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Transcription factor OsHsfC1b regulates salt tolerance and development in *Oryza sativa* ssp. *japonica*

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Abstract

Background and aims Salt stress leads to attenuated growth and productivity in rice. Transcription factors like heat shock factors (HSFs) represent central regulators of stress adaptation. Heat shock factors of the classes A and B are well established as regulators of thermal and non-thermal stress responses in plants; however, the role of class C HSFs is unknown. Here we characterized the function of the OsHsfC1b (Os01g53220) transcription factor from rice.

Methodology We analysed the expression of *OsHsfC1b* in the rice *japonica* cultivars Dongjin and Nipponbare exposed to salt stress as well as after mannitol, abscisic acid (ABA) and H_2O_2 treatment. For functional characterization of OsHsfC1b, we analysed the physiological response of a T-DNA insertion line (*hsfc1b*) and two *artificial micro*-RNA (*ami*RNA) knock-down lines to salt, mannitol and ABA treatment. In addition, we quantified the expression of *small Heat Shock Protein* (*sHSP*) genes and those related to signalling and ion homeostasis by quantitative real-time polymerase chain reaction in roots exposed to salt. The subcellular localization of OsHsfC1b protein fused to green fluorescent protein (GFP) was determined in *Arabidopsis* mesophyll cell protoplasts.

Principal results Expression of *OsHsfC1b* was induced by salt, mannitol and ABA, but not by H_2O_2. Impaired function of *OsHsfC1b* in the *hsfc1b* mutant and the *ami*RNA lines led to decreased salt and osmotic stress tolerance, increased sensitivity to ABA, and temporal misregulation of salt-responsive genes involved in signalling and ion homeostasis. Furthermore, *sHSP* genes showed enhanced expression in knock-down plants under salt stress. We observed retarded growth of *hsfc1b* and knock-down lines in comparison with control plants under non-stress

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conditions. Transient expression of OsHsfC1b fused to GFP in protoplasts revealed nuclear localization of the transcription factor.

Conclusions OsHsfC1b plays a role in ABA-mediated salt stress tolerance in rice. Furthermore, OsHsfC1b is involved in the response to osmotic stress and is required for plant growth under non-stress conditions.

Introduction

Rice represents a major food source for more than half of the world's population. Among crops, rice exhibits the least, wheat a moderate and barley the strongest tolerance to salt stress (Munns and Tester 2008). One reason for the low tolerance of rice to salinity is the high permeability of its roots to sodium ions. Sodium ions can easily enter the apoplast and subsequently rapidly lead to toxic intracellular concentrations. Since an increasing land area is affected by high salinity, understanding the molecular mechanisms underlying salt tolerance of crops is of great societal and economic interest (Yan *et al.* 2005; Obata *et al.* 2007; Hadiarto and Tran 2011). The response to salt stress includes expressional changes of stress-related genes, which among others encode protein kinases, ion transporters and transcription factors. In rice, several transcription factor families (e.g. MYB, NAC, bZIP and AP2/ERF) contribute to stress adaptation by regulating the expression of stress-responsive genes (Hu *et al.* 2006, 2008; Ma *et al.* 2009; Wang *et al.* 2009; Hossain *et al.* 2010; Park *et al.* 2010; Takasaki *et al.* 2010; Mallikarjuna *et al.* 2011; Song *et al.* 2011).

Heat shock factors (HSFs) are transcription factors that can structurally be classified into three classes: A, B and C. They consist of an N-terminal DNA-binding domain, an adjacent oligomerization domain (HR-A/B) and an additional class A-specific C-terminal activation domain containing aromatic, hydrophobic and acidic amino acid residues (AHA motif). In the HR-A/B domain, HSFs of the classes A and C harbour an inserted sequence of 21 and seven amino acid residues, respectively, which is absent from class B HSFs (Nover *et al.* 2001). In contrast to class A HSFs, class B HSFs act as transcriptional repressors while no clear activation or repression has been shown for class C HSFs (Ikeda *et al.* 2011). The number of HSF-encoding genes varies between plant species. The genome of the green alga *Chlamydomonas reinhardtii* contains two, *Arabidopsis thaliana* 21 and rice 25 *HSF* genes (Nover *et al.* 2001; Schulz-Raffelt *et al.* 2007; Guo *et al.* 2008). In rice, 13 HSFs can be assigned to class A (including the subclasses A1, A2 and A4), eight HSFs to class B and four HSFs to class C (Guo *et al.* 2008). Heat shock factors control gene expression by binding to the heat shock element, an inverted 5-bp repeat of the sequence 'nGAAn', found in the promoter regions of many heat-inducible genes (Barros *et al.* 1992; Sun *et al.* 2002). Heat shock factors also function as regulators of other *HSF* genes, demonstrated by HsfA1d and HsfA1e from *A. thaliana*, which are involved in the expressional control of *HsfA2* (Nishizawa-Yokoi *et al.* 2011). Several HSFs of the classes A and B have been shown to play a role in the response to abiotic and biotic stresses. In *Arabidopsis*, next to heat stress adaptation, HsfA2 controls the response to salt, osmotic stress, anoxia and submergence (Ogawa *et al.* 2007; Banti *et al.* 2010). HsfA1 in tomato functions as a master regulator of induced thermotolerance that cannot be replaced by any other HSF (Mishra *et al.* 2002). HsfB1 and HsfB2 from *Arabidopsis* demonstrate the relevance of class B members in stress tolerance, as the knock-out of *HsfB2* and the double knock-out of both *HSF* genes result in improved pathogen resistance (Kumar *et al.* 2009). The role of rice HSFs in stress adaptation is poorly understood. To date, two class A HSFs, i.e. OsHsfA2e and OsHsf7, have been functionally characterized *in vivo*. Transgenic *Arabidopsis* plants overexpressing *OsHsfA2e* are more tolerant to heat and salt stress than control plants (Yokotani *et al.* 2008), and overexpression of *OsHsf7* in *Arabidopsis* results in an increased thermotolerance (Liu *et al.* 2009). The role of class C HSFs in stress response is currently unknown; however, expression patterns of class C *HSF* genes from rice suggest, in addition to a role in the heat shock response, a participation in non-thermal stress responses such as salt, drought and oxidative stress (Hu *et al.* 2009; Mittal *et al.* 2009; Wang *et al.* 2009). In particular, *OsHsfC1b* and *OsHsf2b* are highly responsive to salt and drought stress (Hu *et al.* 2009).

Besides stress, there is evidence for a role of HSFs in development. In animals and yeast, HSFs are involved in various non-stress processes, e.g. cell cycle progression, embryo development, cell differentiation and spermatogenesis (Pirkkala *et al.* 2001). Loss of the transcriptional activator HSF1 in mice results in increased prenatal lethality, retarded growth and female sterility (Xiao *et al.* 1999). The corresponding homologue in *Schizosaccharomyces pombe* is required for growth under control conditions (Gallo *et al.* 1993), whereas in

Drosophila melanogaster HSF regulates oogenesis and larva development (Jedlicka *et al.* 1997). Transgenic *Arabidopsis* plants overexpressing *HsfA2* exhibit increased cell proliferation (Ogawa *et al.* 2007), which demonstrates the function of HSFs in plant growth control.

In this study, we functionally characterized the class C *HSF* gene *OsHsfC1b* from rice, which has been shown to be salt-responsive (Hu *et al.* 2009). By using transgenic lines we show that it plays a dual role in both growth and tolerance to non-thermal stresses.

Materials and methods

BLAST search and multiple sequence alignment

Proteins homologous to OsHsfC1b were identified by BLAST searches on http://www.phytozome.net/. Multiple sequence alignment was done with Clustal X 2.0 (Larkin *et al.* 2007).

Plant growth conditions and stress treatments

Seeds of transgenic rice lines (*hsfc1b*, *ami*-7.1, *ami*-13.3 and empty-vector control) and wild-type (Dongjin) plants were placed on hydroponic boxes with full-strength Yoshida medium (Yoshida *et al.* 1971) using styrofoam adaptors. Plants grown for 3 or 4 weeks at (day/night) 26/22 °C, 75/70 % relative humidity with a day length of 12 h and a light intensity of 700 μmol m^{-2} s^{-1} were subjected to salt stress by adding either 50 or 100 mM NaCl (final concentration) to the growth medium. Furthermore, hydroponically grown plants were treated with 100 mM mannitol, 5 μM abscisic acid (ABA) or 5 mM H_2O_2. T_1 seeds of *ami*RNA and empty-vector lines were selected on 40 mg/L hygromycin. Leaves and roots were harvested separately. For estimation of biomass accumulation, fresh and dry weights of shoots and roots of five replicates each (from four plants in each experiment) were measured. After measurement, tissues were oven dried at 70 °C for 3 days, and the dry weight of each sample was measured. Subsequently, the relative biomass of the samples was calculated as the percentage of non-stressed plants.

Germination assays

Dehulled seeds were surface sterilized with 12 % NaOCl for 5 min and washed five times with distilled water. Subsequently, seeds were sown on vertical plates with Murashige–Skoog (MS) medium (3 % sucrose) containing NaCl (50 or 100 mM), ABA (1 or 5 μM) or mannitol (100 mM). Plates were incubated at (day/night) 26/22 °C, 75/70 % relative humidity with a day length of 12 h. Light intensity was set to 700 μmol m^{-2} s^{-1}.

Isolation of T-DNA insertion line

The T-DNA insertion line 1B-09127.R (rice ssp. *japonica* cv. Dongjin) was ordered from the POSTECH RISD database (Jeon *et al.* 2000; http://www.postech.ac.kr/life/pfg/risd/). Homozygous T_2 plants were confirmed by polymerase chain reaction (PCR) using the primers 5′-CTCCTCCATGCCCCTCTG-3′ and 5′-TTGGGGTTTCTACAGGACGTAAC-3′ for detection of the mutant allele, and the primers 5′-CTGCTCTCATACGGAGGAGG-3′ and 5′-AAGACAGCAGCAACGGAAAG-3′ for wild-type allele detection. Seeds of homozygous plants were propagated two times and T_4 seeds were used for further analysis.

Constructs and rice transformation

For the construction of an *artificial micro*-RNA (*ami*RNA) specific to *OsHsfC1b*, primers harbouring *attB* sites were designed in WMD2 (http://wmd2.weigelworld.org) and multi-step PCR was performed as described (Warthmann *et al.* 2008). Subsequently, the *ami*RNA construct was cloned into the pC5300 OE vector using BP clonase (Invitrogen, Darmstadt, Germany). pC5300 OE was constructed by inserting an *attP1-ccdB-attP2* Gateway^R cassette into the multiple cloning site of pC1300intA.Ubi-tnos (also named IRS154) between the maize ubiquitin promoter/first exon/first intron sequence and the *NOS* polyadenylation sequence (J. C. Breitler, CIRAD, Montpellier, France, unpubl. res.). The backbone vector pC1300intA was originally constructed by Ouwerkerk *et al.* (2001) (GenBank accession number: AF294976). Rice calli of the Nipponbare cultivar were co-cultured with *Agrobacterium tumefaciens* strain EHA105 containing recombinant or empty pC5300 according to Sallaud *et al.* (2003).

Subcellular localization of OsHsfC1b

For the subcellular localization study, the full-length CDS of *OsHsfC1b* was amplified by PCR from root cDNA (cv. Nipponbare) using two oligonucleotides (5′-CACCATGATGGGCGGCGAGTGCAA-3′ and 5′-CTAGTAGAACACTTGGCCAAGAA-3′) and cloned into pENTR vector (Invitrogen). Subsequently, the CDS was recombined at the N-terminus with green fluorescent protein (GFP) by Gateway transfer into the vector pK7WGF2.0 (Karimi *et al.* 2005). *Arabidopsis* mesophyll cell protoplasts were obtained and transformed with the GFP fusion construct according to Wu *et al.* (2009). Fluorescence imaging of the protoplasts was performed using a confocal laser scanning microscope (SP5; Leica Microsystems CMS, Mannheim, Germany).

Expression analysis of stress-related genes

RNA isolation from rice roots and leaves, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

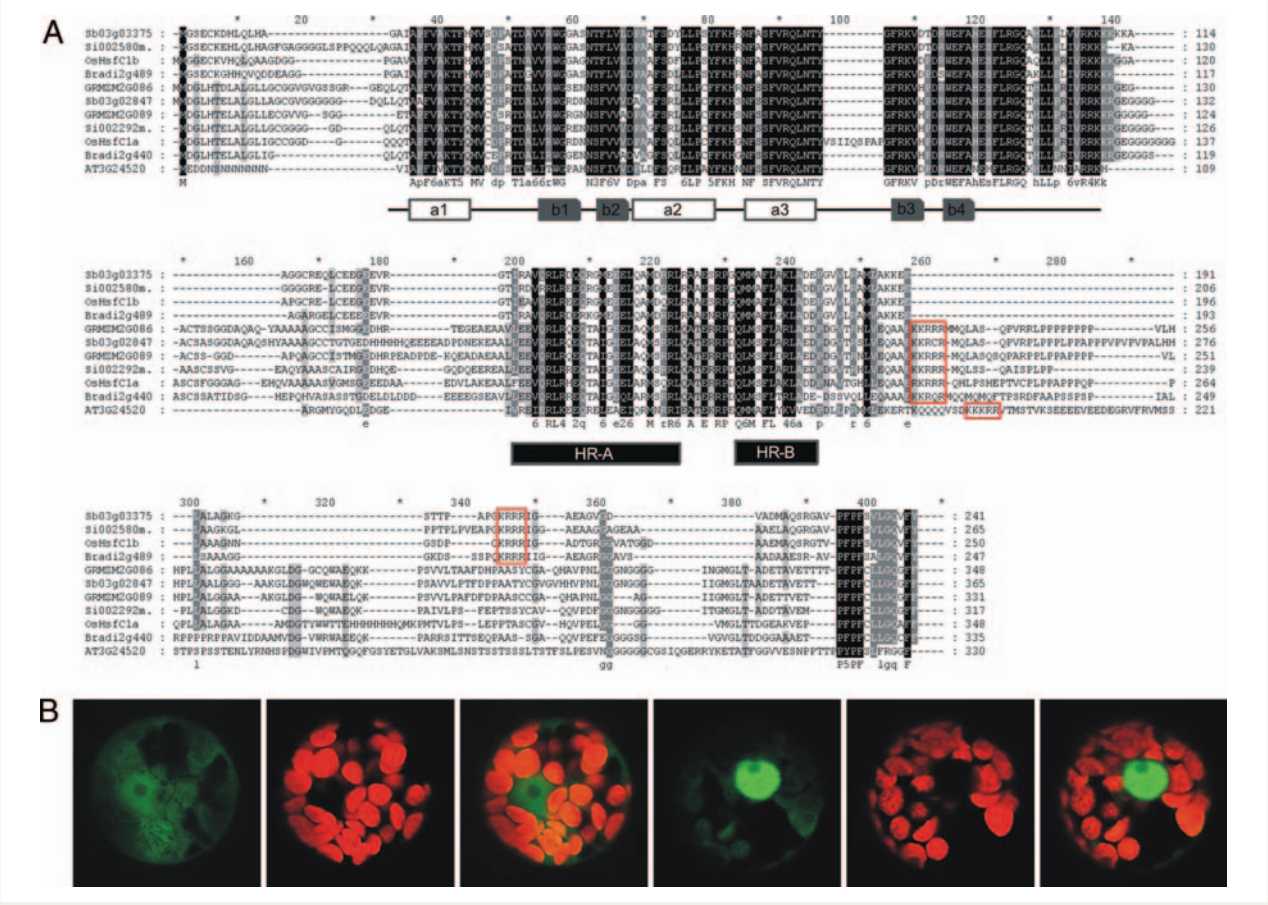

Fig. 1 Phylogenetic analysis and subcellular localization of OsHsfC1b. (A) Multiple sequence alignment of OsHsfC1b and homologous proteins. The DNA-binding domain consists of three α-helices (a1–a3) and four β-sheets (b1–b4). The black boxes represent the HR-A/B domain. All proteins harbour a putative NLS (red boxes). Protein sequence data were derived from phytozome (www.phytozome.net) under the following accession numbers: *O. sativa* OsHsfC1b (Os01g53220) and OsHsfC1a (Os01g43590); *Brachipodium distachyon* Bradi2g489 and Bradi2g440; *Sorghum bicolor* Sb03g03375 and Sb03g02847; *Zea mays* GRMZM2G086 and GRMZM2G089; *Setaria italica* Si002580m and Si002292m; and *Arabidopsis thaliana* AT3G24520. (B) Transient expression of GFP–OsHsfC1b fusion protein in *Arabidopsis* mesophyll cell protoplasts. From left to right: GFP signal of non-transformed control; chlorophyll autofluorescence of the same protoplast; overlay of GFP and autofluorescence signal; GFP signal of GFP–OsHsfC1b fusion protein in transformed protoplast; chlorophyll autofluorescence of the same protoplast; overlay of GFP–OsHsfC1b and autofluorescence signal.

were performed as described (Caldana *et al.* 2007). Three biological replicates (with four plants each) were used for each experiment. The efficiency of cDNA synthesis was estimated by qRT-PCR using *ACTIN* (Os03g50885, forward primer 5′-CTCCCCCATGCTATCCTTCG-3′ and reverse primer 5′-TGAATGAGTAACCACGCTCCG-3′) as reference gene. For expression analysis of *OsHsfC1b*, the oligonucleotides 5′-GCAGCTCAACACCTACGGATTC-3′ and 5′-TTCTTCTTCTTGCGCACGATCC-3′ were used. Oligonucleotide sequences used for expression profiling of signalling and ion homoeostasis components are listed separately [see Additional Information—File 1]. Oligonucleotide sequences for expression profiling of 12 *sHSP* genes were designed with QuantPrime (Arvidsson *et al.* 2008) and are listed in Additional Information (File 1).

Results

Phylogenetic analysis and subcellular localization of OsHsfC1b

BLAST search and multiple sequence alignment of OsHsfC1b (Os01g53220) revealed homologous proteins in other monocots such as maize, sorghum and *Brachypodium*, but also in the dicot *Arabidopsis* (Fig. 1A). In rice, OsHsfC1b shares highest similarity with OsHsfC1a,

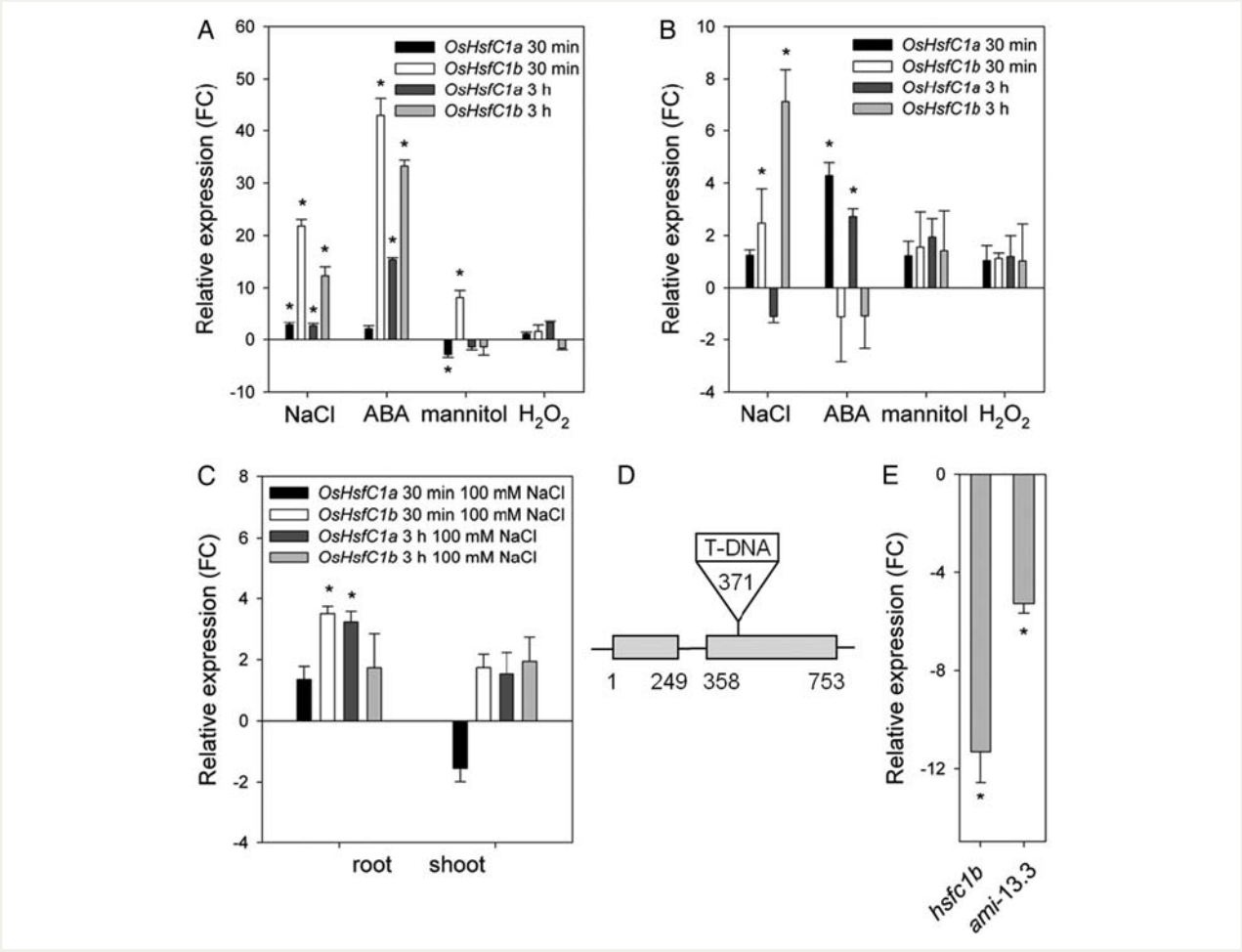

Fig. 2 Expression pattern of *OsHsfC1a* and *OsHsfC1b* under different treatments. (A) Relative expression of *OsHsfC1a* and *OsHsfC1b* in Nipponbare roots treated with 100 mM NaCl, 5 μM ABA, 100 mM mannitol or 5 mM H_2O_2 for 30 min or 3 h, respectively. (B) Relative expression of *OsHsfC1a* and *OsHsfC1b* in Nipponbare leaves treated with 100 mM NaCl, 5 μM ABA, 100 mM mannitol or 5 mM H_2O_2 for 30 min or 3 h, respectively. (C) Relative expression of *OsHsfC1a* and *OsHsfC1b* in Dongjin roots and leaves treated with 100 mM NaCl for 30 min or 3 h, respectively. (D) Insertion site of T-DNA in *hsfc1b* mutant. The *OsHsfC1b* gene consists of two exons, the second exon is disrupted in the mutant by the T-DNA insertion. Numbers indicate nucleotide positions counted from the translation start site. (E) Relative expression of *OsHsfC1b* in *hsfc1b* and *ami*-13.3 lines compared with Dongjin and empty-vector Nipponbare controls, respectively. Expression data in (A)–(C) and (E) represent means of three biological replicates (four plants each) ± SE. A star (*) indicates significant difference to expression under control conditions ($P \leq 0.05$). FC, fold change.

another member of the four class C HSFs identified in rice (Guo *et al.* 2008). All proteins contain a well-conserved N-terminal DNA-binding domain consisting of three α-helices and four β-sheets, and a highly conserved oligomerization domain, also known as HR-A/B domain. In addition, a putative nuclear localization signal (NLS) upstream of the oligomerization domain was identified in all proteins. To confirm targeting of OsHsfC1b to the nucleus, we performed a subcellular localization study, in *Arabidopsis* mesophyll cell protoplasts (Fig. 1B). The fluorescence signal of the GFP–OsHsfC1b fusion protein was detectable mainly in the nucleus and to a lesser extent in the cytosol, as expected for a nuclear protein; the signal of the GFP control was equally distributed over both compartments.

Expression profile of *OsHsfC1b* in rice roots and leaves exposed to salt, mannitol, ABA or H_2O_2

We examined the expression of *OsHsfC1b* in roots and leaves of 4-week-old hydroponically grown rice plants (cv. Nipponbare) exposed to 100 mM NaCl, 100 mM mannitol, 5 μM ABA or 5 mM H_2O_2 for 30 min or 3 h (Fig. 2A and B). *OsHsfC1b* was significantly induced in roots after 30 min treatment with salt, mannitol and

ABA. In addition, *OsHsfC1b* was also significantly upregulated in leaves after 30 min of salt treatment. After 3 h, the expression level of *OsHsfC1b* in roots was significantly increased by salt and ABA, but not by mannitol. Again, salt stress resulted in an upregulation of expression in leaves. Remarkably, the ABA-triggered induction in roots was ~2-fold higher than that triggered by salt, reaching an ~43-fold and ~33-fold induction after 30 min and 3 h ABA treatment, respectively, as compared with non-stress conditions. H_2O_2 had no effect on *OsHsfC1b* transcript level.

In addition to Nipponbare plants, we tested the salt-dependent expression of *OsHsfC1b* in rice plants of the Dongjin cultivar (Fig. 2C). In contrast to Nipponbare plants, *OsHsfC1b* was only induced in roots after 30 min of salt stress, showing an upregulation by ~3.5-fold. We compared the expression profile of *OsHsfC1b* under the different treatments with the paralogous gene *OsHsfC1a* (Fig. 1). As observed for *OsHsfC1b*, *OsHsfC1a* was upregulated in Nipponbare roots exposed to salt stress for 30 min and 3 h, and to ABA treatment for 3 h (Fig. 2A). Unlike its counterpart, however, *OsHsfC1a* was not induced by salt stress in Nipponbare leaves (Fig. 2B). Moreover, it was downregulated by mannitol treatment in roots and induced by ABA in leaves. As shown for *OsHsfC1b*, *OsHsfC1a* was significantly induced by salt stress in Dongjin roots (Fig. 2B).

Identification of the T-DNA insertion line *hsfc1b* and establishment of *ami*RNA lines

For functional characterization of *OsHsfC1b*, we identified a homozygous T-DNA insertion line (1B-09127.R) in the Dongjin background and named it *hsfc1b*. The insertion site is located in the second exon of *OsHsfC1b* (Fig. 2D). Additionally, we generated *ami*RNA lines in the Nipponbare background and selected two independent lines, *ami*-7.1 and *ami*-13.3, for further characterization. Transgenic plants of the T_4 (*hsfc1b*) and T_1 generation (*ami*RNA lines) were analysed regarding the expression of *OsHsfC1b* under non-stress conditions. In *hsfc1b* roots, we observed an 11-fold reduction of *OsHsfC1b* expression, while in *ami*-13.3 roots the expression of *OsHsfC1b* was decreased by ~5-fold as compared with control plants (Fig. 2E). The transcript of *OsHsfC1b* was not detectable in roots of the *ami*-7.1 line. Notably, during salt stress *OsHsfC1a* expression in the insertion line was similar to that in the wild type, suggesting that *OsHsfC1b* and *OsHsfC1a* act independently during the stress response [see Additional Information—File 2].

Growth of *hsfc1b* and *ami*RNA lines under control conditions

Transgenic plants (*hsfc1b*, *ami*-7.1 and *ami*-13.3 lines) showed stunted growth under non-stress conditions, visible 7 days after sowing (DAS) and at the age of 3 weeks (Fig. 3A and D). Root length and shoot height of seedlings germinated on MS medium were measured 4 and 7 DAS (Fig. 3B). Shoot length of the *hsfc1b* mutant was ~75 and ~80 % of that of the wild type (Dongjin) at 4 and 7 DAS, respectively (Fig. 3B). Furthermore, at 7 DAS, root length of *hsfc1b* was ~85 % of that of the Dongjin wild type, whereas at 4 DAS no difference was observed between roots of the insertion line and the wild type. The growth retardation was also observed in the *ami*-7.1 and *ami*-13.3 lines established in the Nipponbare background (Fig. 3B). At 4 DAS, both lines had significantly shorter root and shoot lengths than control plants containing the empty vector. Furthermore, at 7 DAS *ami*-7.1 plants displayed a significantly shorter shoot length, and *ami*-13.3 plants showed a significantly shorter root length. Besides this, we observed differences in biomass accumulation between *hsfc1b* and Dongjin wild-type plants (Fig. 3C). Both shoot fresh weight (FW) and dry weight (DW) of 4-week-old *hsfc1b* plants were reduced by one-third, and root FW and DW were ~60 % of that of wild-type plants. A similar observation was made for *ami*-13.3 and *ami*-7.1 lines, where shoot and root FW and DW were reduced by ~50 and ~75 %, respectively, suggesting that OsHsfC1b functions as a positive regulator of vegetative growth.

Growth of *hsfc1b*, *ami*-7.1 and *ami*-13.3 lines under salt stress, osmotic stress or ABA treatment

We examined the salt tolerance of *hsfc1b* plants. Seeds of the insertion line and the Dongjin wild type were germinated in the presence of either 50 or 100 mM NaCl, and subsequently shoot and root length were determined at 4 and 7 DAS. Under mild stress, shoot length of *hsfc1b* was significantly more reduced than that of the wild type at both time points (Fig. 4A). At 100 mM NaCl, a stronger reduction of both shoot and root length as compared with the stressed wild type was observed. These results suggest a requirement of OsHsfC1b for the response to both mild and severe salt stress. For the *ami*-7.1 line, we consistently observed a 20 % reduction of shoot length at 100 mM NaCl (4 DAS) as compared with stressed empty-vector control plants (Fig. 4B), while line *ami*-13.3 did not differ largely from the empty-vector control with respect to shoot and root growth at both time points. We also tested the response of the transgenic lines after 3 weeks of growth in hydroponic culture

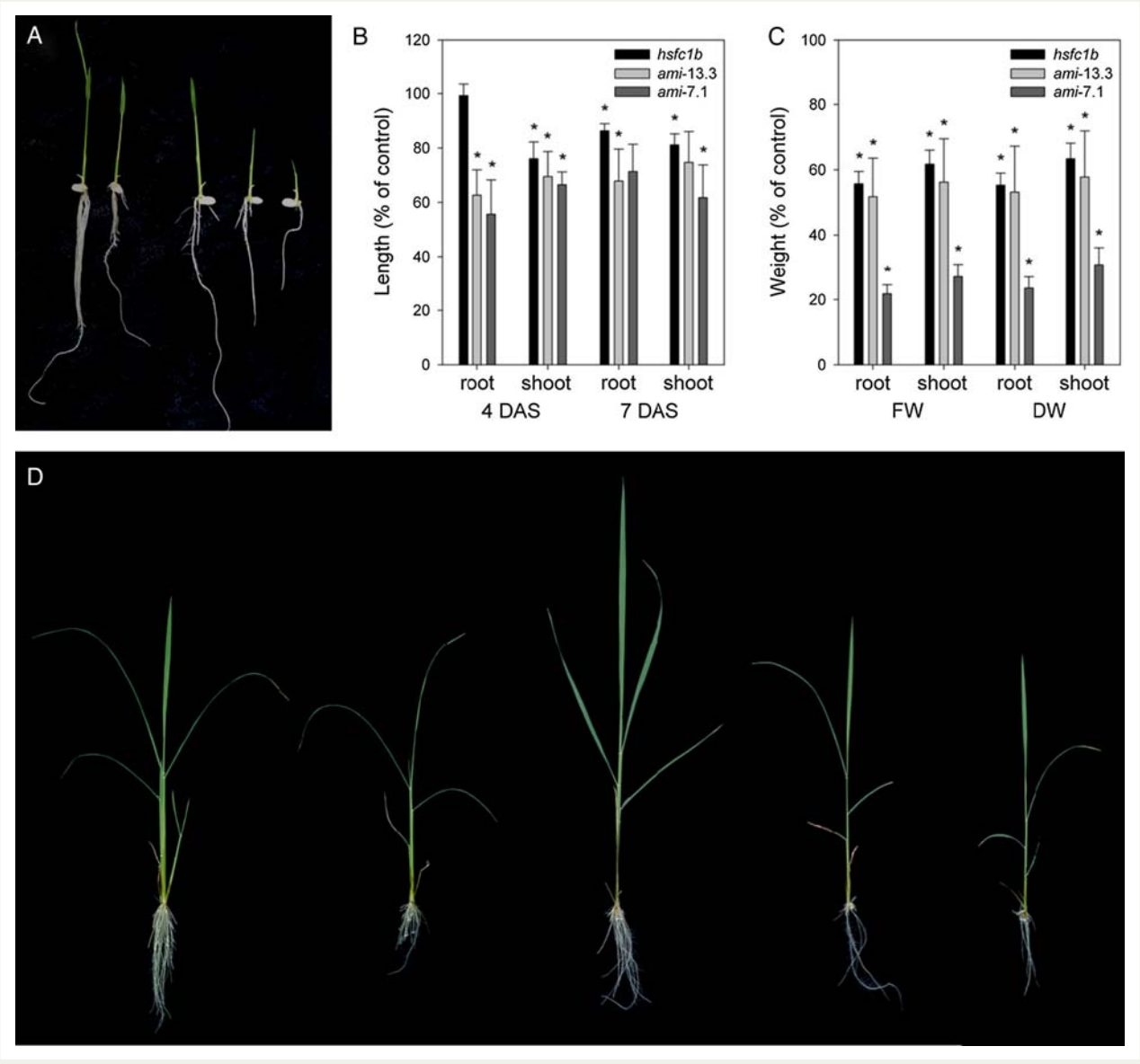

Fig. 3 Impact of OsHsfC1b on vegetative growth under normal conditions. (A) Seven-day-old seedlings grown on MS medium. From left to right: Dongjin wild type, *hsfc1b*, empty-vector control line (Nipponbare background), *ami*-13.3 line, *ami*-7.1 line. (B) Root and shoot length of *hsfc1b*, *ami*-13.3 and *ami*-7.1 lines at 4 and 7 DAS relative to Dongjin wild-type and Nipponbare empty-vector control seedlings, respectively. Data are means of three independent experiments ($n = 12$). A star (*) indicates significant difference to control ($P \leq 0.05$). DAS, days after sowing. (C) Fresh and dry weight of 4-week-old *hsfc1b*, *ami*-13.3 and *ami*-7.1 plants relative to Dongjin wild-type and Nipponbare empty-vector control plants. Data are means of five independent experiments ($n = 4$). A star (*) indicates significant difference ($P \leq 0.05$). FW, fresh weight; DW, dry weight. (D) Growth retardation of 4-week-old plants. From left to right: Dongjin wild type, *hsfc1b*, empty-vector control line (Nipponbare background), *ami*-13.3 line, *ami*-7.1 line.

and subsequent exposure to 50 mM NaCl for 8 days. The *hsfc1b* insertion line accumulated significantly less FW and DW (shoot and root) as compared with the stressed wild type (Fig. 4C). Likewise, *ami*-7.1 and *ami*-13.3 plants showed a significantly stronger reduction of FW and DW of both shoot and root than empty-vector control plants (Fig. 4C). Interestingly, Dongjin wild-type and empty-vector control plants (Nipponbare background) also differed regarding their salt tolerance. Whereas the shoot FW and DW were similarly reduced under stress conditions, empty-vector Nipponbare plants were more strongly affected

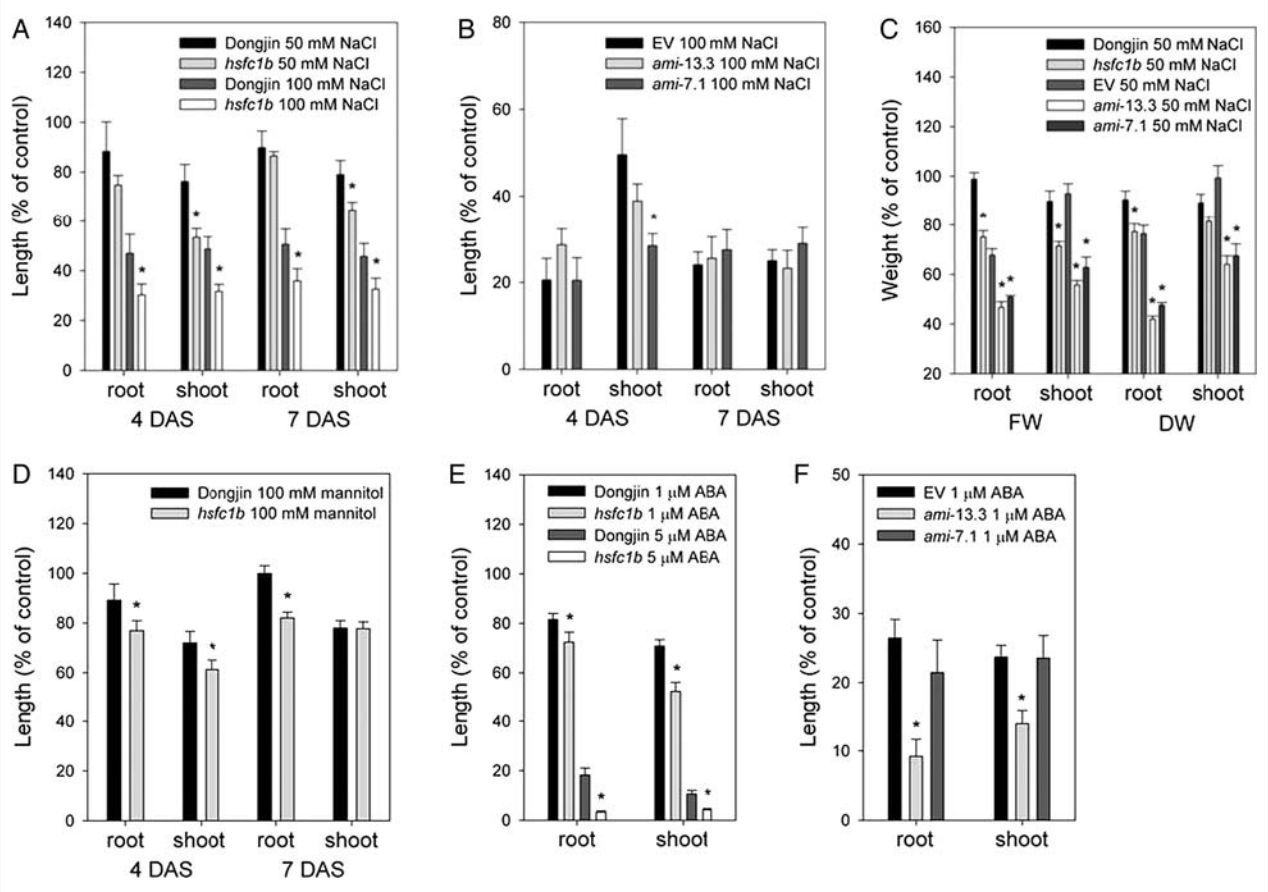

Fig. 4 Impact of OsHsfC1b on growth under stress conditions. (A) Root and shoot length of *hsfc1b* and Dongjin wild-type seedlings at 4 and 7 DAS grown on MS medium containing 50 or 100 mM NaCl, respectively, relative to non-stressed seedlings. (B) Root and shoot length of *ami*-13.3, *ami*-7.1 and Nipponbare empty-vector control (EV) seedlings at 4 and 7 DAS grown on MS medium containing 100 mM NaCl, relative to non-stressed seedlings. (C) Fresh and dry weight of 3-week-old hydroponically grown *hsfc1b*, Dongjin wild-type, *ami*-13.3, *ami*-7.1 and empty-vector control (EV) plants subjected to 50 mM NaCl for 8 days relative to non-stressed plants. (D) Root and shoot length of *hsfc1b* and Dongjin wild-type seedlings at 4 and 7 DAS grown on MS medium containing 100 mM mannitol relative to control seedlings. (E) Root and shoot length of *hsfc1b* and Dongjin wild-type seedlings at 4 and 7 DAS grown on MS medium containing 1 or 5 μM ABA, respectively, relative to untreated seedlings. (F) Root and shoot length of *ami*-13.3, *ami*-7.1 and Nipponbare empty-vector (EV) seedlings at 4 and 7 DAS grown on MS medium containing 1 μM ABA relative to untreated seedlings. Data in (A)–(F) are means of three independent experiments each ($n = 12$). A star (*) indicates significant difference to control ($P \leq 0.05$). DAS, days after sowing.

in root FW and DW than Dongjin wild-type plants (Fig. 4C).

Osmotic stress alone or in combination with salt or drought stress leads to diminished cell growth (Munns and Tester 2008). *OsHsfC1b* expression is induced in Nipponbare roots exposed to mannitol (Fig. 2A). For this reason, we tested the tolerance of *hsfc1b* plants to osmotic stress (Fig. 4D). At 4 DAS, *hsfc1b* plants grown on MS medium containing 100 mM mannitol exhibited significantly greater reduced shoot and root lengths, and at 7 DAS a significantly greater reduced root length as compared with the stressed wild type was observed, suggesting a role of OsHsfC1b in the response to osmotic stress (Fig. 4D).

Abscisic acid is involved in the response to many abiotic and biotic stresses, and exogenous ABA mimics effects caused by environmental stresses (Zhu 2002). The expression of *OsHsfC1b* is ABA-inducible (Fig. 2A), suggesting a potential role in ABA signalling and/or response. Seedlings of *hsfc1b* showed hypersensitivity towards ABA (1 and 5 μM), visible by diminished shoot and root length at 7 DAS (Fig. 4E); this hypersensitivity was more prominent at 5 μM ABA. Seedlings of *ami*-7.1 and *ami*-13.3 had major problems in growing on 5 μM ABA, making a quantitative analysis impossible (data not shown). Therefore, we examined these parameters at 1 μM ABA (Fig. 4F). Whereas the *ami*-7.1 and the empty-vector control line showed a similar reduction

of shoot and root length, those of *ami*-13.3 were significantly more affected (Fig. 4F).

Expression profiling of genes related to the salt stress response

The data described above indicated that OsHsfC1b contributes to the response to salt and osmotic stress. Next, we wanted to know whether genes known to be salt-responsive in the wild type are affected by the knock-down of *OsHsfC1b*. We therefore tested the expression of 80 salt-responsive genes involved in salt signalling and ion homeostasis in rice; these genes respond within 24 h of salt stress, with different induction time points and courses (R. Schmidt, MPIMP, Golm, Germany, unpubl. res.). The transgenic plants (*hsfc1b* and line *ami*-13.3) were exposed to 100 mM NaCl for 30 min or 3 h, and gene expression was analysed by qRT-PCR. We selected the *ami*-13.3 line since the reduction in plant size and weight is comparable to that of the T-DNA insertion line (Fig. 3). Interestingly, under control conditions, i.e. in the absence of stress, various salt-responsive genes were already differentially expressed in *hsfc1b* and *ami*-13.3 lines, compared with the controls (Table 1). In *hsfc1b* roots, we found a significant upregulation of *MAP2K.6*, the ATPases *ECA1*, *AHA1* and *AHA2* as well as *VHA-c4*, the cation transporters *HKT7* and *HKT8*, and *GLR2.8*. Additionally, *GLR2.7* and *TIP2-1* showed a significant downregulation as compared with Dongjin roots. The differentially expressed genes in *hsfc1b* (Dongjin background) under control conditions differed from those of *ami*-13.3 (Nipponbare background), possibly indicating cultivar differences. Here the six genes with a change in expression encode MAP3K.4, MAP3K.18, calcineurin-B-like protein CBL7, CAMK1, HAK4 and a protein kinase (Os06g43030) (Table 1). Remarkably, the expression of *MAPK3K.18* under non-stress conditions was drastically reduced in *ami*-13.3, showing a >130-fold lower expression than in roots of plants transformed with the empty vector.

After 30 min of salt stress, 13 genes were differentially expressed in Dongjin, six of which matched genes that were also differentially expressed in *hsfc1b* compared with the wild type under control conditions, including *ECA1*, *AHA1*, *VHA-c4*, *HKT8*, *GLR2.7* and *TIP2-1*, suggesting that the salt stress-associated gene regulatory network (GRN) is already in part activated in *hsfc1b* even in the absence of salt stress. A similar observation was made when *ami*-13.3 was compared with empty-vector control plants. After 30 min of salt stress, 15 genes responded in empty-vector control plants including four genes, i.e. *MAP3K.4*, *MAP3K.18*, *CBL7* and *HAK4*, which were differentially expressed in *ami*-13.3 under control conditions (Table 1). Exposure of *hsfc1b* roots to salt stress for 30 min induced nine genes which did not overlap with the genes responding in Dongjin wild-type roots at this time point. After 3 h of salt stress, seven and 13 genes were differentially expressed in Dongjin and *hsfc1b* roots, respectively. The only overlapping gene at this time point was *TIP3-2*; five other genes responding in *hsfc1b* under salt stress overlapped with the 30-min time point for wild-type plants (*MAP3K.23*, *CBL7*, *HKT8*, *CaCA*/Os11g01580 and *TIP2-1*). Furthermore, *CNGC2* (Os03g55100) showed a strong induction in Dongjin roots after 3 h, but was already induced in *hsfc1b* after 30 min of salt stress. For *ami*-13.3, we observed diverging expression profiles at both time points of salt stress as compared with the empty-vector control. After 30 min of salt stress, nine genes were differentially expressed in *ami*-13.3 roots, of which three genes, i.e. *AHA1*, *HKT8* and *CaCA* (Os12g42910), showed a similar response in the empty-vector control plants. Similarly, after 3 h of salt stress, we found 16 and 21 genes to be differentially expressed in *ami*-13.3 and empty-vector control, respectively. Eight genes shared a similar behaviour, including e.g. *MPK15*, *MAP3K.15*, *CIPK4*, *TIP2-1* and *TIP4-2* (Table 1). Overall, these results suggest a temporal misregulation of the expressional network in *hsfc1b and ami*-13.3 lines.

Irrespective of the duration of salt stress, we observed only a small overlap between the salt stress-associated GRNs of *hsfc1b*, *ami*-13.3, Dongjin wild type and the Nipponbare empty-vector line (Fig. 5A). Of the 19 and 17 genes that responded to salt stress in *hsfc1b* and Dongjin wild type, respectively, seven genes were in common. Similarly, of the 21 and 29 genes affected by salt stress in the *ami*-13.3 and empty-vector control lines, respectively, 10 genes showed a similar response. However, when comparing Dongjin wild-type and Nipponbare empty-vector control plants, an overlap of only seven salt-responsive genes was observed, of which four genes showed contrasting responses (Table 1), suggesting divergent expressional responses of Dongjin and Nipponbare to salt stress. Furthermore, 29 genes responded to salt stress in Nipponbare control roots, which is almost twice the number of genes differentially expressed in Dongjin wild-type roots.

Expression profiling of sHSP genes

Recently, 12 *sHSP* genes were found to respond to salt stress in rice (Sarkar *et al.* 2009). Since HSFs potentially regulate *sHSP* gene expression under stress conditions, we tested the expression of these 12 genes in *ami*-13.3 roots (Fig. 5B). In the absence of salt stress, *Hsp23.6-MII* was already induced in *ami*-13.3 compared to the Nipponbare empty-vector line. After 30 min of salt stress, *Hsp18.1-CII*, *Hsp23.2-ER* and *Hsp23.6-MII* were

Table 1 Expression of genes encoding signalling and ion homeostasis components in *hsfc1b* and *ami*-13.3 lines. Comparison of expression levels (log_2FC) in roots of *hsfc1b*, *ami*-13.3 and their respective controls under non-stress and salt stress conditions (100 mM NaCl). Values are presented as the relative expression level (log_2FC) of three biological replicates. The values that are significantly different from control ($\Delta\Delta C_T \leq -1$ or $\geq +1$) by Student's *t*-test are in bold ($P \leq 0.05$).

				100 mM NaCl							
		Control		30 min				3 h			
Locus	Name	*hsfc1b*	*ami*-13.3	Dongjin	*hsfc1b*	EV	*ami*-13.3	Dongjin	*hsfc1b*	EV	*ami*-13.3
Os11g17080	MPK15	0.29	0.30	−0.06	−0.36	0.23	0.79	−0.12	−1.60	**1.97**	**2.29**
Os03g12390	MAP2K.6	**1.09**	−0.43	0.50	**1.15**	1.08	**−2.99**	−0.69	**1.03**	0.08	−0.37
Os01g50370	MAP3K.4	0.15	**1.33**	0.39	0.72	**2.55**	−0.77	−0.21	0.53	0.97	−0.42
Os01g50410	MAP3K.6	0.16	0.60	0.11	0.46	**2.34**	−0.19	−0.49	1.01	**1.14**	0.37
Os03g15570	MAP3K.12	0.62	0.12	−0.03	**1.28**	−0.78	−1.50	−0.11	1.20	0.30	0.70
Os03g55560	MAP3K.15	0.53	0.04	0.33	0.38	−0.03	0.84	−0.16	0.23	**1.40**	**1.87**
Os05g46750	MAP3K.18	0.02	**−7.06**	0.69	1.90	**−4.54**	−0.61	−0.28	**2.09**	−0.06	−0.25
Os05g46760	MAP3K.19	0.15	−0.48	0.15	**1.33**	0.34	−1.46	0.09	**1.78**	−0.91	−0.22
Os10g04010	MAP3K.23	1.59	0.40	**1.39**	−0.92	1.52	−1.51	0.39	**1.18**	−0.89	0.30
Os06g43030	Protein kinase	0.58	**1.95**	0.97	−0.14	0.03	−0.88	0.66	**1.01**	**1.84**	−0.88
Os02g18880	CBL7	0.03	**1.78**	**−1.53**	−0.41	**2.21**	−0.38	−1.44	**−1.35**	**2.00**	0.47
Os02g18930	CBL8	−0.28	0.12	−1.55	0.16	−1.72	−0.71	−0.69	0.41	**2.35**	0.88
Os01g51420	CBL10	0.56	−0.47	0.75	**1.15**	−1.25	−1.93	−0.24	1.06	−0.28	0.23
Os02g03410	CPK4	−0.50	0.77	0.02	**1.59**	**1.81**	0.44	−0.45	0.18	1.23	0.02
Os02g58520	CPK6	0.37	0.75	0.69	1.20	1.21	−0.28	0.62	0.17	**2.24**	0.56
Os12g41090	CIPK4	−0.27	0.79	−0.52	0.10	−0.27	−0.42	0.64	−1.24	**1.46**	**2.97**
Os08g34240	CIPK6	0.23	−0.25	**1.10**	0.30	−2.19	**−3.26**	−0.55	−0.06	−1.84	**−1.58**
Os05g26820	CIPK18	−1.12	1.31	−0.43	1.37	−1.14	−1.22	−0.30	−0.15	**1.11**	0.31
Os03g20380	CIPK31	0.58	−0.64	−0.82	1.81	−0.58	0.50	−0.25	−0.40	1.33	**2.08**
Os03g25070	CAMK1	0.87	**2.13**	0.67	−2.15	−0.92	−1.23	−0.70	**3.15**	1.42	−1.46
Os07g44710	CAMK_like.36	0.81	0.22	0.10	−0.10	−0.96	0.11	0.13	0.20	**1.29**	0.77
Os03g17310	ECA1	**1.64**	1.21	**1.74**	0.03	−0.44	0.04	−0.93	2.09	**2.34**	**1.36**
Os03g48310	AHA1	**1.34**	−0.38	**1.91**	1.05	**−3.45**	**−2.20**	2.89	1.37	−0.72	**−1.45**
Os07g09340	AHA2	**1.21**	0.02	−1.12	0.54	1.22	**−1.16**	−0.14	**−1.08**	−1.68	−1.46
Os02g07870	VHA-A2	−0.43	0.04	−0.02	−0.21	0.69	−1.00	0.10	**−1.39**	0.61	−0.07

Os01g73130	VHA-c4	**3.00**	−0.99	**2.56**	0.73	−2.17	−2.04	0.61	2.44	−2.25	−0.30
Os06g08080	OVP2	0.63	0.03	0.22	0.23	**−1.63**	−0.64	1.20	−0.37	0.76	0.92
Os06g48810	HKT1	−0.86	0.36	**−2.76**	−1.49	−1.08	−0.26	**−1.49**	−0.37	0.41	−0.55
Os02g07830	HKT6	1.04	−0.39	0.22	0.72	0.70	n.d.	0.62	0.13	**1.77**	n.d.
Os04g51830	HKT7	**1.81**	1.89	1.07	−0.36	−0.20	−4.70	−0.33	0.91	−1.02	0.64
Os01g20160	HKT8	**1.27**	−2.15	**1.35**	−0.62	**2.23**	**2.89**	−0.65	**2.48**	**2.49**	**4.64**
Os03g55100	CNGC2	−0.43	−0.66	0.69	**2.76**	1.74	3.38	**2.67**	1.29	3.98	3.41
Os04g49570	GLR3.1	0.21	0.58	0.79	1.04	0.57	0.19	0.59	−0.79	1.79	**2.28**
Os06g08930	GLR 2.8	**2.54**	0.39	1.91	1.31	0.05	−2.89	**1.83**	1.89	−0.37	−1.65
Os06g08880	GLR 2.7	**−1.52**	1.04	**−2.54**	−1.02	**2.43**	0.78	−1.33	−1.58	**2.87**	**1.74**
Os09g30446	Transporter	0.10	6.60	**−1.95**	−0.98	−0.01	−8.39	**−2.16**	−0.21	−1.32	−9.66
Os02g14840	KAT1	2.26	−1.42	**3.48**	2.72	−1.48	−1.89	**2.99**	1.20	−1.20	−0.10
Os11g01580	CaCA	0.59	−1.18	**1.94**	2.59	−1.65	**−1.51**	−1.36	**4.25**	−0.23	0.58
Os12g42910	CaCA	0.56	0.06	0.47	0.63	**−3.42**	**−4.13**	**1.03**	1.59	**−2.93**	−2.69
Os08g36340	HAK4	−1.37	**3.79**	n.d.	2.21	**1.94**	−0.87	n.d.	n.d.	**3.98**	0.84
Os01g70660	HAK6	0.25	0.63	1.82	**3.45**	0.19	2.35	3.31	n.d.	−1.20	0.95
Os06g45940	HAK13	−0.23	0.85	−1.85	0.37	−0.20	−0.73	0.00	0.39	**1.62**	−0.35
Os09g38960	HAK18	0.77	0.04	0.30	0.73	−2.09	**−1.48**	0.08	1.01	0.39	−0.08
Os06g15910	HAK24	−0.25	0.23	−0.33	0.42	**−4.31**	−1.78	0.78	0.63	0.80	0.60
Os02g13870	NIP1-1	0.67	−0.69	0.65	0.74	**−1.85**	−0.78	0.02	1.83	**−1.07**	−1.11
Os06g12310	NIP2-2	−0.47	−1.26	0.20	−0.72	−1.48	−1.57	0.90	−2.16	−0.37	**−1.56**
Os02g44080	TIP2-1	**−2.43**	0.46	**−1.61**	0.24	−0.08	1.96	−0.07	**−4.54**	**−1.88**	**−2.15**
Os01g13130	TIP4-2	0.65	−0.64	1.38	**2.60**	0.36	0.68	0.29	0.78	**3.14**	**1.83**
Os04g44570	TIP3-2	1.71	0.06	2.90	**1.59**	−0.29	−1.64	**2.61**	**2.65**	0.86	−1.13
Os02g57720	PIP1-3	0.02	0.28	0.02	0.06	**1.22**	−0.44	−0.30	0.00	0.97	0.34
Os07g26690	PIP2-1	−0.41	0.56	−0.32	0.75	0.17	**−1.18**	−0.65	−0.81	−1.59	**−1.32**
Os02g41860	PIP2-2	−0.60	0.68	−0.99	0.13	0.64	0.29	0.01	−1.63	**1.53**	0.86
Os07g26630	PIP2-4	−0.21	0.83	−0.01	0.81	−0.97	−0.40	−0.63	−1.42	−0.52	**−1.59**
Os07g26640	Aquaporin	−0.39	0.34	−0.62	0.53	**1.45**	−0.25	−0.79	−1.24	−0.67	**−1.44**

FC, fold change; EV, empty-vector control; n.d., not determined.

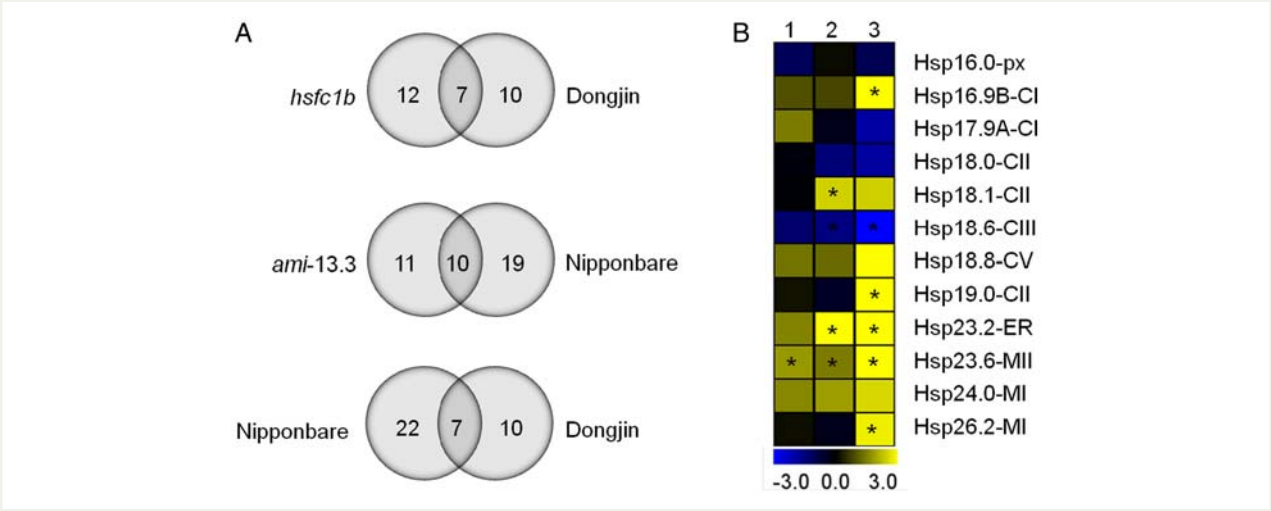

Fig. 5 Expressional response of salt stress-responsive genes in the different genotypes. (A) Venn-diagram presentation of the overlap of differentially expressed genes in *hsfc1b*, *ami*-13.3 and corresponding controls under salt stress. (B) Differentially expressed sHSP genes: (1) expression in *ami*-13.3 roots compared with Nipponbare empty-vector control roots in the absence of salt stress; (2) expression in empty-vector control roots exposed to salt stress (100 mM NaCl, 30 min) compared with non-stressed roots; (3) expression in *ami*-13.3 roots exposed to salt stress (100 mM NaCl, 30 min), compared with non-stressed roots. Values are presented as the relative expression level ($\log_2$FC) of three biological replicates. The values that are significantly different (≤ -1 or $\geq +1$) from the respective control by Student's *t*-test ($P \leq 0.05$) are marked with a star (*).

significantly induced in empty-vector roots, whereas *Hsp18.6-CIII* was downregulated. In addition to *Hsp23.2-ER* and *Hsp23.6-MII*, *Hsp16.9B-CI*, *Hsp19.0-CII* and *Hsp26.2-MI* also showed an increased expression in *ami*-13.3 roots under salt stress. Like in empty-vector control plants, *Hsp18.6-CIII* was downregulated in *ami*-13.3 under salt stress, whereas *Hsp18.1-CII* was not significantly affected. For *Hsp18.6-CIII* and *Hsp23.6-MII*, we observed a stronger response in *ami*-13.3 under salt stress than in empty-vector control roots. There was no significant change in the expression of *Hsp16.0-px*, *Hsp17.9A-CI*, *Hsp18.8-CV* and *Hsp24.0-MI* in *ami*-13.3 or empty-vector control roots under salt stress.

Discussion

OsHsfC1b is localized in the nucleus

OsHsfC1b possesses a nuclear localization signal upstream of the HR-A/B domain, but lacks a nuclear export signal (Wang *et al.* 2009). We cloned the open reading frame of OsHsfC1b downstream of GFP. When transiently expressed in *Arabidopsis* mesophyll cell protoplasts, the fluorescence signal was primarily detected in the nucleus and to a lesser extent in the cytoplasm (Fig. 1B), consistent with a transcription factor function of OsHsfC1b. The nuclear localization of OsHsfC1b in the absence of stress indicates that a stress-dependent modification is not required for nuclear accumulation. In addition, unlike LpHsfA2 from tomato, whose physical interaction with LpHsfA1 is mandatory for its translocation from the cytosol to the nucleus (Scharf *et al.* 1998), nuclear translocation of OsHsfC1b may not be complex dependent, as transient expression of *35S::GFP:OsHsfC1b* in *Arabidopsis* protoplasts eliminates possible interactions with other rice HSFs. However, due to high sequence similarities between rice and *Arabidopsis* HSFs (Wang *et al.* 2009), we cannot exclude heteromeric complex formation of OsHsfC1b with *Arabidopsis* HSFs.

OsHsfC1b is required for normal growth in rice

Heat shock factors have been shown to regulate developmental processes in animals (Pirkkala *et al.* 2001). In addition, HSF1 in mice has an impact on the immune system (Inouye *et al.* 2004), indicating a dual role of HSFs in development and survival. In *S. pombe*, the disruption of HSF results in growth defects under normal temperature; furthermore, it can be functionally replaced by the single HSF present in *D. melanogaster*, suggesting a general role of HSFs for the regulation of growth or development of eukaryotic organisms under non-stress conditions (Gallo *et al.* 1993). Transgenic *Arabidopsis* plants overexpressing *OsHsfA2e* from rice,

AtHsfA2 or *AtHsfA3* from *Arabidopsis*, or *BhHsf1* from *Boea hygrometrica* display impaired growth phenotypes (Ogawa *et al.* 2007; Yokotani *et al.* 2008; Yoshida *et al.* 2008; Zhu *et al.* 2009). Interestingly, we observed retarded root and shoot growth for *hsfc1b* and knock-down lines of *OsHsfC1b* and a decreased biomass at the age of 4 weeks as compared with control plants under normal conditions (Fig. 3). Thus, in contrast to class A HSFs, OsHsfC1b acts as a positive regulator of growth under standard growth conditions. We therefore propose that class C HSFs play an opposite role to class A members in plant growth control. The *hsfc1b* T-DNA insertion line and the *ami*RNA lines were established in the rice *japonica* cultivars Dongjin and Nipponbare, respectively. Irrespective of the genetic background, all lines exhibited stunted growth, indicating a conserved role of OsHsfC1b in growth control in the different cultivars.

OsHsfC1b positively regulates salt and osmotic stress tolerance

Heat shock factors represent interaction points connecting multiple stress response pathways. Many of them display an expressional response to various stresses and for some HSFs, mainly class A members, an involvement in stress adaptation has been shown (Ogawa *et al.* 2007; Banti *et al.* 2010; Chauhan *et al.* 2011). *OsHsfC1b* has been reported to be induced by heat stress (Hu *et al.* 2009; Wang *et al.* 2009). Remarkably, of all class C genes, *OsHsfC1b* shows the strongest expression under cold stress, suggesting an involvement of class C HSFs in the cold stress response, with OsHsfC1b playing a prominent role (Mittal *et al.* 2009).

In this study, we examined the physiological role of OsHsfC1b in the response to non-thermal stress. We show that *OsHsfC1b* is salt-responsive in both Nipponbare and Dongjin backgrounds. Furthermore, the decrease of *OsHsfC1b* expression level results in a decreased salt tolerance, as determined by the growth inhibition in the *hsfc1b* and *ami*-7.1 lines under salt stress (Fig. 4A and B). Hence, OsHsfC1b contributes to salt stress tolerance, which is conserved between the two tested *japonica* cultivars. Its role in the response to salt stress is not restricted to the seedling stage, as 4-week-old *hsfc1b*, *ami*-13.3 and *ami*-7.1 plants accumulated less biomass than wild-type or empty-vector control plants under salt stress (Fig. 4C).

In addition to ion toxicity, osmotic stress develops in the course of salt stress. Seedlings of *hsfc1b* subjected to mannitol displayed a stronger reduction of shoot and root growth than the Dongjin wild type (Fig. 4D). Since *OsHsfC1b* is induced by mannitol (Fig. 2A), it might function as a regulator of an osmotic stress response by itself or in combination with salt stress, as shown for HsfA2 in *Arabidopsis* (Ogawa *et al.* 2007). Previously, it was reported that *OsHsfC1b* expression is induced by drought stress (Hu *et al.* 2009), which causes osmotic imbalance as well.

Abscisic acid acts as an important integrator of abiotic stress responses. For salt and osmotic stress, ABA-dependent and ABA-independent signalling pathways are known. The expressional network related to salt stress partially overlaps with those for drought, cold and ABA. The decreased expression of *OsHsfC1b* results in ABA hypersensitivity (Fig. 4E and F). Interestingly, the promoter of *OsHsfC1b* contains three putative ABA-response elements (ABREs; Mittal *et al.* 2009) and we showed that its expression is highly induced by ABA (Fig. 2A). These findings strongly support a role of OsHsfC1b in ABA response pathways or signalling. Abscisic acid hypersensitivity is often accompanied by hypersensitivity to salt and osmotic stress (Borsani *et al.* 2001; Pandey *et al.* 2005; Zhu *et al.* 2010). Thus, the salt and osmotic stress tolerance conferred by OsHsfC1b is most likely ABA-dependent. In addition to stress tolerance, ABA is involved in the regulation of plant growth and development. Abscisic acid hypersensitivity can cause growth retardation even in the absence of external ABA, as demonstrated by the *brx-2* mutant in *Arabidopsis*, which displays retarded root growth, and *ABF3* and *ABF4* overexpressers, which show general growth defects under non-stress conditions (Kang *et al.* 2002; Rodrigues *et al.* 2009). Possibly, the impaired growth of *hsfc1b*, *ami*-13.3 and *ami*-7.1 lines under normal conditions is a consequence of an increased sensitivity to ABA. In the case of the *ami*-7.1 line, the relative response to ABA was similar to that of the control, which might be due to the severe growth retardation under control conditions.

OsHsfC1b is required for an adequate temporal expression of salt stress-associated genes

We performed expression profiling of 80 salt stress-related genes encoding signalling and ion homeostasis components in *hsfc1b* and *ami*-13.3 lines (Table 1). Interestingly, decreased expression of *OsHsfC1b* already resulted in differential expression of salt-responsive genes under control conditions. Thus, in *hsfc1b*, eight genes were induced and two genes were repressed in the absence of stress, relative to Dongjin. Notably, seven of them responded to salt stress in Dongjin wild-type roots. Similarly, in *ami*-13.3 roots, six genes were differentially expressed compared with empty-vector control plants in the absence of stress, of which five genes were affected by salt stress in the control plants. These findings indicate that decreased expression of

OsHsfC1b causes the constitutive activation of some genes of the salt stress-associated GRN. Nonetheless, although the reduced *OsHsfC1b* expression in *hsfc1b* and the *ami*RNA lines resulted in the misregulation of salt stress-responsive genes, we observed only a small overlap of genes responding similarly in *hsfc1b* and *ami*-13.3 (Fig. 5A). One possible explanation for this difference is that Dongjin and Nipponbare (here represented by the empty-vector line) themselves differed regarding their expression response to salinity. In both cultivars we observed a differential expression of previously identified salt stress markers (R. Schmidt, MPIMP, Golm, Germany, unpubl. res.); however, there was only an overlap of seven genes with a similar response. Moreover, the number of salt stress-responsive genes in Nipponbare was almost twice as high as in Dongjin. Cultivars differing in stress tolerance exhibit contrasting expression profiles, as demonstrated for salt and drought stress, where increasing sensitivity is positively correlated with an increasing number of differentially expressed stress-related genes (Walia *et al.* 2005; Degenkolbe *et al.* 2009). Among *japonica* cultivars, Dongjin is considered to be more salt tolerant than Nipponbare (Oh *et al.* 2003; Ferdose *et al.* 2009) and we observed a greater reduction of root FW and DW of empty-vector control plants in the Nipponbare background as compared with Dongjin wild-type plants under salt stress (Fig. 4C), fitting the observed expression of stress marker genes.

MAP3K.18 expression is induced by salt, drought, cold, and fungal and viral pathogens, and is therefore thought to be relevant for multiple stress responses (Jung *et al.* 2010). Remarkably, *MAP3K.18* exhibited a >130-fold downregulation in *hsfc1b* compared with Dongjin wild type under control conditions, whereas in Dongjin its expression was downregulated by ~20-fold under salt stress. *TIP2-1*, encoding a tonoplast-located aquaporin, was constitutively repressed in *hsfc1b* roots under control conditions. To a lesser extent, *TIP2-1* was found to be downregulated in Dongjin wild-type roots after 30 min of salt stress. Moreover, after 3 h of salt stress, *TIP2-1* expression in *hsfc1b* roots was >20-fold reduced, whereas no altered expression was observed in Dongjin roots. Additionally, after 3 h of salt stress, *TIP2-1* was more strongly repressed in *ami*-13.3 than in Nipponbare empty-vector control plants. Consistent with our data, *TIP2-1* expression appears to be correlated with salt tolerance, as salt tolerant cultivars display an eight times higher expression level of *TIP2-1* in roots than salt-sensitive cultivars (Cotsaftis *et al.* 2011). Interestingly, we observed upregulation of *PIP2-2* expression in the empty-vector control line but not in *ami*-13.3 upon salt stress, which might be causative for the differences between both lines regarding salt tolerance, as transgenic *Arabidopsis* plants overexpressing rice *PIP2-2* are more tolerant to salt and osmotic stress (Guo *et al.* 2006). Besides *PIP2-2, PIP1-3* was found to be induced in the empty-vector control after 30 min of salt stress, but was not changed in *ami*-13.3 under stress conditions. Like *PIP2-2*, *PIP1-3* might confer salt tolerance, since its expression is induced by salt, and rice plants overexpressing *PIP1-3* display an enhanced chilling and drought stress tolerance (Lian *et al.* 2004; Guo *et al.* 2006; Matsumoto *et al.* 2009). In addition to aquaporins, we tested the expression of ion transporters. HKT1 (OsHKT2;1) functions as a high-affinity Na^+ transporter in rice roots and root sodium influx during salt stress is prevented by downregulation of *HKT1* expression (Golldack *et al.* 2002; Horie *et al.* 2007). However, in contrast to Dongjin wild-type roots, *HKT1* expression was not downregulated in *hsfc1b* roots upon salt stress, which might cause an accumulation of sodium ions in the root. Similar to a previous study (Cotsaftis *et al.* 2011), we did not find a correlation between *HKT8* (*OsHKT1;5*) expression and salt tolerance, as *HKT8* expression was induced in both Dongjin wild-type and *hsfc1b* roots under salt stress. Still, *HKT8* was more strongly induced under salt stress in *ami*-13.3 than the empty-vector control line, which might reflect the increased sensitivity of *ami*-13.3 to salt stress. Furthermore, we observed a stronger reduction of *CIPK6* expression in *ami*-13.3; in accordance with this, a positive correlation between *CIPK6* expression and salt tolerance was previously reported (Cotsaftis *et al.* 2011). Thus, in addition to the temporal misregulation of genes upon salt stress, the expression levels of individual marker genes strongly support the reduced salt tolerance observed for the *hsfc1b* and *ami*RNA lines.

The heat shock element (HSE) is found in promoters of *sHSP* genes, suggesting a transcriptional control via HSFs (Scharf *et al.* 2001). Furthermore, HsfA2 in *Arabidopsis* has been shown to regulate the expression of several *sHSP* genes by binding to HSEs in the target gene promoters (Schramm *et al.* 2006). We tested the expression of 12 previously identified salt-responsive rice *sHSP* genes (Sarkar *et al.* 2009) in roots of salt-stressed *ami*-13.3 plants (Fig. 5B). Interestingly, we observed altered *sHSP* gene expression within 30 min of salt stress, indicating a rapid expressional response of the heat-shock network to non-thermal stresses. Similar to salt stress-related genes encoding signalling and ion homeostasis components, decreased *OsHsfC1b* expression causes a partial activation of the heat-shock expression network in the absence of stress (Fig. 5B). Under salt stress, *ami*-13.3 plants showed more and stronger responding *sHSP* genes. The increased response of *sHSP* genes in

ami-13.3 indicates a regulatory function of OsHsfC1b in the heat-shock expressional network; however, whether OsHsfC1b interacts with these *sHSP* genes in a direct or an indirect manner has to be determined. Our findings suggest that an enhanced response of the heat-shock expressional network under salt stress does not necessarily improve stress tolerance.

Conclusions and forward look

OsHsfC1b is, to our knowledge, the first class C HSF characterized *in planta*. Its dual role in salt tolerance and plant growth illustrates the contribution of class C HSFs to these important aspects. Transgenic rice overexpressing *OsHsfC1b* might be relevant for future breeding strategies, as decreased expression of *OsHsfC1b* causes salt sensitivity and impaired growth under normal conditions. Identifying direct interactions between OsHsfC1b and *cis*-regulatory elements of stress-related target genes is of further interest, as OsHsfC1b has been reported not to bind to the typical HSE element (Mittal *et al.* 2011). In addition to its eminent role as a food source, rice serves as an important model plant. Thus, the knowledge gained here for a class C HSF transcription factor could form the basis for the characterization of similar regulators in other monocotyledonous species. Of note, the relevance of OsHsfC1b in stress adaptation and growth demonstrated for two rice cultivars suggests that the function of class C HSFs is conserved within, and possibly also between, species.

Additional information

The following additional information is available in the online version of this article –

File 1. Table. Primer sequences for qRT-PCR-based expression profiling of rice genes.

File 2. Figure. Expression of *OsHsfC1a* in the *hsfc1b* mutant under control and salt stress conditions.

Accession numbers

Sequence data from this article can be found in the Michigan State University Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu; Ouyang *et al.* 2007) by using the MSU accession numbers given in Table 1 and [Additional Information—File 1].

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Contributions by the authors

R.S., J.H.M.S. and B.M.R. designed the research. R.S., J.H.M.S. and A.W. conducted the research. D.M. and E.G. did the rice transformations. R.S., J.H.M.S. and B.M.R. wrote the paper.

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Conflict of interest statement

None declared.

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