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

Romy Schmidt\(^1,2\)\(^\dagger\), Jos H.M. Schippers\(^1,2\)\(^\dagger\), Annelie Welker\(^1\), Delphine Mieulet\(^3\), Emmanuel Guiderdoni\(^3\) and Bernd Mueller-Roeber\(^1,2\)*

\(^1\) Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Str. 24–25, 14476 Potsdam, Germany
\(^2\) Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, 14476 Potsdam, Germany
\(^3\) CIRAD, UMR AGAP, Avenue Agropolis, 34398 Montpellier, Cedex 5, France

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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\(_2\)O\(_2\) treatment. For functional characterization of OsHsfC1b, we analysed the physiological response of a T-DNA insertion line (hsfc1b) and two artificial micro-RNA (amiRNA) 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\(_2\)O\(_2\). Impaired function of OsHsfC1b in the hsfc1b mutant and the amiRNA 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 conditions.

* Corresponding author’s e-mail address: bmr@uni-potsdam.de
\(^\dagger\) These two authors have contributed equally to this work.

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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 ‘nGAAAn’, 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 thermo-tolerance (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 H₂O₂. T₁ seeds of amiRNA 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 T₂ plants were confirmed by polymerase chain reaction (PCR) using the primers 5’-CTCTCTATGCCCTCTG-3’ and 5’-TGGGTTTCTACGGACGTAA-3’ for detection of the mutant allele, and the primers 5’-CTGGCTCTCATAGGAGG-3’ and 5’-AAGACA-GCAGCAACGGGA-3’ for wild-type allele detection. Seeds of homozygous plants were propagated two times and T₄ seeds were used for further analysis.

Constructs and rice transformation
For the construction of an artificial micro-RNA (amiRNA) 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 amiRNA construct was cloned into the pc5300 OE vector using BP clonase (Invitrogen). pc5300 OE was constructed by inserting an attP1-ccdB-attP2 Gateway® 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’-CACCATGATGGCGGGCGAGTGCAA-3’ and 5’-CTAGTAGAACACTTGGCC-3’) 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 pk7WGFW2.0 (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)
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′-CTCCCCATGCTATCCTTCG-3′ and reverse primer 5′-TGAATGAGTAACCACGCTCCG-3′) as a reference gene. For expression analysis of OsHsfC1b, the oligonucleotides 5′-GCAGCTCAACACCTACGGATTC-3′ and 5′-TTCTTCTTCTTGGCAGATCC-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 (Os01g35220) 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,
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 H₂O₂

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 H₂O₂ 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$_2$O$_2$ 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 amiRNA lines

For functional characterization of OsHsfC1b, we identified a homozygous T-DNA insertion line (18-09127.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 amiRNA 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 (amiRNA 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 amiRNA 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.
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
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 grown on MS medium containing 100 mM mannitol to osmotic stress (Fig. 4D). At 4 DAS, hsfc1b plants 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 in root FW and DW than Dongjin wild-type plants (Fig. 4C).

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 ≤ 0.05). DAS, days after sowing.
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, MPI-M, 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 MAP3K.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, CaCaA/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 CaCaA (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 expression 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
Comparison of expression levels (log2FC) in roots of \( {\text{hsfc1b, ami}-13.3} \) and their respective controls under non-stress and salt stress conditions (100 mM NaCl). Values are presented as the relative expression level (log2FC) 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 (\( P \leq 0.05 \)).

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FC, fold change; EV, empty-vector control; n.d., not determined.
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 amiRNA 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 lines 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, thesalt 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 amiRNA 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 amiRNA 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 expression 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 expression 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 expression 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].

Sources of funding
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Obata T, Kitamoto HK, Nakamura A, Fukuda A, Tanaka Y. 2007. Rice Shaker potassium channel OsKAT1 confers tolerance to...


