2007). It is argued that *OsWRKY45-2* is also a transcription factor. ABA is an important signalling molecule when a plant faces abiotic stress (Verslues and Zhu, 2005). The two alleles differentially regulate the expression of

ABA synthesis-related *NCED* genes, which encode 9-*cis*-epoxycarotenoid dioxygenases and function in the ABA biosynthetic pathway (Nambara and Marion-Poll, 2005). The opposite expression patterns of *NCED2* and *NCED5* in *OsWRKY45-1-oe* and *OsWRKY45-1-KO* plants suggest that the two genes may be more closely associated with the negative role of *OsWRKY45-1* in ABA signalling. In contrast, the opposite expression patterns of *NCED4* in *OsWRKY45-2-oe* and *OsWRKY45-2-RNAi* plants suggest that this gene may be more closely associated with the positive role of *OsWRKY45-2* in ABA signalling. Thus, *OsWRKY45* transcriptionally regulates ABA biosynthesis, which in turn may influence the expression of ABA-responsive genes and abiotic stress-responsive genes involved in salt stress response.

This hypothesis is supported by the different expression patterns of *RD22*, *Rab16D*, and *Rab21* in *OsWRKY45-1* and *OsWRKY45-2* transgenic plants. The *Arabidopsis* homologues of rice *RD22*, *Rab16D*, and *Rab21* are well-documented ABA- and abiotic stress-responsive genes (Yamaguchi-Shinozaki and Shinozaki, 1993; Nylander *et al.*, 2001). Rice *Rab16D*, encoding a dehydrin-like protein, was rapidly induced by ABA application and abiotic stresses (Rabbani *et al.*, 2003; Buchanan *et al.*, 2004). The opposite expression patterns of *Rab16D* in *OsWRKY45-1* and *OsWRKY45-2* transgenic plants suggest that *OsWRKY45-1* is a transcriptional activator and *OsWRKY45-2* is a transcriptional repressor of *Rab16D*.

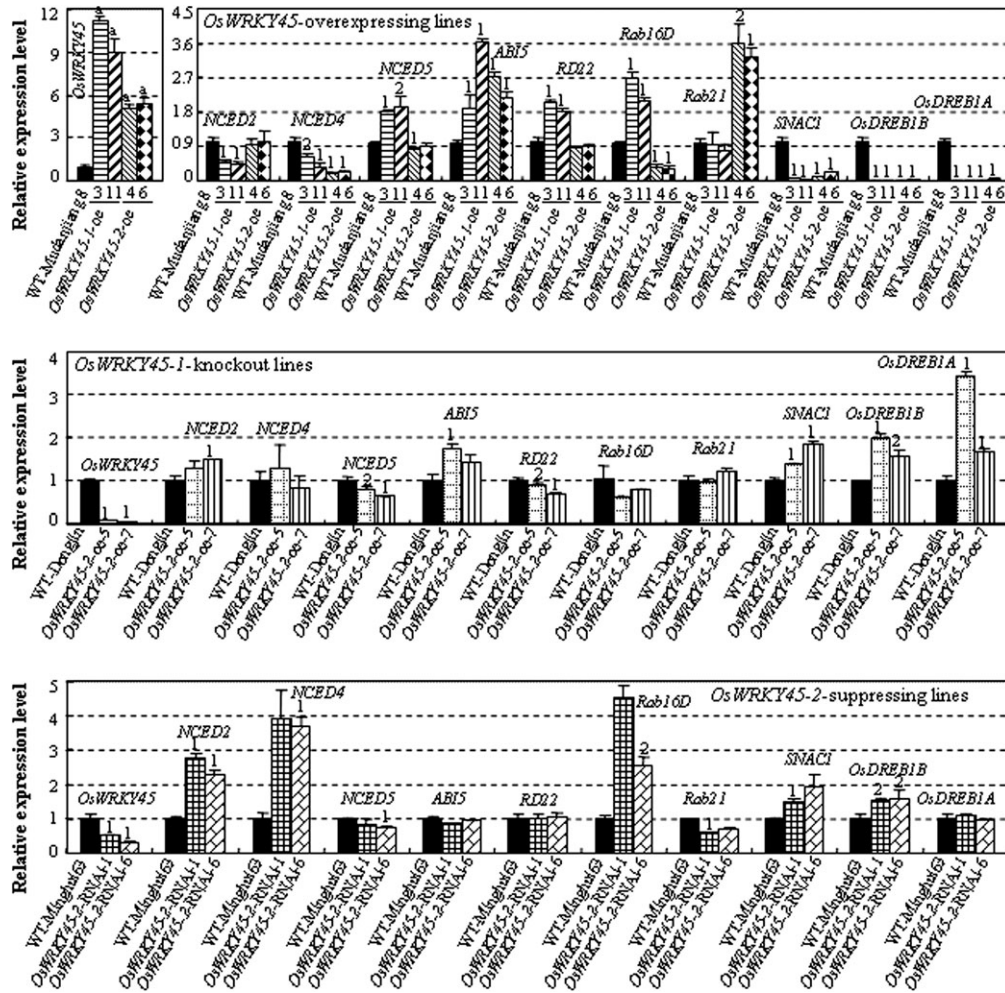


Fig. 6. Modulating *OsWRKY45-1* and *OsWRKY45-2* expression influenced the expression of ABA- and abiotic stress-related genes. Bars represent the mean (three technical replicates) \pm standard deviation. The '1' or '2' indicates that a significant difference was detected between a transgenic line and its corresponding wild-type (WT) plant at $P < 0.01$ or $P < 0.05$, respectively.

Although *Rab21*, which also encodes a dehydrin-like protein, was transcriptionally responsive to abiotic stress, its expression level was reduced in transgenic plants showing enhanced drought tolerance (Hou *et al.*, 2009). The result is consistent with this previous report. Transcriptional modulation of *OsWRKY45-2* but not *OsWRKY45-1* influenced *Rab21* expression; the suppression of *OsWRKY45-2* accompanied by enhanced abiotic stress tolerance was associated with suppressed *Rab21* expression (Fig. 6). The induction of *RD22*, encoding a BURP domain-containing protein, was associated with enhanced drought tolerance in transgenic plants (Hou *et al.*, 2009). The present result is also consistent with this report. Transcriptional modulation of *OsWRKY45-1* but not *OsWRKY45-2* influenced *RD22* expression; activation of *OsWRKY45-1* accompanied by enhanced ABA stress tolerance and non-sensitivity to salt stress was associated with induced *RD22* expression (Fig. 6). Although the definite functions of the three genes in adaptation to abiotic stresses remain to be elucidated in rice, the present results suggest that these genes may be involved in the different roles of *OsWRKY45-1* and *OsWRKY45-2* in ABA signalling and the response to salt stress.

OsWRKY45-1 and *OsWRKY45-2* may regulate abiotic stress responses via different pathways

Transgenic rice plants overexpressing *SNAC1*, which encodes a transcription factor, showed improved drought and salt tolerance but increased ABA sensitivity (Hu *et al.*, 2006). Overexpressing *OsDREB1B*, which encodes a transcription factor, enhanced rice tolerance to drought, salt, and cold stresses (Ito *et al.*, 2006). *OsDREB1B* is under the regulation of *SNAC1*, and the activation of *SNAC1* transcriptionally induced *OsDREB1B*, suggesting that *SNAC1* and *OsDREB1B* may function in the same pathway (Fig. 6). Although both *OsWRKY45-1* and *OsWRKY45-2* suppressed the expression of *SNAC1* and *OsDREB1B*, the phenotypes of transgenic plants suggest that *OsWRKY45-2* negatively regulates rice responses to cold, drought, and salt stresses, and *OsWRKY45-1* negatively regulates rice responses to cold and drought stresses but it does not obviously influence the response to salt stress. These results

suggest that both OsWRKY45-1 and OsWRKY45-2 negatively regulate rice responses to cold, drought, and salt stresses through the pathway involving SNAC1 and OsDREB1B. In addition, OsWRKY45-1 may positively regulate a salt tolerance pathway that could compensate for the suppression of the pathway involving SNAC1 and OsDREB1B in salt stress response. This hypothesis is supported by evidence that OsWRKY45-1 and OsWRKY45-2 differentially regulate the expression of *RD22*, *Rab16D*, and *Rab21*, whose transcript levels are associated with rice adaptation to abiotic stresses (Rabbani *et al.*, 2003; Buchanan *et al.*, 2004; Hou *et al.*, 2009). However, further research is required to examine this hypothesis.

OsWRKY45-1 and OsWRKY45-2 differentially mediate the cross-talk between abiotic and biotic stress defence pathways

Plants are constantly exposed to diverse abiotic and biotic stresses and have evolved efficient ways to reallocate metabolic resources rapidly among different physiological signalling pathways in order to adapt to a changing environment. Convergence points of the cross-talk between abiotic and biotic stress signalling are emerging (Fujita *et al.*, 2006; Park *et al.*, 2007; Qiu *et al.*, 2008), but our understanding of the molecular mechanisms underlying this cross-talk in rice remains preliminary. Different proteins, including transcription factors, kinases, and other enzymes, have been reported to play roles in this cross-talk (Xiong and Yang, 2003; Anderson *et al.*, 2004; Park *et al.*, 2007; Qiu *et al.*, 2008). Abiotic and biotic stress signalling pathways frequently interact antagonistically (Fujita *et al.*, 2006) because the proteins functioning in the convergence points of the cross-talk have two functions. For example, the proteins may function as positive regulators in abiotic stress tolerance but as negative regulators in disease resistance, such as rice OsMPK5 kinase (Xiong and Yang, 2003) and *Arabidopsis* AtMYC2 transcription factor (Anderson *et al.*, 2004), or they may function as negative regulators in abiotic stress tolerance but as positive regulators in disease resistance, such as rice OsWRKY13 transcription regulator (Qiu *et al.*, 2007, 2008). However, the *Arabidopsis* protein WES1, a putative GH3-type auxin-conjugating enzyme, functions as a positive regulator in both abiotic stress tolerance and disease resistance (Park *et al.*, 2007).

Adding to the short list of proteins known to regulate cross-talk, here it is shown that OsWRKY45-1 and OsWRKY45-2 are also players in the cross-talk between abiotic and biotic stress signalling. Previously, it was reported that the two alleles had opposite roles in rice–bacterium interactions and had the same role in rice–fungus interactions (Tao *et al.*, 2009). Based on the present and previous findings (Tao *et al.*, 2009), OsWRKY45-1 negatively regulates bacterial pathogen resistance, cold and drought tolerance, and ABA-mediated signalling, and positively regulates fungal pathogen resistance. In contrast, OsWRKY45-2 positively regulates bacterial and fungal

pathogen resistance and ABA-mediated signalling, and negatively regulates cold, drought, and salt tolerance. The different roles of the *OsWRKY45* alleles in the cross-talk may result at least partly from their differential regulation of ABA-mediated signalling. ABA is extensively involved in plant responses to abiotic stresses and is also implicated in plant–pathogen interactions (Verslues and Zhu, 2005; Bari and Jones, 2009).

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Alignment of the promoter regions of *OsWRKY45-1* and *OsWRKY45-2*.

Figure S2. Modulating *OsWRKY45-1* and *OsWRKY45-2* expression influenced rice response to abscisic acid (ABA) treatment.

Figure S3. Modulating the expression of *OsWRKY45-2* but not *OsWRKY45-1* markedly influenced rice response to salt stress.

Figure S4. Modulating *OsWRKY45-1* and *OsWRKY45-2* expression influenced rice response to cold stress.

Figure S5. Modulating *OsWRKY45-1* and *OsWRKY45-2* expression influenced rice response to drought stress.

Table S1. Primers used for PCR amplification

Table S2. The numbers of known abiotic stress-responsive *cis*-acting elements in the promoters of *OsWRKY45-1* and *OsWRKY45-2*.

Acknowledgements

We thank Mr Jun You and Professor Lizhong Xiong of Huazhong Agricultural University for providing ABA- and abiotic stress-treated RNA samples. This work was supported by grants from the National Natural Science Foundation of China (30930063, 30921091).

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