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ORIGINAL PAPER

Heat shock factor OsHsfB2b negatively regulates drought and salt tolerance in rice

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Abstract
Key message **Expression of *OsHsfB2b* was strongly induced by heat, salt, ABA and PEG treatments. Drought and salt tolerances were significantly decreased by *OsHsfB2b* overexpression, but were enhanced by RNA interference.**
Abstract Plants have more than 20 heat shock factors (Hsfs) that were designated class A, B, and C. Many members of Class A Hsfs were characterized as activators of transcription, but the functional roles of class B and C Hsfs have not been fully recognized. OsHsfB2b is a member of class B Hsfs in rice (*Oryza sativa*). Expression of *OsHsfB2b* was strongly induced by heat, salt, abscisic acid (ABA) and polyethylene glycol (PEG) treatments but was almost not affected by cold stress. Drought and salt tolerances were significantly decreased in *OsHsfB2b*-overexpressing transgenic rice, but were enhanced in the *OsHsfB2b*-RNAi transgenic rice. Under drought stress, the *OsHsfB2b*-overexpressing transgenic rice exhibited increased relative electrical conductivity (REC) and content of malondialdehyde (MDA) and decreased proline content compared with the wild type, while the lower REC and MDA content and increased proline content were found in the *OsHsfB2b*-RNAi transgenic rice. These results suggest that OsHsfB2b functions as a negative regulator in response to drought and salt stresses in rice, with its existing B3 repression domain (BRD) that might be necessary for the repressive activity. The present study revealed the potential value of *OsHsfB2b* in genetic improvement of rice.

Keywords Rice · OsHsfB2b · Heat shock factor · Drought tolerance · Salt tolerance

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Introduction

Plants cannot escape from unfavorable environmental conditions and thus have developed numerous physiological and biochemical changes to respond and adapt to the adverse conditions during their growth and development. The major modes of plant response to various stresses are perception and transduction of the stress signals through signaling components, which result in expression of a great quantity of stress-related genes and synthesis of diverse functional proteins. Finally, a variety of physiological and metabolic responses are produced in plants to adapt to the stresses (Hirayama and Shinozaki 2010; Lata and Prasad 2011; Tang et al. 2012; Yamaguchi-Shinozaki and Shinozaki 2006; Zhu 2002).

Heat shock proteins (Hsps) in plants function as molecular chaperones which regulate cellular homeostasis

and promote plants survival under stressful conditions (Hartl and Hayer-Hartl 2002; Haslbeck and Buchner 2002). The expressions of *Hsps* are regulated by heat shock factors (Hsfs) through their interaction with heat shock elements (HSE) presented in the *Hsp* promoter region (Baniwal et al. 2004; Nover et al. 2001). Plants have more than 20 Hsfs, with *Arabidopsis* and rice having 21 and 25 members, respectively (Guo et al. 2008; Nover et al. 2001). Based on structural characteristics and phylogenetic comparison, plant Hsfs are subdivided into three classes (A, B and C). Class A Hsfs possess the exclusive C-terminal activation domain with AHA motifs, while class B and class C Hsfs are characterized by lacking an activation domain (Baniwal et al. 2004; Nover et al. 2001). In *Arabidopsis*, the members of the HsfA1 group (HsfA1a, HsfA1b, HsfA1d and HsfA1e) function as the master regulators of heat stress response (HSR) and participate in other abiotic stress responses as important components (Busch et al. 2005; Liu et al. 2011; Lohmann et al. 2004). Overexpression of the *Arabidopsis* gene *AtHsfA1b* improved resistance to chilling in transgenic tomato (Li et al. 2003). HsfA1 was characterized as master regulator of thermotolerance in tomato (Mishra et al. 2002). Overexpression of *GmHsfA1* significantly enhanced thermotolerance in soybean by the activation of GmHsp70 (Zhu et al. 2006). The HsfA2 was reported as a key regulator in the induction of the defence system under several types of environmental stresses and enhanced tolerance to heat, high light, salt, oxidative, osmotic and anoxia stresses in *Arabidopsis* (Banti et al. 2010; Charng et al. 2007; Li et al. 2005; Nishizawa-Yokoi et al. 2011; Nishizawa et al. 2006; Ogawa et al. 2007; Schramm et al. 2006). Overexpressing rice gene *OsHsfA2e* highly expressed certain stress-associated genes under unstressed conditions and improved thermotolerance and salt stress tolerance in transgenic *Arabidopsis* (Yokotani et al. 2008). Furthermore, HsfA3, HsfA4a and HsfA7 play an important role in abiotic stress tolerance in *Arabidopsis*, rice and wheat (Larkindale and Vierling 2008; Liu et al. 2013; Schramm et al. 2008; Shim et al. 2009; Yoshida et al. 2008).

In contrast to class A Hsfs, there was no evidence that class B and C Hsfs functioned as transcription activators on their own due to lack of the activation domain (Czarnecka-Verner et al. 2000, 2004; Kotak et al. 2004; Nover et al. 2001). Tomato HsfB proteins were suggested to be coactivators of HsfAs (Bharti et al. 2004; Hahn et al. 2011). OsHsfB4b showed interaction with OsHsfA2c and activated the target genes (Singh et al. 2012). Recent researches show that some members of class B Hsfs are active transcriptional repressors. The repressive activity of HsfB1 and HsfB2b was reported in *Arabidopsis* and soybean, and a repression domain, designated the B3 repression domain (BRD), was found in HsfB1 and HsfB2b at the C terminus (Czarnecka-Verner et al. 2000, 2004; Ikeda et al. 2011; Ikeda and Ohme-Takagi 2009; Kumar et al. 2009). HsfB1 and HsfB2b were shown to regulate the expression of defensin genes, and the disease resistance were significantly improved in mutant lines compared with the wild type (Kumar et al. 2009), suggesting that HsfB1 and HsfB2b may negatively regulate the disease response. HsfB1 and HsfB2b suppressed the general heat shock response under non-heat-stress conditions and in the attenuating period via direct repression of the expression of the heat stress inducible HsfA2 (Ikeda et al. 2011). On the other hand, HsfB1 and HsfB2b exhibited cell death-inducing activity in *Nicotiana benthamiana*, and the cell death symptom by HsfB1 and HsfB2b required both their repression activity and their nuclear localization activity (Zhu et al. 2012). The Hsfs interaction showed that HsfB1 and HsfB2b might be involved in complex regulatory networks, which were linked to stress responses and signaling processes (Li et al. 2010).

In this paper, we report the functional analysis of *OsHsfB2b*, a member of class B Hsfs in rice. Overexpression of *OsHsfB2b* significantly decreased drought and salt tolerance in transgenic plants, while the *OsHsfB2b*-RNAi plants enhanced tolerance under these stresses. Our results indicate *OsHsfB2b* may negatively regulate the abiotic stress tolerance in rice.

Materials and methods

Generation of transgenic rice plants

The full-length cDNA clone of *OsHsfB2b* (AK101700) was obtained from the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan). To create the overexpression construct of *OsHsfB2b*, the *OsHsfB2b* coding region was PCR amplified with *Bam*HI-*Pst*I (indicated by underline) linker primers 5′-CGGGATCCTGGCATGG CTGACCAGACCGCT-3′ and 5′-AACTGCAGCGAGA CGGGTCTAACACACAAC-3′ based on the template of the cDNA clone AK101700. The PCR fragment of *OsHsfB2b* was digested by *Bam*HI and *Pst*I and ligated into the *Bam*HI and *Pst*I sites of the binary expression vector pCAMBIA1301-Multi (modified from pCAMBIA1301) under the control of the CaMV 35S promoter. To make double-strand RNA interference (RNAi) construct, the antisense fragment *OsHsfB2b*-A was PCR amplified with *Xho*I-*Kpn*I (indcated by underline) linker primers 5′-CCGCTCGAGGTTTACCAGCCCATCCG-3′ and 5′-G GGGTACCAACTCGATCTGATCTTTTATGC-3′, the sense fragment *OsHsfB2b*-S was PCR amplified with *Pst*I-*Xba*I (indcated by underline) linker primers 5′-AACTG CAGGTTTACCAGCCCATCCG-3′ and 5′-GCTCTAGA

AACTCGATCTGATCTTTTATGC-3′, using the cDNA clone AK101700 as the template. The *OsHsfB2b*-A fragment was digested by *Xho*I and *Kpn*I and cloned into pBSK-3Dlinker (containing 128 bp intron sequence of *RAmy3D* between *Xho*I and *Pst*I of pBluescript SK, pBS-*OsHsfB2b*-A-*ΔRAmy3D*). The *OsHsfB2b*-S fragment was digested by *Pst*I and *Xba*I and cloned into pBS-*OsHsfB2b*-A-*ΔRAmy3D* (pBS-*OsHsfB2b*-A-*ΔRAmy3D-OsHsfB2b*-S). Finally, pBS-*OsHsfB2b*-A-*ΔRAmy3D-OsHsfB2b*-S was digested with *Kpn*I and *Xba*I and cloned into pCAMBIA1301-Multi to yield the 35S::RNAi *OsHsfB2b*. Both of the constructs were transformed into the *japonica* rice Nipponbare by the *Agrobacterium*-mediated transformation method (Toki et al. 2006).

Stress treatment of plant materials

To check the expression level of *OsHsfB2b* gene under various abiotic stresses or phytohormone treatment, Nipponbare rice plants were grown in sandy soil (for heat and cold stress) or hydroponic culture medium (1/2 MS, for others) for about 3 weeks in a climate chamber at 28 °C, 14 h/10 h (light/dark) and 75 % relative humidity. The seedlings at four-leaf stage were used for the following treatments: osmotic stress (20 % PEG), salt stress (200 mM NaCl), heat stress (exposing plants to 42 °C), cold stress (seedlings were transferred to a growth chamber at 4 °C), and phytohormone treatment (0.1 mM ABA). Samples were taken after treatment for 0, 0.5, 1, 2, 4, 8 and 24 h separately. Ten shoots were pooled together as one biological replicate and each treatment was repeated three times.

To detect the transcript levels of *OsHsfB2* in different tissues, Nipponbare rice plants were grown in field or the climate chamber and tissue samples of roots, stems, leaves, sheaths and spikes were collected essentially as described previously (Zou et al. 2009). Seedlings were obtained from the two-week-old rice seedlings grown in a climate chamber. Three replications were performed in this study.

To test various abiotic stress tolerances of transgenic plants at the seedling stage, positive T2 transgenic lines were selected by germinating seeds in 1/2 MS medium containing 50 mg/L hygromycin; the wild type was also germinated in normal 1/2 MS medium. The one-week-old rice seedlings (15 plants each, three repeats) were grown in an equational separation manner (*OsHsfB2b*-overexpressing:wild type:*OsHsfB2b*-RNAi = 1:1:1) in plastic pots filled with sandy soil. After 2 weeks growing in the growth chamber, the seedlings were used for abiotic stress treatments. For drought stress treatment, irrigation was withheld for 1 week followed by recovery for 1 week. For salt stress treatment, irrigated with 200 mM NaCl solution for 1 week followed by recovery for 1 week. For heat or cold stress treatment, the seedlings were transferred to a growth chamber at 45 °C for 5 h/day continued for 5 days or 4 °C for 5 days separately, followed by recovery at 28 °C for 1 week.

For testing the osmotic or salt stress tolerance of transgenic seed germination, the homozygous T3 transgenic lines and the wild type (40 seeds each, three repeats) were germinated in 1/2 MS medium containing a gradient concentration of mannitol (0, 50, 100, and 150 mM) or NaCl (0, 50, 100, and 150 mM), and the germination rate of the treated seeds was calculated after 10 days.

For testing the osmotic or salt stress tolerance of transgenic seedling, the homozygous T3 transgenic and wild-type seeds were geminated in normal 1/2 MS medium for 4 days, and the seedlings were then transferred to medium containing 150 mM mannitol or 150 mM NaCl, respectively. The shoot heights were measured at 10 days after being transferred.

Drought testing at the reproductive stage was conducted in plastic pots. One positive transgenic plant and one wild-type plant each were planted per plastic pot (10 plants each). Drought stress was initiated at the booting stage by discontinuing watering of plants. After 2 weeks of water deprivation, a period that can normally cause serious drought stress (almost all leaves became completely rolled and some of the leaves died) in this environment, the plants were recovered with irrigation for 2 weeks.

To measure the water loss rate under dehydration conditions, leaves of positive transgenic plants and wild-type plants (10 leaves each, three repeats) were cut, exposed to air at room temperature (approximately 25 °C). The leaves were weighed at 0, 15, 30, 45 min, 1, 2, 3, 6, 12, and 24 h after being cut down.

RNA isolation, semi-quantitative PCR and quantitative PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The DNaseI-treated (Fermentas) RNA was reversed transcribed using ReverTra Ace® RT Kit (Toyobo). Semi-quantitative RT-PCR analysis was performed according to Zou et al. (2009), the rice *Actin1* gene (Os03g0718100) was used as the endogenous control. The following gene-specific primes were used. *OsHsfB2b*: 5′-CAGACAACGAGGAGGGAATGA-3′ and 5′-CGGCTCGGTCGTACTCGTAT-3′. *Actin1*: 5′-CTTCAACACCCCTGCTATG-3′ and 5′-TCCATCAGGAAGCTCGTAG-3′. Real-time PCR was performed on an ABI 7300 real-time PCR system (Applied Biosystems) using SYBR® Premix Ex Taq™ II (TaKaRa) according to the manufacturer's instructions. The PCR thermal cycles were as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 31 s.

Real-time PCR of *OsHsfB2b* was performed using primer pair 5′-GCACGGCAAGGACGAACA-3′ and 5′-CGGCTC GGTCGTACTCGTAT-3′. The rice *Actin1* gene was used as the endogenous control with primers 5′-ATCGCCCTGG ACTATGAC-3′ and 5′-GAAACGCTCAGCACCAAT-3′. Dates represent three biological replicates and three technical replicates. The relative expression levels were quantified using the $2^{-\Delta\Delta C_t}$method (Livak and Schmittgen 2001). The data were expressed as mean ± standard error.

Measurements of relative electrical conductivity (REC), malondialdehyde (MDA) and proline content

The transgenic plants and wild-type plants at the booting stage were used for drought stress treatment. The flag leaves (5 plants each, three repeats) were harvested at the beginning and at third day of discontinuing watering of plants for the measurements of REC, MDA and proline content. The leaf REC was determined as described previously (Yu et al. 2006). MDA content was measured as the method described previously (Kuk et al. 2003). Free proline content was measured in acidic extracts and quantified spectrophotometrically using the acid-ninhydrin reagent with proline as a standard (Bates et al. 1973).

Results

Expression pattern of *OsHsfB2b* under different stresses

The previous study revealed that *OsHsfB2b* (Os08g 0546800) was strongly induced in heat-tolerant rice cultivar 996 (*indica*) under heat stress (Zhang et al. 2012). The induction level of this gene was significantly higher than those of the other members of class B Hsfs, class C Hsfs and some members of class A Hsfs. Therefore, we isolated the full-length cDNA of *OsHsfB2b* (AK101700) from Nipponbare (*japonica*) for further functional analysis.

The expression pattern of *OsHsfB2b* under different abiotic stresses and abscisic acid (ABA) treatment as well as at different tissues was measured by quantitative PCR (qPCR). The expression of *OsHsfB2b* was induced by salt, polyethylene glycol (PEG), and ABA treatments after 2 h, and kept increasing at 24 h (Fig. 1a). The expression of *OsHsfB2b* was strongly induced by heat stress especially at very early stage and gradually reduced to lower increased level, but the expression was almost not affected by low-temperature treatment (Fig. 1b). The tissue-specific expression analysis indicated that *OsHsfB2b* expression was high in sheaths and panicles, medium expression in stems and leaves, but very low in roots and seedlings (Fig. 1c). The strong induction of *OsHsfB2b* gene by abiotic stresses prompted us to check its promoter sequence (2,000 bp upstream the transcription start site) by searching the promoter sequence against the PLACE database (http://www.dna.affrc.go.jp/PLACE/) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The results revealed that the promoter contains many putative stress response-related *cis*-elements, such as ABRE (4 hits), DRE (2 hits), MYB recognition site (MYBRS, 5 hits), MYC recognition site (MYCRS, 6 hits), and HSE (1 hit) (Fig. 1d).

OsHsfB2b negatively regulates drought tolerance in rice

The strong induction of *OsHsfB2b* expression by abiotic stress and ABA treatment suggested its possible involvement in stress response in rice. To observe the effect of *OsHsfB2b* on stress tolerance, *OsHsfB2b* overexpression (Fig. 2a) and RNAi (Fig. 2b) vectors were constructed and were separately transformed into *japonica* cultivar Nipponbare. The expression level of *OsHsfB2b* in transgenic plants was analyzed by reverse transcription (RT)-PCR (Fig. 2c, d). Altogether 22 positive *OsHsfB2b* overexpression lines and 17 positive RNAi lines were obtained. Under normal condition, there was no significant difference in plant morphology (such as root depth, plant height, and numbers of tillers and spikelets) and yield between the transgenic lines and the wild type (data not shown). These results suggested that overexpression or RNA interference of *OsHsfB2b* had no detrimental effect on the growth and development of rice. Three independent overexpression lines (OE1, OE10 and OE12) and three independent RNAi lines (Ri1, Ri5 and Ri8) were chosen for stress tolerance test.

Three-week T2 transgenic and non-transgenic control seedlings were tested for drought tolerance. After 5 days of water-withholding, almost all leaves of *OsHsfB2b*-overexpressing plants became completely rolled, while the leaves of wild-type plants were partially rolled and only a small portion of the leaves of *OsHsfB2b*-RNAi plants were slightly rolled. After water-withholding for 7 days, almost all leaves of the transgenic plants and the wild-type plants were completely rolled. One week after rewatering, more than 80 % of the *OsHsfB2b*-RNAi plants recovered, only about 40 % of the wild-type plants recovered, whereas the survival rate of *OsHsfB2b*-overexpressing plants fell to about 10 % (Fig. 3a, b). The results indicated that overexpressing *OsHsfB2b* significantly decreased drought tolerance while RNA interference of this gene improved drought tolerance in transgenic rice at the seedling stage.

Seed germination experiment of transgenic lines under osmotic stress was performed by adding mannitol in the medium to mimic dehydration stress. After 10 days of seed imbibition, the *OsHsfB2b*-overexpressing seeds had only

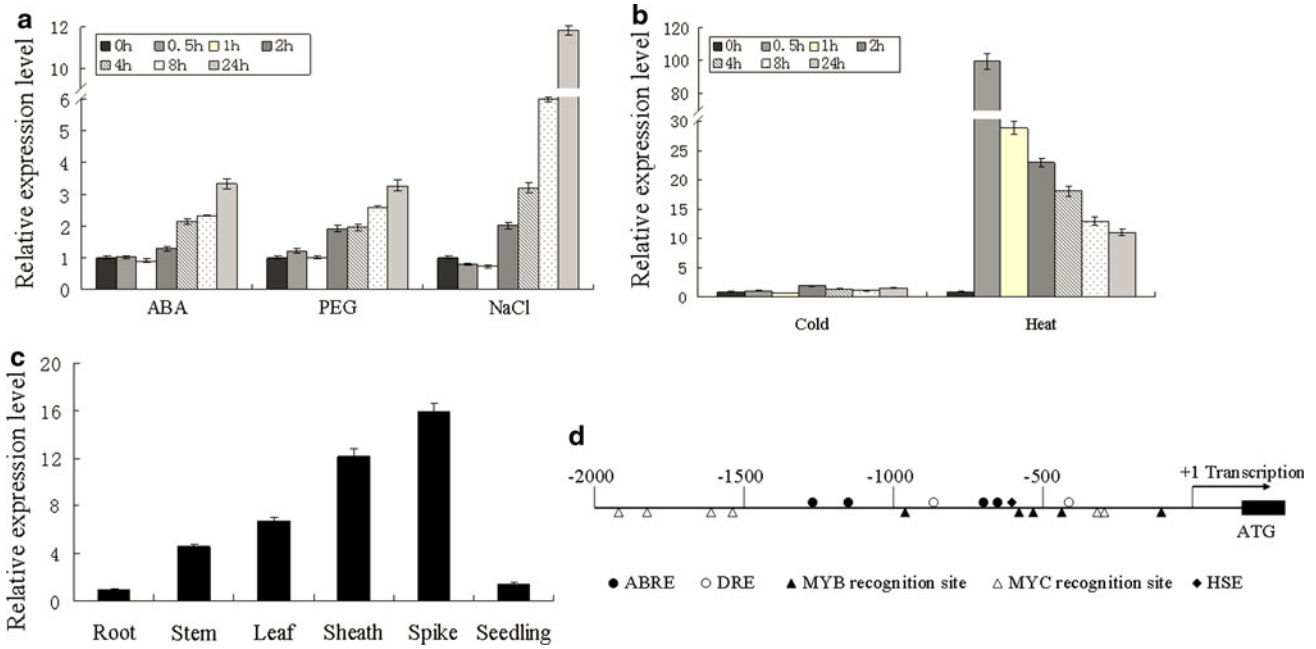

Fig. 1 Expression analysis of the *OsHsfB2b* gene by real-time PCR. **a** Expression level of *OsHsfB2b* under salt, PEG and ABA treatments (time course 0, 0.5, 1, 2, 4, 8 and 24 h). **b** Expression level of *OsHsfB2b* under cold and heat stresses (time course 0, 0.5, 1, 2, 4, 8 and 24 h). **c** Expression level of *OsHsfB2b* in different tissues. **d** Distribution of major stress-related *cis*-elements in the promoter region of *OsHsfB2b*

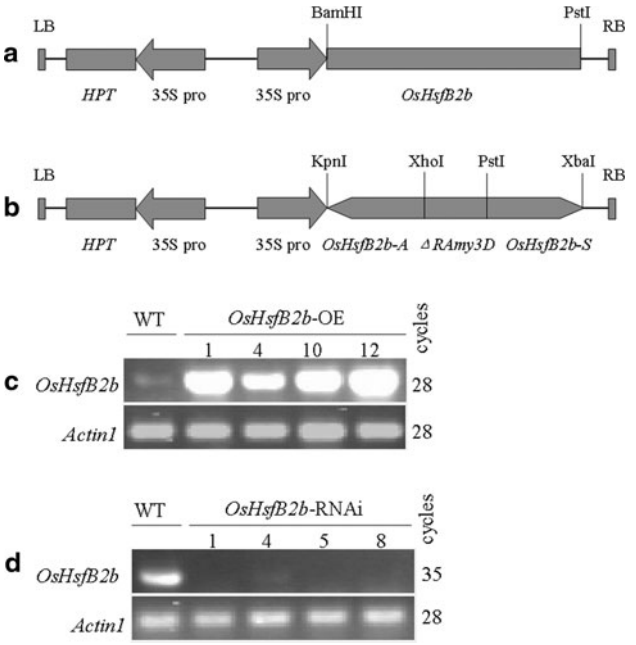

Fig. 2 Expression analysis of *OsHsfB2b* in transgenic rice lines and wild type under normal condition. **a** Diagram of the CaMV35S promoter::*OsHsfB2b* construct. **b** Diagram of the CaMV35S promoter::RNAi *OsHsfB2b* construct. **c** Expression level of *OsHsfB2b* in overexpressing *OsHsfB2b* (*OsHsfB2b*-OE) lines and wild type under normal condition. Rice *Actin1* gene was included as a control for constitutive expression in the assays. **d** Expression level of *OsHsfB2b* in *OsHsfB2b*-RNAi lines and wild type under normal condition. Rice *Actin1* gene was included as a control for constitutive expression in the assays

about 75 % of the relative germination rate under 100 mM mannitol treatment, while the *OsHsfB2b*-RNAi seeds and wild type showed more than 90 % of the relative germination rate. Under 150 mM mannitol treatment, the relative germination rates of the *OsHsfB2b*-RNAi and wild-type seeds were 82 and 67 %, respectively, but that of *OsHsfB2b*-overexpressing seeds reduced to about 45 % (Fig. 3c). There was no difference between the germination rates of transgenic lines and wild type in normal medium, suggesting that overexpression or RNA interference of *OsHsfB2b* does not affect seed germination under normal condition. The lower germination rate of overexpression lines and the higher germination rate of RNAi lines than that of wild-type seeds under osmotic stress suggested that *OsHsfB2b* may have a negative regulatory effect on osmotic stress tolerance in rice. This effect was further confirmed in the postgermination stage rice seedling. In this experiment, transgenic and wild-type rice seedlings geminated in 1/2 MS medium, were transferred to the medium containing 150 mM mannitol for 10 days and the shoot heights were compared. No shoot height difference was observed for the transgenic and wild-type seedlings growing in the medium without mannitol. Under mannitol treatment, the shoot heights of *OsHsfB2b*-overexpressing seedlings were shorter while that of the *OsHsfB2b*-RNAi seedlings were longer compared with those of the wild type (Fig. 3d).

Drought stress was also performed at the booting stage by discontinuing watering for 2 weeks and all leaves of

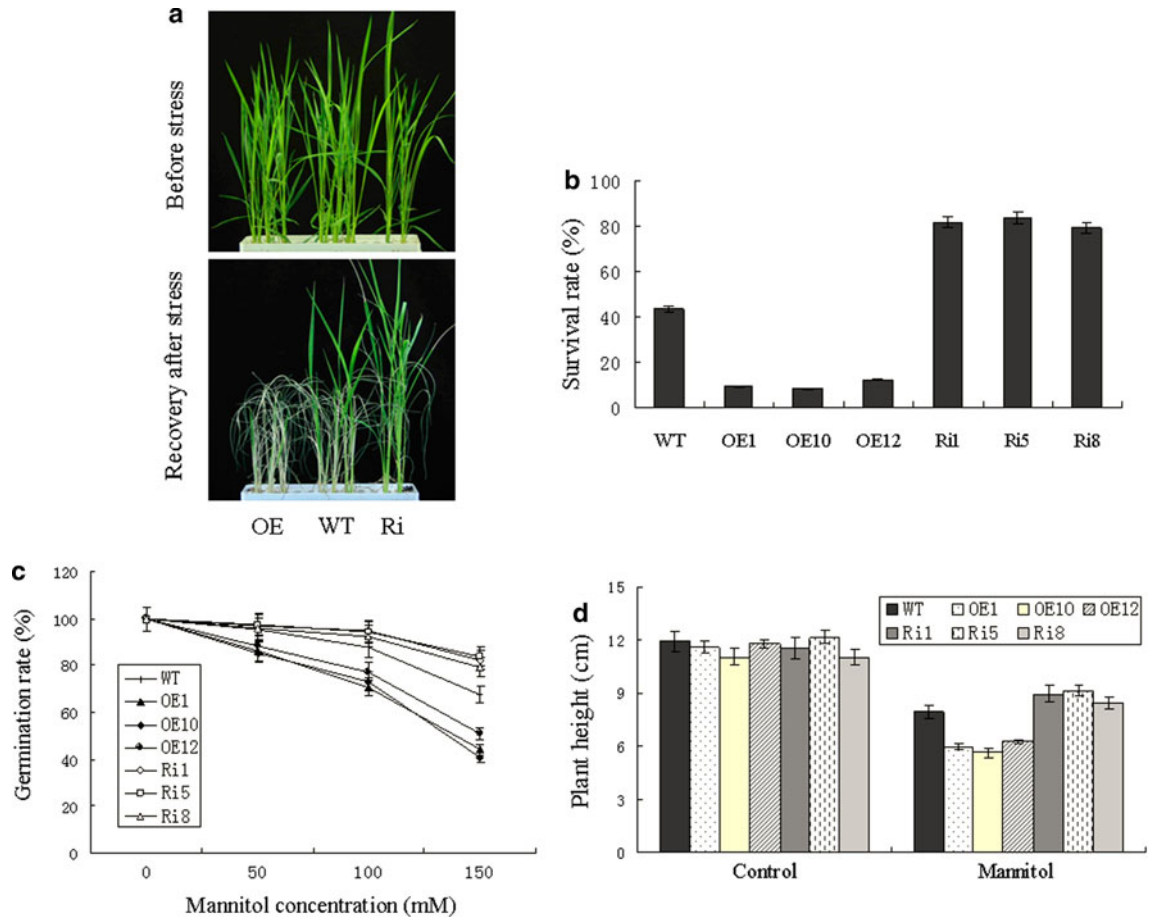

Fig. 3 Drought stress tolerance testing of *OsHsfB2b*-overexpressing and *OsHsfB2b*-RNAi transgenic rice at the seedling stage. **a** Phenotype of the *OsHsfB2b*-overexpressing (*OE*) plants, the *OsHsfB2b*-RNAi (*Ri*) plants and wild-type (*WT*) plants before drought stress (*upper figure*) and recovery after drought stress (*lower figure*) at the seedling stage. **b** Survival rates of the *OsHsfB2b*-overexpressing plants (OE1, OE10 and OE12), the *OsHsfB2b*-RNAi plants (Ri1, Ri5 and Ri8), and wild-type plants recovered for 7 days after drought stress. *Error bars* indicate SE based on three replicates. **c** Germination rate of the *OsHsfB2b*-overexpressing lines, the *OsHsfB2b*-RNAi lines, and wild-type seeds in 1/2 MS medium with a gradient concentration of mannitol. *Error bars* indicate SE based on three replicates. **d** Plant heights of the *OsHsfB2b*-overexpressing lines, the *OsHsfB2b*-RNAi lines, and wild type grown in normal or mannitol-containing (150 mM) medium. Plant height was measured after the seedlings growing for 10 days. Values are the mean ± SD ($n = 10$)

transgenic and wild-type plants became completely rolled. After recovering with irrigation for 2 weeks, the overexpression lines failed to be recovered and died, while all the RNAi lines and part of the wild type got recovered (Fig. 4a). The detached leaves of transgenic and wild-type plants were exposed to air to compare the water loss rate in leaves. The results showed that the leaves from the overexpression plants had higher water loss rate, while the leaves from the RNAi plants had lower water loss rate than wild type (Fig. 4b).

Proline enrichment in the stressed plants is a general response to various abiotic stresses and serves as effective adaptation for stress tolerance identification (Akram et al. 2007). In contrast, REC of electrolyte leakage can be used as an indicator of the cell membrane penetrability. MDA, a product of lipid peroxidation, is associated with oxidative degradation of cell membrane lipids and its abundance serves as an indicator of cell membrane damage. The flag leaves of transgenic and wild-type plants at the booting stage were harvested at the beginning and at the third day of discontinuing watering for the measurements of REC, MDA and proline content. There were no significant differences in REC, MDA content and proline content between the transgenic lines and wild type before drought stress. However, after withholding water for 3 days, the REC and the MDA content of the *OsHsfB2*b-overexpressing lines were significantly higher than that of the wild type, but those of the *OsHsfB2b*-RNAi lines significantly lower than that of the wild type (Fig. 4c, d). The accumulation of proline in the *OsHsfB2b*-RNAi lines was higher than that of wild type and the *OsHsfB2b*-overexpressing lines (Fig. 4e). Lower REC and the MDA content indicate less membrane damage, and proline can improve osmotic adjustment ability of plants. Therefore,

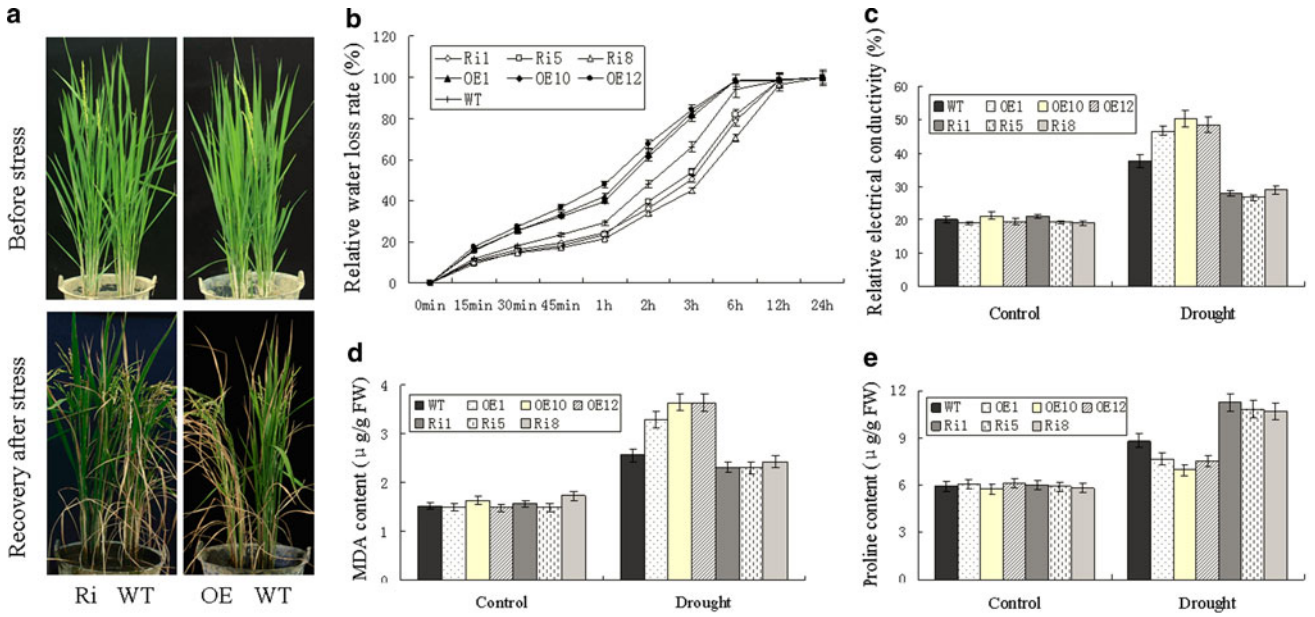

Fig. 4 Drought stress tolerance testing of *OsHsfB2b*-overexpressing and *OsHsfB2b*-RNAi transgenic rice at the booting stage. **a** Phenotype of the *OsHsfB2b*-overexpressing (*OE*) plants, the *OsHsfB2b*-RNAi (*Ri*) plants and wild-type (*WT*) plants before drought stress (*upper figure*) and recovery after stress (*lower figure*) at the booting stage. **b** Water loss rates in leaves detached from the *OsHsfB2b*-overexpressing plants (OE1, OE10 and OE12), the *OsHsfB2b*-RNAi plants (Ri1, Ri5 and Ri8), and wild-type plants. *Error bars* indicate SE based on three replicates. **c** Relative electrical conductivity (C1/C2) of leaves. C1, conductance of the water solution with leaves in room-temperature water for 30 min; C2, conductance of water solution with leaves in boiled water for 15 min. Values are the mean ± SD ($n = 10$). **d** MDA content of the *OsHsfB2b*-overexpressing plants, the *OsHsfB2b*-RNAi plants, and wild-type plants under normal and drought stress conditions. Values are the mean ± SD ($n = 10$). **e** Proline content of the *OsHsfB2b*-overexpressing plants, the *OsHsfB2b*-RNAi plants, and wild-type plants under normal and drought stress conditions. Values are the mean ± SD ($n = 10$)

the results may partially explain the reason that overexpression of *OsHsfB2b* resulted in decreased drought tolerance, while RNA interference of this gene improved stress tolerance in rice.

OsHsfB2b negatively regulates salt stress tolerance in rice

To check salt tolerance of the *OsHsfB2b* transgenic rice, three-week-old plants were irrigated with water containing 200 mM NaCl, and leaf death rate was checked after 7 days of treatment. The results showed that the *OsHsfB2b*-overexpressing plants had less green leaf area (approximately 63 %) than the wild-type control (approximately 80 %) and the *OsHsfB2b*-RNAi plants (approximately 88 %; Fig. 5a, b), suggesting that overexpression of *OsHsfB2b* in rice can also decrease salt tolerance, while RNA interference of this gene can slightly improve salt tolerance of transgenic rice at seedling stage.

The germination ability evaluation of the transgenic lines under salt stress condition revealed that, after 10 days of germination in the medium containing 100 mM NaCl, only about 38 % of the *OsHsfB2b*-overexpressing seeds were poorly germinated, whereas more than 84 % of wild-type seeds and *OsHsfB2b*-RNAi seeds were well germinated (Fig. 5c). Under the treatment with 150 mM NaCl, the relative germination rate of the *OsHsfB2b*-overexpressing seeds fell to 20 %, but that of wild type and *OsHsfB2b*-RNAi seeds were 66 and 85 %, respectively. In the normal medium, the germination rates of transgenic lines and wild-type had no significant difference. The results indicated that overexpression of *OsHsfB2b* significantly decreased seed germination, while RNA interference of this gene improved seed germination under salt stress condition.

Transgenic and wild-type rice seedlings geminated in 1/2 MS medium, were transferred to the medium containing 150 mM NaCl for 10 days and the shoot heights were compared. The *OsHsfB2b*-overexpressing plants had shorter shoots than the *OsHsfB2b*-RNAi plants and the wild-type plants, and the *OsHsfB2b*-RNAi plants were taller than the wild-type plants, while no obvious difference was observed for transgenic and wild-type seedlings grown in the medium without NaCl (Fig. 5d). These results indicated that *OsHsfB2b* negatively regulated salt stress tolerance at postgermination stage.

In addition, three-week-old seedlings of the transgenic rice and wild type with similar vigor were used for high (45 °C) and low (4 °C) temperature treatments. No significant difference was observed between the transgenic lines

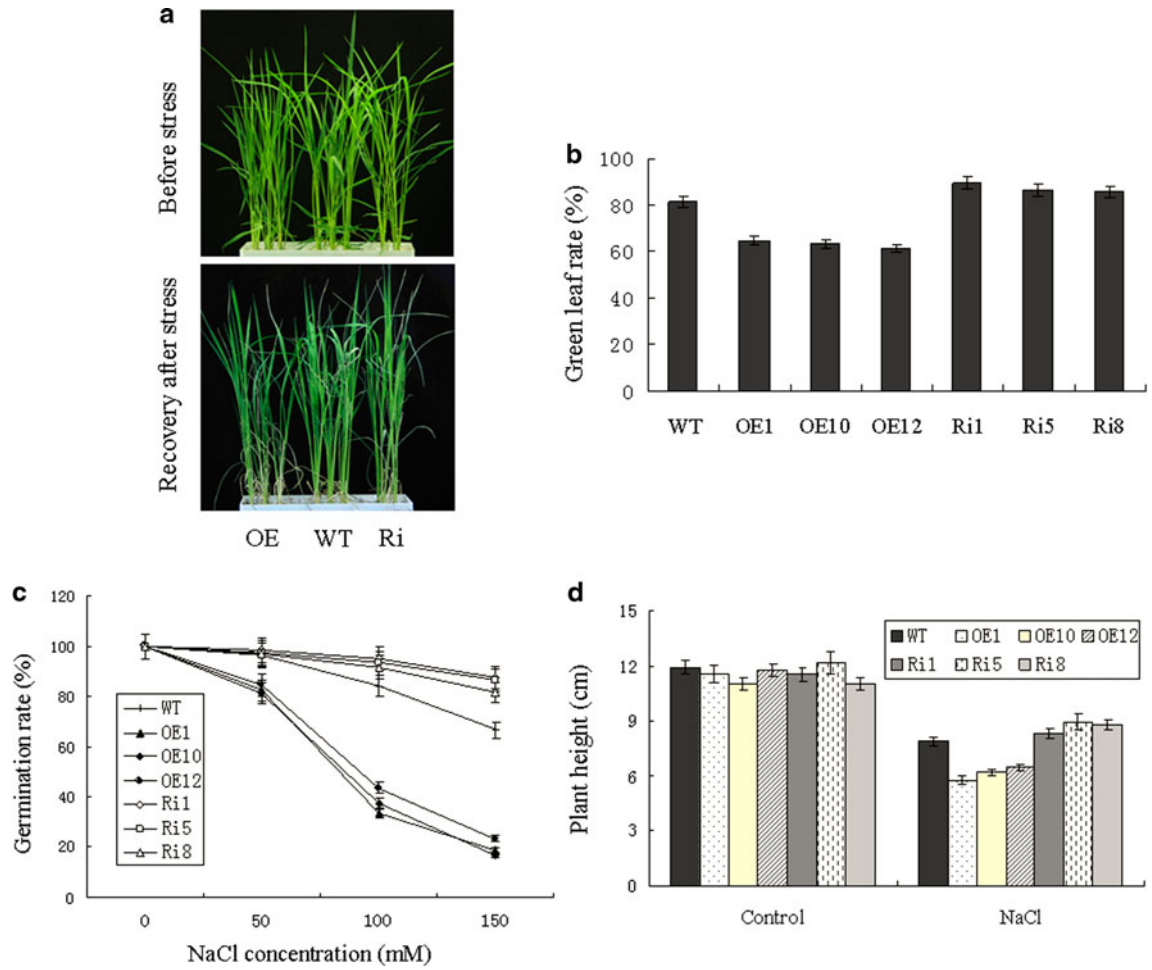

Fig. 5 Salt stress tolerance testing of *OsHsfB2b*-overexpressing and *OsHsfB2b*-RNAi transgenic rice at the seedling stage. **a** Phenotype of the *OsHsfB2b*-overexpressing (*OE*) plants, the *OsHsfB2b*-RNAi (*Ri*) plants and wild-type (*WT*) plants before (*upper figure*) and recovery after salt stress (*lower figure*) at the seedling stage. **b** Percentage of green leaves of the *OsHsfB2b*-overexpressing plants (OE1, OE10 and OE12), the *OsHsfB2b*-RNAi plants (Ri1, Ri5 and Ri8), and wild-type plants recovered for 7 days after salt stress. *Error bars* indicate SE based on three replicates. **c** Germination rate of the *OsHsfB2b*-overexpressing lines, the *OsHsfB2b*-RNAi lines, and wild-type seeds in 1/2 MS medium with a gradient concentration of NaCl. *Error bars* indicate SE based on three replicates. **d** Plant heights of the *OsHsfB2b*-overexpressing lines, the *OsHsfB2b*-RNAi lines, and wild type grown in normal or NaCl-containing (150 mM) medium. Plant height was measured after the seedlings growing for 10 days. Values are the mean ± SD ($n = 10$)

and the wild type after treatment. After recovery for 1 week, both the transgenic plants and the wild-type plants remained alive and produced new leaves (data not shown). These results showed overexpression or RNA interference of *OsHsfB2b* gene had no significant effect on temperature tolerance.

Discussion

Plant Hsfs play important roles not only in heat stress, but also in other biotic and abiotic stresses. Class A Hsfs contain the exclusive C-terminal activation domain and are reported as positive regulators in stress tolerance. The recently reported OsHsfC1b played an important role in ABA-mediated salt and osmotic stress tolerance in rice (Schmidt et al. 2012). HsfB1 and HsfB2b had repressive activities and negatively regulated stress tolerance in *Arabidopsis* and soybean (Czarnecka-Verner et al. 2000, et al. 2004; Ikeda et al. 2011; Ikeda and Ohme-Takagi 2009; Kumar et al. 2009). In this study, we report the characterization of OsHsfB2b, which is a member of class B Hsfs in rice. Our data suggest that OsHsfB2b may be a negative regulator for affecting rice tolerance under drought and salt stresses.

OsHsfB2b expression can be induced by abiotic stress and ABA treatment and have tissue specificity

We investigate the expression pattern of *OsHsfB2b* under different abiotic stresses and ABA treatment, and the

accumulation patterns of *OsHsfB2b* transcripts in different tissues. *OsHsfB2b* is strongly up-regulated by heat stress like the other members of Hsfs, which agrees with our previous microarray data very well (Zhang et al. 2012), and is also induced by NaCl, PEG and ABA treatments. The induced expression of *OsHsfB2b* by heat, high-salinity, drought and oxidative stress and ABA treatment were also reported in previous studies (Chauhan et al. 2011; Mittal et al. 2009). The stress-related *cis*-acting elements presented in the 2-kb promoter region of *OsHsfB2b*, including ABRE, DRE, MYBRS, MYCRS, and HSE, may be responsible for the responses under abiotic stress and ABA treatment. ABRE, MYBRS, and MYCRS can be recognized by the AREB/ABF, MYB, and MYC transcription factors, respectively. These *cis*-elements and the corresponding transcription factors have important roles in ABA signaling and abiotic stress responses (Yamaguchi-Shinozaki and Shinozaki 2005; Yamaguchi-Shinozaki and Shinozaki 2006). Such an enriched presence of various stress responsive *cis*-acting elements in the *OsHsfB2b* promoter may suggest an important role of this gene in stress tolerance. We also found the *OsHsfB2b* exhibited diverse expression in different tissues, although expression could be detected in almost all tissues of rice we checked. The transcript abundances of *OsHsfB2b* in sheath and spike were significantly higher than those in other tissues. This result is similar to the previous study that higher transcript level of *OsHsfB2b* was noted at seed developmental stage (Mittal et al. 2009), suggesting *OsHsfB2b* may be of great importance for seed development.

OsHsfB2b is a negative regulator of drought and salt tolerance

Although *OsHsfB2b*-overexpressing transgenic plants lead to an abundance of mRNA accumulation and *OsHsfB2b*-RNAi transgenic plants lead to a reduction of mRNA accumulation, there was no detectable effect on transgenic rice growth and development under normal condition. But overexpression and RNAi transgenic plants showed opposite phenotypes in stress tolerance. The seedlings of the *OsHsfB2b*-overexpressing lines showed significantly decreased drought and salt tolerance, while the *OsHsfB2b*-RNAi plants had higher stress tolerance compared with the wild type. This is also true for the osmotic and salt stress tolerance tested at both germination and postgermination stages. Furthermore, drought stress at booting stage revealed that *OsHsfB2b*-overexpressing lines exhibited significantly poorer drought tolerance than the wild type, but the *OsHsfB2b*-RNAi lines enhanced drought tolerance.

Leaf REC, MDA and proline content act as three important physiological parameters for evaluating plant stress tolerance. Under drought stress condition, leaf REC and MDA content were higher in the *OsHsfB2b*-overexpressing lines than those in the wild-type and the *OsHsfB2b*-RNAi lines, and the accumulation of proline in the *OsHsfB2b*-overexpressing lines was lower compared with the wild type and the *OsHsfB2b*-RNAi lines. On the other hand, leaf REC and MDA content were lower in the *OsHsfB2b*-RNAi lines than those in the wild type, and proline content in the *OsHsfB2b*-RNAi lines was higher compared with the wild type. Electrolyte leakage is an indirect effect of damage done to plant cell membranes (Bajji et al. 2002), and the higher REC in *OsHsfB2b*-overexpressing lines indicates that more membrane damage occurred, while the lower REC in *OsHsfB2b*-RNAi lines means less membrane damage occurred. MDA is one of the end products of lipid peroxidation damage from free radicals (Marnett 1999). The higher level of MDA in the *OsHsfB2b*-overexpression lines means more lipids damage occured, while the lower level of MDA in the *OsHsfB2b*-RNAi lines indicated less lipids damage. Proline accumulation in response to environmental stress plays an important role in the osmoregulation and also exhibits many protective effects (Szabados and Savoure 2010). The decreased proline content in *OsHsfB2b*-overexpressing lines and increased proline content in the *OsHsfB2b*-RNAi lines indicated negative regulation of OsHsfB2b on proline accumulation under drought stress. The REC, MDA and proline content are related to plants stress tolerance that have also been found in OsHsp17.0, OsHsp23.7 and OsHsfA7 overexpression rice plants under drought and salt stress at seedling stage (Liu et al. 2013; Zou et al. 2012). These findings provided physiological evidences for the negative regulation of OsHsfB2b in stress tolerance.

Recently, HsfB2b has been reported to act as a transcriptional repressor in *Arabidopsis*. The single mutant *hsfb2b* and the double mutant *hsfb1 hsfb2b* showed significantly improved pathogen resistance levels compared with the wild type, and the double mutant *hsfb1 hsfb2b* enhanced expression of the defensin genes (Kumar et al. 2009). It was also reported that the double mutant *hsfb1 hsfb2b* exhibited higher thermotolerance and the reason may be that HsfB1 and HsfB2b repress expression of the HS-inducible genes under non-HS conditions, which are important for stress tolerance (Ikeda et al. 2011; Nishizawa et al. 2006). In the similar way, we speculate that OsHsfB2b may act as a transcriptional repressor in rice and repress expression of HS-inducible genes for OsHsfs, some OsHSPs and defensin genes under normal condition. When the transgenic rice plants and the wild-type plants are exposed to drought and salt stresses, the *OsHsfB2b*-overexpressing plants exhibit lower stress tolerance than the wild type, while *OsHsfB2b*-RNAi plants exhibit higher stress tolerance, probably due to the different expression level of genes for HSPs and defensin genes repressed by

OsHsfB2b. The target genes of OsHsfB2b and the interaction manner of OsHsfB2b and class A Hsfs in rice stress response system will be conducted in a further study.

In our study, there were no detectable changes in temperature stress tolerance for both the *OsHsfB2b*-overexpressing and the *OsHsfB2b*-RNAi pants at seedlings stage. This is reminiscent of *Arabidopsis*. The single mutant *hsfb2b* showed no obvious effect on the heat stress response (Kumar et al. 2009), but the double mutant *hsfb1 hsfb2b* enhanced expression of the heat-inducible genes for Hsfs and small HSPs under non-heat-stress conditions and exhibited higher thermotolerance (Ikeda et al. 2011). And in tomato, when the expression of LbHsfB1 was compromised in a transgenic approach, plants showed no obvious effect on the heat stress response (Mishra et al. 2002). These results suggested that HsfB2b may act as a functionally redundant factor to HsfB1 in heat stress response.

BRD may be a critical component of OsHsfB2b acting as transcriptional repressor

In *Arabidopsis* leaves, HsfB1 and HsfB2b exhibited strong repressive activities in a yeast Gal4 DNA-binding domain (GAL4-DB) fusion transient expression assay (Ikeda and Ohme-Takagi 2009). The core BRD sequence L/VR/KLFGVXM/V/L is conserved in the orthologs of HsfB1 in various plant species and in all five members of *Arabidopsis* class B Hsfs (Czarnecka-Verner et al. 2004; Ikeda et al. 2011; Ikeda and Ohme-Takagi 2009). When the BRD was mutated, the repressive activity of HsfB1 was abolished (Ikeda et al. 2011). The OsHsfB2b protein contains BRD in the C-terminal amino acid sequence (Fig. 6a). The core BRD sequence P/AR/KLFGVS/CIG, was conserved in HsfB2b orthologs of a variety of plant species (Fig. 6b) and is similar to the core BRD sequence of HsfB1 in *Arabidopsis*. The core BRD sequence of OsHsfB2b is also conserved in all eight members of rice class B Hsfs (Fig. 6c). We presumed that BRD is necessary for the OsHsfB2b acting as transcriptional repressor just like the HsfB1 in *Arabidopsis*.

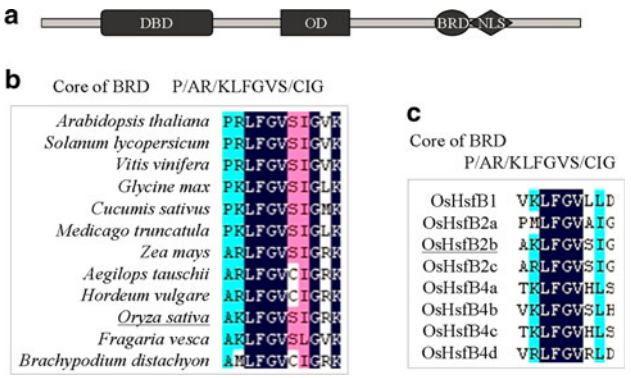

Fig. 6 Identification of the repression domain of OsHsfB2b. **a** Schematic representation of the OsHsfB2b. DNA-binding domain (*DBD*), oligomerization domain (*OD*, *HR-A/B*), nuclear localization signal (*NLS*), B3 repression domain (*BRD*) are shown. **b** An alignment of the core sequence of BRD of HsfB2b orthologs from 12 varieties of plants including rice OsHsfB2b. **c** An alignment of the core sequence of BRD of rice class B Hsfs

In conclusion, this study showed that OsHsfB2b can be induced by various abiotic stresses and ABA treatment. OsHsfB2b negatively regulates drought and salt stress tolerance in rice, and BRD might be necessary for the repressive activity of OsHsfB2b. Our results also suggest that RNA interference of the *OsHsfB2b* gene may have a promising utility in improving stress tolerance of crops.

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