Title:
The heat-stress factor HSFA6b connects ABA signaling and ABA-mediated heat responses

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Running title:
HSFA6b in ABA Signaling and Thermoprotection
ABSTRACT

Heat stress response (HSR) is a conserved mechanism developed to increase the expression of heat shock proteins (HSPs) via a heat shock factor (HSF)-dependent mechanism. Signaling by the stress phytohormone abscisic acid (ABA) is involved in acquired thermotolerance as well. Analysis of Arabidopsis thaliana microarray databases revealed that the expression of HSFA6b, a class-A HSF, extensively increased with salinity, osmotic, and cold stresses, but not heat. Here, we show that HSFA6b plays a pivotal role in the response to ABA and in thermotolerance. Salt-inducible HSFA6b expression was downregulated in ABA insensitive and deficient mutants; however exogenous ABA application restored expression in ABA-deficient, but not insensitive plants. Thus, ABA signaling is required for proper HSFA6b expression. A transcriptional activation assay of protoplasts revealed that ABA treatment and coexpression of an ABA-signaling master effector, ABA-RESPONSIVE ELEMENT-BINDING PROTEIN1, could activate the HSFA6b promoter. In addition, HSFA6b directly bound to the promoter of DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN2A and enhanced its expression. Analysis of ABA responses in seed germination, cotyledon greening, and root growth as well as salt and drought tolerance in HSFA6b-null, overexpression, and dominant-negative mutants revealed that HSFA6b is a positive regulator participating in ABA mediated salt and drought resistance. Thermoprotection tests showed that HSFA6b was required for thermotolerance acquisition. Our study reveals a network in which HSFA6b operates as a downstream regulator of the ABA-mediated stress response and is required for HS resistance. This new ABA-signaling pathway is integrated into the complex HSR network in planta.
When plants are exposed to high temperatures, heat stress (HS) causes cellular damage, leading to severe growth retardation and possible death (Larkindale and Vierling, 2008). Organisms can survive under HS through basal thermotolerance (BT). However, organisms with acquired thermotolerance (AT) can endure lethal HS through metabolic and cellular adjustments induced during an acclimation period at a moderately high but survivable temperature before HS (Vierling, 1991; Larkindale et al., 2005). The HS response (HSR), activated by HS, is the general mechanism preventing stress-caused damage and helping organisms overcome the lethal stress (Krishna, 2004). During HSR, plants accumulate HS proteins (HSPs), which primarily function as molecular chaperones to prevent protein aggregation and facilitate appropriate refolding of the heat-damaged proteins (Parsell and Lindquist, 1993). The expression of HSPs during the HSR is primarily regulated by HS transcription factors (HSFs) that bind to the HS elements (HSEs; nGAAAnnTTCn) located in the promoter of HSFs and HSPs (Rabindran et al., 1993; Schöffl et al., 1998; Nover et al., 2001). When stress is relieved, the HSR is attenuated by excess HSP70 and other proteins that repress the transcriptional activity of HSFs by binding to them and converting HSFs back to the original inactive form. AtHSBP, an HSF-binding protein in Arabidopsis thaliana (Arabidopsis), can reduce the DNA binding capacity of HSFs and function as a negative regulator of the HSR (Hsu et al., 2010).

That multiple HSFs exist among different eukaryotic organisms suggests the importance of the backup and diversification of HSFs. Vertebrates contain 4 HSFs and Drosophila and Caenorhabditis elegans contain 1 each (Nover et al., 2001). Plants have predicted HSF families that are more diverse compared to animals: 24 HSFs and 3 HSF-like genes were identified in tomato, 25 in rice, 30 in maize, and 52 in soybean (Kotak et al., 2004; Scharf et al., 2012). In Arabidopsis, the HSF family is small but more defined, with 21 members: 15 are in class A, 5 in class B, and 1 in class C. The class-A members, HSFAs,
contain the conserved oligomerization, DNA-binding, and AHA-type activation domains, as well as nuclear localization and export signal motifs; and they function as transcription activators. The class B and C HSFs lack a defined activation domain; class B HSFs may serve as co-regulators or repressors of the HSFAs, while class C HSF functions remain unclear (Boscheinen et al., 1997; Czarnecka-Verner et al., 2000; Czarnecka-Verner et al., 2004; Kotak et al., 2007; von Koskull-Doring et al., 2007; Ikeda et al., 2011). The large number of HSFs in higher plants suggests that compared with animals, plants have a more complex and highly regulated system to respond to HS for survival in a broader temperature range (Nover et al., 2001; Kotak et al., 2007).

In Arabidopsis, *HSFA1a* and *HSFA1b* double-knockout (KO) mutants are significantly impaired in early transient mRNA accumulation of *HSPs* (Lohmann et al., 2004). The *HSFA1a/b/d/e* quadruple-KO mutant showed greatly decreased BT and AT, and developmental defects; it was less tolerant to NaCl, mannitol, and H2O2 stress (Liu et al., 2011; Yoshida et al., 2011). Thus, we can conclude that class-A1 HSFs may function as the master regulators of HSR. HS-inducible *HSFA2* is the dominant HSF in thermotolerant cells. Analysis of the *HSFA2*-KO mutant revealed that HSFA2 controls the expression of *HSPs* under prolonged HS and recovery (Busch et al., 2005; Schramm et al., 2006; Charng et al., 2007). In addition, *HSFA2*-KO plants were sensitive to oxidative stress, high light, and anoxia as compared with overexpression (OE) lines, which were more resistant to salt, osmotic, oxidative, and anoxia stresses (Ogawa et al., 2007; Banti et al., 2010). *HSFA3* is induced by HS and drought, and its expression is dependent on the transcription factor DEHYDRATION-RESPONSIVE ELEMENT (DRE; A/GCCGAC core motif)-BINDING PROTEIN2A (DREB2A). DREB2A-KO and OE lines showed reduced and increased thermotolerance respectively, as the result of altered *HSFA3* and *HSP* genes expression (Sakuma et al., 2006; Schramm et al., 2006; Lim et al., 2007; Schramm et al., 2008; Yoshida et al., 2008; Qin et al., 2011). HS-induced DREB2A expression depends on HSFA1s, but HSFA1s could not mediate
the drought responsiveness of DREB2A (Yoshida et al., 2011). Overexpression of HSFA2 and HSFA3 did not affect DREB2A expression, therefore these genes do not function in regulating DREB2A expression (Nishizawa et al., 2006; Yoshida et al., 2008). HSFA1d, HSFA2, and HSFA3 are key factors in regulating oxidative-response gene ASCORBATE PEROXIDASE2 (APX2) expression under diverse stress conditions (Panchuk et al., 2002; Jung et al., 2013). APX2 is highly responsive to HS and plays an important role in the acquisition of thermotolerance (Shi et al., 2001; Panchuk et al., 2002; Larkindale and Huang, 2004; Schramm et al., 2006; Suzuki et al., 2013); a mutant with constitutively higher expression of APX2 showed enhanced tolerance to drought and high abscisic acid (ABA) levels (Rossel et al., 2006).

Other HSFs, HSFA4a and HSFA8, may act as reactive oxygen species (ROS) sensors (Davletova et al., 2005), whereas HSFA5 acts as a specific repressor of HSFA4 isoforms to form a HSFA4–HSFA5 complex that negatively regulates this pathway. The pathway might be connected with controlled cell death triggered by pathogen infection (Baniwal et al., 2007). HSFA7a and HSFA7b are HSR factors (Liu et al., 2011). Moreover, HSFA9 acts as a master regulator of the expression of HSPs during seed development and displays a synergism between HSFA9 and ABA-responsive transcription factor ABA INSENSITIVE3 (ABI3) (Kotak et al., 2007). HSFA4c was found to be involved in root circumnutation, gravitropic response, and hormonal control of differentiation (Fortunati et al., 2008).

Recently, HSFB2a was found to be involved in gametophyte development and HSFB2b was found to be important for accurate circadian rhythms following elevated temperature and salt treatment (Kolmos et al., 2014; Wunderlich et al., 2014). Consequently, a crosstalk exists between HS and other abiotic stress-signaling cascades mediated by HSFs (Kotak et al., 2007). HSFA6a and HSFA6b transcript levels are particularly expressed in response to salt, osmotic, and cold stress (von Koskull-Doring et al., 2007; Hwang et al., 2014). Overexpression of HSFA6b, but not HSFA6a, could regulate the transcription of DREB2A,
which suggests non-redundant functions (Yoshida et al., 2011); however, the molecular mechanism of *HSFA6b* still needs to be elucidated upon.

With regards to osmotic stress signaling due to salinity, drought, and cold stress, endogenous ABA levels increase, and ABA responsive transcription acts through an ABA-RESPONSIVE ELEMENT (ABRE; PyACGTGG/TC)-BINDING PROTEINS/FACTORS (AREBs/ABFs) via multiple ABREs or combinations of ABREs with coupling elements (Busk and Pages, 1998; Fujita et al., 2011; Nakashima and Yamaguchi-Shinozaki, 2013). ABA deficient and insensitive mutants are sensitive to HS, whereas *AREB/ABF-OE* plants show enhanced thermotolerance (Larkindale and Knight, 2002; Kim et al., 2004; Larkindale et al., 2005; Suzuki et al., 2016). Thus, ABA plays a role in the thermotolerance response.

Analysis of an Arabidopsis microarray database (AtGenExpress consortium; https://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp) showed *HSFA6b* had the highest expression induced by salinity, osmotic, and cold stress among all *HSFs*, but *HSFA6a* had little response to the stresses; notably, both genes were not significantly upregulated by HS. Transcriptome analysis of the Arabidopsis *AREB1/AREB2/ABF3* triple-KO mutant showed strongly reduced expression of *HSFA6a* and *HSFA6b* under salt stress, dehydration stress, and ABA treatment (Yoshida et al., 2010).

The HSR depends on a complex regulatory network involving *HSF* and *HSP* families, and *HSFs* and *HSPs* respond to various abiotic stresses besides HS (Swindell et al., 2007). However, the role of the 21 Arabidopsis *HSFs* under HS and non-HS conditions is not well understood, and the types of stress that interact most strongly with HSF and HSR pathways remain unclear. In this study, we investigated the function of *HSFA6b* genetically with T-DNA insertional KO and 35S-driven OE mutants. We used a dominant-negative HSFA6b fused to an ETHYLENE-RESPONSIVE ELEMENT-BINDING FACTOR-associated Amphiphilic Repression (EAR)-motif repression domain SRDX (Hiratsu et al., 2003) to diminish the activities of endogenous and functionally redundant factors. The results, including our
microarray data, revealed that HSFA6b acts as a positive regulator downstream of ABA signaling, mediates salinity and drought-stress responses, and is required for establishing thermotolerance. Thus, HSFA6b functions in ABA signaling cascades in the complex regulatory networks of the HSR.
RESULTS

Arabidopsis Class-A HS Transcription Factor HSFA6b Is Induced by Salt, Osmotic, and ABA but not Heat Stress

Analysis of the AtGenExpress database revealed the expression of 21 Arabidopsis HSFs responding to heat, salt, osmotic, drought, and cold stress (Supplemental Figure S1A and B): among the 15 class-A HSF genes, HSFA6b expression was extensively induced by salt (NaCl), osmotic (mannitol), and cold, but not HS. We analyzed the gene expression of HSFA6b and its potential paralog HSFA6a in response to HS, NaCl, and mannitol as well as the osmotic stress signal mediator ABA using RT-PCR (Figure 1) or qRT-PCR (Supplemental Figure S1C). HSFA6b expression was markedly induced by NaCl, mannitol, and ABA in a dose-dependent manner, but not with HS; this was supported by the microarray findings. The expression of HSFA6b occurs earlier and is stronger than that of HSFA6a for all treatments.

Nuclear and Cytosol Localization of HSFA6b

The protein structure of HSFA6b shares conserved HSF signatures with HSFA1a and HSFA2 (Nover et al., 2001), as it harbors the predicted nuclear localization and export signal peptides (Supplemental Figure S2). HSFA6b was fused to either the N- or C-terminal yellow fluorescent protein (YFP) reporter and transiently expressed in Arabidopsis protoplasts for subcellular localization analysis (Figure 2A). It was localized to the nucleus and cytosol, with partial but not complete translocation to the nucleus. Both 2-h treatments of 10 μM ABA and 20 nM Leptomycin B only slightly enhanced the nuclear enrichment. Meanwhile, HSFA6a, with a predicted nuclear importin but no exportin, was specifically localized to the nucleus (Figure 2B).

HSFs form homo- or hetero-trimers, resulting in altered nuclear localization, and then bind to their own promoters and/or to promoters of other HSFs genes, enhancing or
suppressing their promoter transcription (Miller and Mittler, 2006). We used a co-immunoprecipitation (Co-IP) and bimolecular fluorescence complementation (BiFC)
assay to investigate the interaction of HSFA6b with itself and HSFA6a, as well as HS related HSFs, such as HSFA (1a, 1b, 2, 3, 7a, 7b) and HSFB (1, 2a, 2b) in Arabidopsis protoplasts (Supplemental Figure S3A and B). HSFA6b neither interacts with itself, even with the 10-μM ABA treatment, nor interacts with HSFA6a. It interacts with the well-known HSFA1a, HSFA1b, and HSFA2 in the nucleus.

Expression of HSFA6b Was Impaired in ABA Deficient and Insensitive Mutants under Salt Treatment
HSFA6b was the most NaCl responsive gene among all the HSFs (Supplemental Figure S1), which implies a potential function in salt-stress signaling. We investigated the expression levels of HSFA6b and AREB1 in ABA deficient (aba2-1 and aba3-1) and insensitive (abi1-1 and abi2-1) mutants under a 200-mM NaCl or 20-µM ABA treatment (Figure 3). HSFA6b expression was greatly impaired in ABA-deficient mutants under NaCl treatment; however ABA treatment notably restored expression (Figure 3A). HSFA6b expression was likewise equally impaired in ABA-insensitive mutants under both NaCl and ABA treatment (Figure 3B). The AREB1 expression profile was similar to that of HSFA6b (Figure 3C), and NaCl and drought responsive marker genes RESPONSIVE TO DEHYDRATION29A (RD29A) and RD29B were used as references (Figure 34). Therefore, we suggest that NaCl induced HSFA6b gene expression could be downstream of ABA perception and regulated by the ABA signal.

ABA Treatment and AREB1/ABF2 Co-expression Enhanced HSFA6b Promoter Activity

To confirm that HSFA6b expression was related to ABA signaling, we analyzed an upstream 2-kb potential promoter region of HSFA6b using Plant Promoter Analysis Navigator (PlantPAN 2.0; http://plantpan2.itps.ncku.edu.tw; Chow et al., 2016) and found that the promoter contained ABREs (Figure 4A). After fusing the 1.7-kb promoter region to the reporter gene β-glucuronidase (GUS), the protoplast transactivation assay confirmed that a 10-μM ABA treatment significantly enhanced the transcriptional activity of HSFA6b (Figure 4B). However, a 1.0-kb upstream region of HSFA6b without ABREs, used as a reference, did not respond to ABA treatment. Coexpression with effector AREB1 (i.e., ABF2), an ABRE-dependent master transcription regulator, enhanced the transcriptional activity of HSFA6b, and in the presence of both ABA and AREB1 greatly activated HSFA6b promoter activity (Figure 4C). Thus, the transcription of HSFA6b could be regulated in an ABA-dependent AREB/ABF-ABRE manner.
HSFA6b Mutants Were Screened and Confirmed

To elucidate the function of HSFA6b required for the ABA response, HSFA6b null mutant lines a6b-1 (Col ecotype background) and a6b-2 (Ler ecotype background) were screened and confirmed by RT-PCR (Supplemental Figure S4A). As a reference, the HSFA6a homozygous T-DNA insertion line a6a (Col ecotype background) was screened, and RT-PCR revealed that it is a null mutant (Supplemental Figure S4B).

For HSFA6b-OE lines, a hemagglutinin (HA) tag was fused to the N terminus of HSFA6b, with expression driven by the CaMV 35S promoter (Supplemental Figure S4C). For HSFA6b dominant-negative effect (HSFA6b-RD) lines, an EAR-motif repression domain
SRDX (Hiratsu et al., 2003) was fused to the C-terminus of HA-HSFA6b, with expression driven by the 35S promoter (Supplemental Figure S4D). RT-PCR and immunoblotting results confirmed the overexpression of transgenes in the transgenic plants $HSFA6b$-OE-5-6 ($OE5-6$) and $HSFA6b$-RD-3-6 ($RD3-6$) (Supplemental Figures S4C, D and S5A). These transgenic lines were generated in the ecotype Col background. Transgenic lines with the different expression levels of transgenes $HSFA6b$-OE and $HSFA6b$-RD showed differing phenotype response to 0.75-µM ABA treatment.
During early seedling growth, the overexpression of HSFA6b-OE (gain-of-function transgene) showed a higher response to ABA and overexpression of HSFA6b-RD (dominant-negative effect transgene) showed reduced sensitivity to ABA. We used the representative lines OE5-6, OE13-6 and OE12-6, as well as RD3-5, RD15-3 and RD23-1 for the following experiments.

**HSFA6b Is Required for Proper Responses to Salt, Osmotic, and ABA Stress**

The HSFA6b-mutant lines a6b-1, a6b-2, OE5-6, and RD3-5 as well as the HSFA6a mutant a6a (used as a reference) were treated with NaCl, mannitol, and ABA in order to mimic a downstream mediator of salt, osmotic, and drought stress. ABA inhibited seed germination, cotyledon greening, and root growth were then analyzed (Kreps et al., 2002; Fujii et al., 2007).

Seed germination did not differ between the a6b-1, a6b-2, a6a mutants, and the wild-type (WT) Col and Ler for 1-µM ABA and 200-mM NaCl treatments (Figure 5A). Seed germination rate was markedly reduced in OE5-6 with ABA treatment at days 2 and 3, but was not affected by NaCl treatment; under both treatments, seed germination did not differ between RD3-5 and the Col.

Seeds were planted in growth medium agar plates containing 0.5, 0.75, or 1-µM ABA; post-germination seedling growth for OE5-6 was more sensitive (ABA, 0.5 µM), while growth of RD3-5 was more resistant (ABA, 0.75 µM) when compared to the Col (Figures 5B and 5S). As expected, ABA treatment had no effect on early seedling growth in an ABA-insensitive mutant, abi4-1 (Col ecotype background; an ABA-responsive marker in seeds) that was used as a reference. Mutants of a6b-1 and a6b-2 showed a similar phenotype as the Col and Ler, respectively.

With 150-mM NaCl treatment, the cotyledon greening ratio was lower and higher for OE (5-6 and 13-6) and RD (3-5 and 15-3) lines respectively when compared with the Col,
while no significant difference was observed for $a6b-1$ (Figure 5C), which agreed with the ABA treatment findings (Figure 5B). The cotyledon greening ratio for $a6a$ and the Col was
The relative root elongation rate did not differ between \( a6b-1 \) and the Col under NaCl and mannitol treatments, but \( a6b-1 \) was more resistant to ABA treatment (Figure 6A). However, root elongation was significantly affected in \( RD \) lines (Figure 6B and C): with NaCl, mannitol, and ABA treatments, \( RD \) lines were more resistant growth inhibition. In addition, the ABA-mediated root growth phenotypes of \( a6a \) and the Col were similar. The two \( OE5-6 \) and \( 13-6 \) lines and two \( RD3-5 \) and \( 15-3 \) lines exhibited consistent phenotypic responses to the abiotic stresses.

Thus, misregulation and dysfunction of \( HSFA6b \) interfered in the proper response to salt, osmotic, and ABA treatments, which implies that \( HSFA6b \) contributes to ABA-mediated stress responses.

**Misregulated ABA Biosynthesis and Responsive Gene Expression, in Response to Salt and ABA, in \( HSFA6b \) Mutants**

To confirm that \( HSFA6b \) is involved in ABA signaling, we investigated the effect of NaCl and ABA treatment in \( HSFA6b \) mutants by looking at the regulation of ABA biosynthesis marker genes *Nine-CIS-EPOXYCAROTENOID DIOXYGENASE3* (\( NCED3 \)) and *ARABIDOPSIS ALDEHYDE OXIDASE3* (\( AAO3 \)) (Barrero et al., 2006), as well as \( RD29A \) and \( RD29B \), which are involved in the ABA independent and dependent pathways, respectively (Shinozaki and Yamaguchi-Shinozaki, 2000; Huang et al., 2012) (Supplemental Figure S6).

With NaCl treatment, the expression of \( NCED3 \) was higher than that of \( AAO3 \) in Col plants (Supplemental Figure S6A), which concurs with previous studies (Barrero et al., 2006). \( NCED3 \) expression was suppressed in \( a6b-1 \) and \( RD3-5 \) following NaCl treatment, as compared with the Col, but the expression of \( AAO3 \) was not greatly affected in \( HSFA6b \) mutants. The expression of \( RD29A \) was not markedly affected in \( HSFA6b \) mutants following NaCl and ABA treatments, as compared with the Col (Supplemental Figure S6B). Notably,
the expression of RD29B was increased in OE5-6 and decreased in RD3-5. In addition, 
DREB2A expression was significantly decreased in RD3-5, but the expression profile of 
AREB1 was not affected in mutants. However, the expression of RD22 (Supplemental 
Figure S6B), a drought-responsive marker gene mediated by the transcription activators 
MYB/MYC via MYB/MYC-responsive elements (MYBRE/MYCRE) in an ABA-dependent 
manner (Abe et al., 2003), was unaffected in HSFA6b mutants. Therefore, HSFA6b might 
not contribute to ABA-independent DREB1/CBF-DRE and ABA-dependent 
MYB/MYC-MYBRE/MYCRE regulation.

**HSFA6b Participates in Salt and ABA mediated HSP genes Expression**

The induction and accumulation of HSPs is essential for establishing thermotolerance, 
and HSP gene transcription can also be induced by other abiotic stresses such as salt and
drought (Supplemental Figure S7). Intriguingly, the overexpression of DREB2A and DREB2C greatly induced HSP18.1-CI, HSP26.5-MII, and HSP70 expression, which enhanced heat and drought tolerance (Sakuma et al., 2006; Lim et al., 2007).

With 1-h 37°C HS treatment (Supplemental Figure S8A), the expression of HSPs was upregulated, with no difference in HSFA6b mutants when compared with the Col; in addition, the attenuation of HSP expression remained unaffected during recovery from HS (Supplemental Figure S9A). The expression of HSFs was similar to that of HSPs in response to HS (Supplemental Figure S8A).

Under 150-mM NaCl and 20-μM ABA treatments (Supplemental Figure S8B and C), the expression of HSPs was markedly suppressed in RD3-5 but effectively upregulated in OE5-6, while the expressions of HSP18.1-CI and HSP26.5-MII were both greatly enhanced. However, the treatments had no major effect on HSP expression in OE5-6, whereas those of HSFA6a and class-B HSF genes HSFB1, HSFB2a, HSFB2b, and HSFB4 were downregulated in RD3-5.

Meanwhile, the expression of oxidative stress-responsive genes COPPER-ZINC SUPEROXIDE DISMUTASE (CuZnSOD; CSD1, CSD2, CSD3), ASCORBATE PEROXIDASE (APX1, APX2) and CATALASE (CAT1), key components of the ROS network (Davletova et al., 2005), were largely unaffected in HSFA6b mutants in response to HS, NaCl, and ABA treatments (Supplemental Figure S9B). Notably, the expression of APX2 was significantly upregulated and suppressed in OE5-6 and RD3-5, respectively, in response to HS (Supplemental Figure S9B), NaCl, and ABA (Supplemental S10A). As well, superoxide dismutase (SOD) activity and H₂O₂ accumulation were not affected in HSFA6b mutants under the treatments (Supplemental Figure S9C and D).

HSFA6b Activated Transcriptional Activity of HSP18.1-CI, DREB2A and APX2 Promoters

In a6b-1 mutant protoplasts, HSFA6b overexpression significantly activated the
transcription of HSP18.1-CI and DREB2A promoters (Figure 7A and B), and coexpression of AREB1 had an additive effect on two promoter’s expression. The interaction of HSFA6b with the HS element (HSE) sequence in the DREB2A promoter (Kim et al., 2011) was confirmed by chromatin immunoprecipitation (ChIP) assay with anti-HA antibodies in OE5-6 plants (Figure 7C). NaCl and dehydration treatments significantly enhanced the interaction. Thus, HSFA6b bound to the HSE sequence of the DREB2A promoter effectively enhanced its expression in response to the stresses.

The DREB2A activates HSFA3 expression, and HSFA3 in turn regulates the expression of HS- and oxidative-response genes, including APX2 (Sakuma et al., 2006; Yokotani et al., 2008; Jung et al., 2013). In HSFA3 defective protoplasts (Supplemental Figure S11A), HSFA6b overexpression significantly activated the transcription of HSP18.1-CI and APX2
promoters (Figure 7D; Supplemental Figure S10B), and HSFA3 coexpression had an additively effect on their expression. In HSFA6a and HSFA6b double-KO protoplasts (Supplemental Figure S11B), we confirmed that HSFA6b functions independently of HSFA6a on HSP18.1-CI and APX2 promoter activation (Figure 7E; Supplemental Figure S10C).

**HSFA6b Positively Regulated Thermotolerance**

Basal thermotolerance (BT) and acquired thermotolerance (AT) were analyzed with a well-characterized HS-sensitive reference, the HSP101-mutant line (hsp101; At1g74310, SALK_066374) (Hong and Vierling, 2000). OE5-6 and OE13-6 plants displayed significantly increased BT and AT as compared with Col plants, while a6b-1, RD3-5, and RD15-3 plants showed significantly reduced BT and AT (Figure 8A). The transgenic lines of OE12-6 and RD23-1 (transgene expression similar to that of the Col; Supplemental Figure S5A), used as references, showed similar thermotolerant phenotypes as the Col. RD3-5 also showed an HS-sensitive phenotype to long-term AT (LAT; Supplemental Figure S12), with 37°C HS for 1 h, then 22°C 2-day long-term recovery followed by 44°C 155-min HS (Charng et al., 2006).

We used a 12-h ABA pretreatment before testing ABA mediated BT and AT, and the 44°C HS period was extended to 35 and 205 min, respectively (Figure 8B). Col plants showed ABA enhanced survival of BT and AT as expected; however, RD3-5 plants still displayed significantly reduced BT and AT in comparison. Thus, HSFA6b was required for ABA mediated HSR. BT, but not AT was mildly affected in a6a as compared with Col plants (Supplemental Figure S13).

**HSFA6b Overexpression Conferred Drought and Salt Tolerance**

We grew 200 seedlings in plates (9-cm diameter with 20 ml medium) for 24 days,
then the growth medium was kept dehydrated (Figure 9A; left), or seedlings were grown in high soil salinity stress by watering 2-week-old soil grown plants with 300-mM NaCl
solution over the course of 5 weeks (Figure 9B and C). OE5-6 plants stayed green, but RD3-5 plants showed a phenotype of anthocyanin accumulation as compared with Col
plants. *OE5-6* plants showed higher chlorophyll/anthocyanin ratio while the *RD3-5* plants displayed a lower ratio when compared with the Col; the chlorophyll a/b ratio was not
affected (Figure 9A; right). Although no phenotype appeared at 2 weeks (Figure 9B), the salt-responsive phenotype was observed at 5 weeks (Figure 9C): OE5-6 showed the
salt-resistant phenotype (the survival rate was 38.8 ± 6.6%), but Col, RD3-5, and a6b-1 plants did not survive (0% survival).

Transcription Profiling of HSFA6b-OE5-6 and HSFA6b-RD3-5 Transgenic Plants

We performed a genome-wide expression analysis with a microarray assay to dissect how HSFA6b regulates thermotolerance and confers drought and salt tolerance. We examined differentially expressed genes (DEGs) with levels changed > 6-fold at \( p < 0.10 \) in transgenic plants OE5-6 and RD3-5 after 150 mM NaCl or 37°C HS treatment, as compared with the Col control treatment (Supplementary Table S2). The Venn diagram showed 246 NaCl and 197 HS induced DEGs (Supplementary Figure S14A).

These DEGs were used for gene ontology (GO) enrichment analysis. The OE5-6 DEGs were associated with GO terms of biological process and showed a more complex network than those of RD3-5 under NaCl stress did (Supplementary Figure S15; Supplementary Table S3A). The overrepresented GO terms were related to response to abiotic stimulus of “heat, water, and salt stress” and linked with the response to “ABA stimulus”. The enriched GO item “response to jasmonic acid (JA) stimulus” was also significantly overrepresented for OE5-6 under NaCl treatment. The results agreed with our findings that HSFA6b is downstream of ABA signaling and regulates genes enriched in abiotic stress-response networks including heat, drought, and salt stresses; in addition, HSFA6b might be involved in JA signaling. However, under HS, the networks of enriched GO terms were similar between OE5-6 and RD3-5 (Supplementary Figure S16; Supplementary Table S3B). GO terms of “protein folding, oxidative phosphorylation, and respiratory electron transport chain” were overrepresented.

We highlighted the top 39 DEGs with > 30-fold change in expression at \( p < 0.05 \) in OE5-6 and RD3-5 under NaCl or HS treatment as compared with the Col control treatment (Figure 10A; Supplementary Table S4). We also highlighted the top 25 DEGs of HSFA6b HS
response regulon in RD3-5 with an expression change of < 1.5-fold compared with the Col HS (Figure 10B). The HSFA6b regulon controlled the expression of important transcriptional regulators such as DREB2A, MUTIPROTEIN BRIDGING FACTOR1c (MBF1c, regulates HS-responsive regulon; Suzuki et al., 2011) and three HSFs (A7a, B1, B2a), as well
as HSPs, zinc finger proteins and enzymatic genes. The HSFA6b HS-regulon and NaCl-regulon (Supplementary Figure S14B) showed overlap co-regulation in part with DREB2A and HSFA3 HS-regulons (Sakuma et al., 2006; Yoshida et al., 2008).

This data supports our findings that HSFA6b regulates DREB2A to turn on particular downstream HS-responsive genes via the ABA signal and also might connect with the MBF1c HS-response regulon (Suzuki et al., 2011).

Thus, HSFA6b, with ABA-dependent and HS-independent expression, has a central role in regulating ABA-mediated HSR and oxidative-stress response gene expression and increasing the flexibility to modulate the compatibility of HSR regulatory networks.
DISCUSSION

Some multiple perceptions and signaling pathways in response to stress are specific, while others merge at various steps; these together confer cross-protection against different environmental stresses. Abiotic stresses, especially salinity, drought, and cold, all lead to a similar physiological effect, so that signaling is transmitted through the ABA mediated pathway that involves the expression of stress specific and/or stress related genes to relieve the stress (Shinozaki and Yamaguchi-Shinozaki, 2000; Huang et al., 2012). Studies have revealed that ABA plays an important role in the acclimation of plants to a combination of salt and HS (Suzuki et al., 2016). The \textit{aba1} and \textit{abi1} mutants were more susceptible to the salt and HS combination than the wild type. In addition, the interactions of ABA with ROS signaling plays a key intermediate in the regulation of systemic acquired acclimation of plants to HS (Suzuki et al., 2013; Mittler and Blumwald, 2015). We confirmed that the transcription factor HSFA6b, whose expression depends on the ABA signal is required for ABA–mediated HS resistance.

**HSFA6b, a Nuclear Factor, Responds to ABA Signaling via the AREB/ABF-ABRE Regulon**

Unlike the HSR marker genes \textit{HSP18.1-CI} and \textit{HSFA2} which contain the perfect/active HSE motif (nTTCnnGAAAnnTTCn/nGAAAnnTTCn), \textit{HSFA6a} and \textit{HSFA6b} potential promoter regions only contain one aaAAtcTTCt and aGAAggaTCT sequence (Nover et al., 2001), respectively. This supports the expression data showing that neither \textit{HSFA6a} nor \textit{HSFA6b} respond to HS (Figure 1; Supplemental Figure S1). HSFA6b is a nuclear-localized factor (Figure 2), and during ABA or Leptomycin B treatment the nuclear localization of the YFP-tagged HSHA6b showed partial but not completed translocation to the nucleus, which implies that a limited amount of nuclear-localized HSFA6b is required for its function.

As promoters of ABA-responsive genes, ABREs serve as binding motifs for AREBs/ABFs. Reduced expression of \textit{HSFA6b} in the \textit{areb1}/\textit{areb2}/\textit{abf3} triple mutant suggested that
HSFA6b might function in the ABA-dependent pathway under salt and dehydration (Yoshida et al., 2010). We demonstrated that regulation of HSFA6b transcription could depend on ABA (Figure 3), and the promoter translational activity assay revealed significantly induced transcription of HSFA6b by ABA and the coexpressed AREB1 (Figure 4). Thus, HSFA6b expression is mediated through an AREB/ABF-ABRE pathway, a novel signal pathway regulating HSF gene expression independent of HS.

Phylogenetic analysis showed that HSFA6a is the closest member to HSFA6b (Scharf et al., 2012), and both were induced under abiotic stress and ABA treatments (Figure 1). The AREB/ABF factors positively regulated HSFA6a transcription, which is required for salt and drought stresses (Hwang et al., 2014). However, transactivation assays have shown that HSFA1s function as the main positive regulators in HS-responsive gene expression via the HSE in the DREB2A promoter; also, coexpression of HSFA6b but not HSFA6a resulted in higher reporter gene activity than the control (Yoshida et al., 2011). We confirmed that HSFA6b expression occurs earlier and stronger than that of HSFA6a under abiotic stress and ABA treatments (Figure 1 and Supplemental Figure 1), as well as that HSFA6b mutants affect HSFA6a expression (Supplemental Figures S8 and S11). In addition, HSFA6a and HSFA6b expression showed a differential expression pattern in the hsf1a/b/d/e quadruple mutant under HS stress (Supplemental Figure S17) (Liu and Charng, 2013): the results imply that HSFA1 (a, b, d) are negative regulators on HSFA6b expression but not of HSFA6a. We showed a marked difference in a6b-1 root growth in response to ABA and in AT testing as compared with a6a (Figure 6A; Supplemental Figure S13B), and HSFA6b functioned independently of HSFA6a on HSP18.1-CI and APX2 promoter activation (Figure 7E; Supplemental Figure S10C). Therefore, HSFA6b has an independent specific function in HS and oxidative stresses from HSFA6a.
**Thermotolerance**

*HSFA6b* expression was highly induced by NaCl, mannitol, and ABA treatments (Figure 1). The *a6b-1* was insensitive to ABA in terms of primary root growth as compared with the WT, but displayed no significant differences under salt or drought/osmotic stress (Figure 6A). These results suggest that contribution of *HSFA6b* may be small in response to these stresses, or that potential functional redundancy of *HSFA6* family proteins or other HSFs may compensate for the role of HSFA6b, such as HSFA1s or HSFA2 (Ogawa et al., 2007; Liu et al., 2011; Liu and Charng, 2013). Mutants of RD3-5 displayed decreased ABA-inhibition sensitivity in seed germination, cotyledon greening, and root growth phenotypes, whereas *OE5-6* showed enhanced ABA inhibition sensitivity (Figures 5 and 6). These results suggest that HSFA6b has an impact on the ABA signaling pathway, and HSFA6b plays a positive regulator in ABA-mediated stress responses. *HSFA6b* is crucial; however, we could not determine whether these phenotypes were caused by the ectopic expression of *HSFA6b* or its dominant negative form.

Plants obtain thermotolerance, regulated by an HSF network, by inducing and accumulating HSPs. Overexpression of Arabidopsis *HSFA2* and rice *HSFA2e* conferred increased thermotolerance and salt stress tolerance (Ogawa et al., 2007; Yokotani et al., 2008). ABA plays a role in thermotolerance response (Larkindale and Knight, 2002), and ABA deficient and insensitive mutants are sensitive to HS (Larkindale et al., 2005; Suzuki et al., 2016), with thermotolerance enhanced in *AREB/ABF-OE* plants (Kim et al., 2004). Thus, HSR confers tolerance to HS and other abiotic stresses such as salt, drought, and cold stress due to crosstalk among stress signaling pathways. We also confirmed that the BT, AT, and LAT were severely affected in *HSFA6b* mutants: the thermotolerance was significantly increased in *OE5-6* and 13-6; consistently, RD3-5 and 13-6 showed greatly impaired establishment of thermotolerance, even with ABA pretreatment (Figure 8; Supplemental Figure S12). Again, HSFA6b, a pivotal positive regulator, is required for ABA-mediated HSR.
With loss of function of the major HS-responsive genes HSFA1s and HSFA2, the expression of HSP genes was reduced and thermotolerance lost. Induction and attenuation of HSR genes were not markedly affected in HSFA6b mutants in response to HS (Supplemental Figures S8A and S9A). In addition, expression of oxidative stress-responsive marker genes and activity of ROS detoxification enzymes (SODs) were properly responded in HSFA6b mutants in response to HS (Supplemental Figure S9B and C). However, the NaCl and ABA induced HSR gene expressions were enhanced in OE5-6 but largely impaired in RD3-5. Intriguingly, OE5-6 showed selective upregulation of HSP18.1-CI and HSP26.5-MII expression, in addition to APX2 (Supplemental Figures S8B, C and S9). The results confirmed that ABA-mediated HSFA6b expression is required for proper stress-related gene expression under both salt and ABA treatments.

Photosynthetic pigment content and ratios (i.e., chlorophyll/anthocyanin ratio) are indicators of stress detection and tolerance (Chalker-Scott, 1999); the chlorophyll/anthocyanin ratio is decreased in stressed plants. OE5-6 and RD3-5 mutants retained a higher and lower ratio of chlorophyll/anthocyanin respectively, and HSFA6b overexpression conferred salt tolerance (Figure 9). Thus, HSFA6b is required for drought and salt stress response.

Crosstalk of HSFA6 and DREB2A in HSR Genes Expression and Regulatory Networks

The physiological drought/osmotic stress responsive regulons largely depend on 2 major classes of cis-acting elements, ABRE and DRE. ABRE can perceive ABA-dependent signals, whereas DRE is involved in an ABA-independent pathway. DREB2A, a downstream regulator of both osmotic and HS stress, positively controls osmotic and HS inducible gene expression (Yoshida et al., 2011). Nearly 1000 genes were downregulated after 1-h HS treatment in the Arabidopsis hsfa1a/b/d/e quadruple mutant, with 658 genes HS-upregulated in the WT and 81 genes HS-upregulated in transgenic plants.
overexpressing a constitutive active form of DREB2A (DREB2A-CA). In the top 100
downregulated genes in the hsfa1a/b/d/e mutant, HSE motifs were highly enriched as
compared with the whole genome, and DRE motifs were also enriched in these promoters
(Yoshida et al., 2011). Of these HS-upregulated genes in the WT, 90 contained DRE instead
of the HSE motif, the former of which is suggested to be regulated by DREB2A,
downstream of the HSFA1s. For example, DREB2A harbors one HSE motif and one ABRE-CE
(coupling element) module, but lacks the DRE motif, and its expression depends on HSFA1s
under HS, but not salt or drought stress. Notably, the ABA-mediated HSFA6b positively
regulates the expression of DREB2A (Yoshida et al., 2011). HSFA3 harbors one DRE but
lacks HSE, and its expression depends on DREB2A; thus, overexpression of DREB2A-CA
upregulates HSFA3 but has no effect on HSFA6b expression (Yoshida et al., 2011). HSFA3 in
turn upregulates the expression of HSP18.1-CI (1 DRE and 2 HSEs), HSP26.5-MII (1 DRE and
no HSE), and HSP70 (1 DRE and 1 HSE), which have been shown to enhance
thermotolerance and drought tolerance significantly (Sakuma et al., 2006).

With ABA treatment, the transcription factor AREBs/ABFs that activate DREB2A
transcription depend on ABRE, resulting in only a modest accumulation of DREB2A
transcript, as compared with osmotic stress. However, DREB2A expression in response to
osmotic stress was largely impaired in ABA insensitive and deficient mutants, which
highlights that in addition to ABA independence, the ABA-dependent cascade plays a
positive role in the osmotic stress-responsive expression of DREB2A (Kim et al., 2011).
Notably, a short term ABA accumulation is following a 10-min HS application as reported
by Suzuki et al. (2013), and then the AREBs/ABFs, DREB2A, HSP18.1-CI and APX2 genes
expression was upregulated in 15 min, 30 to 60 min and 1 h after the HS, respectively
(Supplemental Figure S1B). Our ChIP and transactivation assay showed that HSFA6b
directly bound to the DREB2A promoter, then, when combined with AREB1 expression,
additively enhanced DREB2A transcriptional activity in response to NaCl and dehydration
**Figure 7B and C.** HSFA6b overexpression significantly activated the transcription of HSP18.1-CI and APX2 promoters, and coexpression of AREB1 or HSFA3 had an additive effect on their expression (Figure 7A and D; Supplemental Figure S10B). This result appears to support an HSFA6b and AREB/ABF crosstalk with DREB2A transcription connected with ABA-induced thermotolerance. The existence of such multiple and complex regulons regulating AREB/ABF and DREB2A expression may allow plants to precisely and rapidly respond to abiotic stress.

The HSR genes are differentially regulated by the HSFA1s and HSFA2: HSFA1s are involved in thermotolerance and ectopic expression of HSFA2 in hsfa1a/b/d/e quadruple mutant complemented tolerance to different HS regimes, and to hydrogen peroxide, but not to salt and osmotic stresses (Liu and Charng, 2013). Our Co-IP and BiFC assays confirmed the interaction of HSFA6b with HSFA1a, HSFA1b, and HSFA2 in the nucleus (Supplemental Figure S3). Thus, the HSFA6b might cooperate/compete with others HSF such as HSFA1s and/or HSFA2 in hetero-oligomeric complexes for its functions. The HSFA6b might have effect on DREB2A, HSFA3, and downstreamed genes expression (Figure 10) plays a role in ABA-mediated thermotolerance.

The drought inducible genes were upregulated by salt stress, but a few were cold inducible. The activators DREB1/CFB and DREB2 function separately in the cold, drought, and salt signaling pathways. Transcription of the cold-induced marker gene RD29A (1 ABRE and 3 DREs) was through the DREB1/CFB-DRE/CRT regulon in response to low temperature, and was induced under salt, drought, and ABA via ABRE and DRE (as a coupling element of ABRE) motifs cooperating mainly with AREB/ABF and DREB2A (Narusaka et al., 2003). Here, we showed that in HSFA6b mutants, RD29A expression was not affected by either NaCl or ABA treatments (Supplemental Figure S6), so HSFA6b might not contribute to the DREB1/CFB-DRE regulon. Induced expression of RD29B (4 ABREs and 1 DRE), which mainly depends on an ABA pathway, was largely affected in the HSFA6b mutant (Supplemental
However, the expression of the ABA-dependent/drought-responsive marker gene *RD22*, depending on transcription factors MYB2 and MYC2 (Abe et al., 2003), was largely unaffected in *HSFA6b* mutants (Supplemental Figure S6B). *MYB2* and *MYC2* expression was also enhanced by jasmonic acid, which further indicates that abiotic and biotic stress transduction pathways are interconnected. In summary, crosstalk exists in different regulons, such as ABA dependent or independent regulons, and in different stress responsive gene expressions (Qin et al., 2011; Huang et al., 2012).

The top 39 DEGs of 197 DEGs (Supplemental Figure S14) in *HSFA6b* overexpression (OE5-6) and dominant-negative (RD3-5) mutant plants in response to HS showed increased and reduced expression of transcription activators MBF1c, *HSFA7a*, *HSFB1*, and DREB2A under salt stress, respectively, as well as specific protein and enzyme gene expression, such as previously studied in GOLS1, PDX1.2, SR45a and EF1B (Figure 10A). The HS-response regulon has been showed to be involved with the MBF1c HS-response regulon, in addition to the well-known network of HSFs. In addition, the top 25 DEGs of 197 DEGs of *HSFA6b* HS response regulon in RD3-5 showed an expression change of < 1.5-fold when compared with Col HS treatment (Figure 10B), including HSP70, *HSFB2A*, temperature-induced lipocalins (TILs), and enzymatic genes. TILs are involved in the protection from abiotic stresses such as, heat, oxidative and salt stresses (Chi et al., 2009; Levesque-Tremblay et al., 2009; Abo-Ogiala et al., 2013). *HSFB2a* has been reported to be required for gametophyte development (Wunderlich et al., 2014), and we proposed that it might also be required for thermotolerance acquisition. Thus, our data supports that salt and ABA-responsive *HSFA6b* (Figures 1, 3, 4, 7B and 7C) triggers a downstream HS-responsive gene expression that leads to heat thermotolerance.

In conclusion, our data indicates that *HSFA6b* functions are tightly involved in ABA mediated regulons. HSFA6b and AREB1 activate DREB2A expression in concert to mediate a cross talk between ABA dependent and HSR gene expression. A simplified working model.
is shown Figure 11, which proposes a new pathway merging with the complex ABA- and HS-response networks in planta.
MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis HSFA6a-, HSFA6b- and HSFA3-KO lines, a6a (SALK_045608; Columbia ecotype), a6b-1 (GK-513A02; Columbia ecotype), a6b-2 (GT_3_7561; Landsberg erecta ecotype), and hsfa3 (SALK_01117; Columbia ecotype), were obtained from the Arabidopsis Biological Research Center or the Nottingham Arabidopsis Stock Center. Transgenic plants were created in the Columbia (Col) ecotype background by the floral-dip method (Clough and Bent, 1998) and were selected by spraying with 0.4% BASTA herbicide. Seedlings were grown at 22 to 24°C with 16-h light at 60 to 100 μmol m⁻² s⁻¹.

RNA Preparation, cDNA Synthesis and Real-Time Quantitative PCR (qRT-PCR)

Total RNA was prepared with use of TRIZOL reagent (Invitrogen) and TURBO DNA-free Kit (Applied Biosystems). cDNA synthesis involved cDNA Reverse Transcription Kits (Applied Biosystems). PCR primers were designed by use of Primer3 (http://primer3.ut.ee). qRT-PCR reactions were analyzed by using the MyiQTM thermocycler (BIO-RAD) with the PCR mix of iQ SYBR Green Supermix (BIO-RAD). Data analysis involved iQ5 Optical System Software (BIO-RAD), and the internal control for normalization was PP2AA3 (PP2A; At1g13320) (Czechowski et al., 2005).

Generation of Transgenic HSFA6b Mutant Plants

For HSFA6b overexpression, the RT-PCR amplified HSFA6b was cloned into pPE1000 (Hancock et al., 1997) using SalI and PstI to confer a hemagglutinin (HA) tag at the N-terminus, then subcloned into pCambia3300 (CAMBIA) with SacI for CaMV 35S promoter-driven expression. To generate the HSFA6b dominant-negative mutant, HA-HSFA6b was amplified and cloned into p35S::SRDXG (Hiratsu et al., 2003) with SmaI to confer a dominant repression domain SRDX at the C-terminus driven by the 35S promoter,
then subcloned into binary vector pBCKH by Gateway LR reaction (Invitrogen). All gene fragments were sequenced before making the constructs.

**Protoplast Preparation and Transfection**

Protoplast preparation and transfection were as described (Yoo et al., 2007). HSFA6a and HSFA6b were cloned into p35S::EYFP (Kuo et al., 2013) to insert yellow fluorescent protein (YFP) into the N- or C-terminus. To generate the constructs for BiFC analysis, the RT-PCR–amplified HSFs were cloned into a pCR8/GW/TOPO vector and then recombined into pEarleyGate201-YN or pEarleyGate202-YC vectors (Lu et al., 2010). An amount of 2 x 10^4 protoplasts was transfected with 10 to 20 μg DNA, then incubated at 22°C for 16 to 24 h. The reconstituted YFP signals were observed by confocal microscopy (Leica, TCS SP5) as described (Hsu et al., 2010).

**Protoplast Transactivation Assay**

The potential promoters for HSFA6b (1.0 or 1.7 kb) as well as DREB2A, HSP18.1-Cl, or APX2 (1.0 or 0.865 kb) were amplified and cloned into the Pgal4-UASx4::GUS vector. A CaMV 35S promoter was cloned into the Pgal4-UASx4::GUS vector, which resulted in P35S::GUS construct, to be used as a positive control. The transactivation assay involved co-transfection with a mixture of 15 μg effector (P35S::AREB1-3XFLAG, P35S::HSFA3-3XFLAG, P35S::HSFA6a-3XFLAG, or P35S::HSFA6b-3XFLAG) and reporter plasmids, using 5 μg MTC-301 luciferase plasmid as an internal control to normalize the transfection efficiencies (Ehlert et al., 2006; Hsu et al., 2010). The protoplasts were incubated for 12 to 16 h, then treated without or with 10 μM ABA for 12 h. Luciferase activity was analyzed by use of Luciferase Assay Buffer according to the technical manual (Promega) and GUS activity assay was performed as described (Yoo et al., 2007). GUS activity in all samples was normalized against the luciferase internal control.
**Seed Germination, Cotyledon Greening and Root Growth Assay**

Seed germination and cotyledon greening were analyzed on plates with 1/2 MS medium containing NaCl, mannitol or ABA (Kreps et al., 2002; Pandey et al., 2005; Fujii et al., 2007). Germination was scored for 1 to 7 days, when the radicle had emerged from the testa, and cotyledon greening percentage was quantified at day 10. Three-d-old seedlings were transferred to NaCl-, mannitol- or ABA-containing plates, and primary root growth was measured at day 7 to 9 by use of ImageJ (http://imagej.nih.gov/ij).

**Thermotolerance, Superoxide Dismutase (SOD) Activity, H₂O₂ and Pigment Content Determination**

Thermotolerance testing was performed as described (Charng et al., 2006; Hsu et al., 2010). Seedlings were grown at 22 to 24°C with 16-h light for 6 to 9 days before heat treatment. Plates were sealed with plastic electric tape and submerged in a water bath, then heated at 44°C for 25 to 35 min for BT testing. For AT testing, plates were preheated at 37°C for 1 h, then recovered at 22°C for 2 h before 44°C HS for 190 to 205 min. Healthy-growing seedlings were counted 10 days after the end of HS treatment. In-gel SOD activity assay was as described (Chu et al., 2005; Huang et al., 2012). The detection of H₂O₂ involved 3,3’-diaminobenzidine (DAB) staining as described (Thordal-Christensen et al., 1997). Chlorophyll and anthocyanin measurement was as described (Porra et al., 1989; Neff and Chory, 1998).

**Co-immunoprecipitation (Co-IP) assay**

An amount of 4 x 10⁵ protoplasts was transfected with 20 μg each of HSFA6b-3XFLAG and tester (HSFs)-YFP plasmids, then incubated at 22°C for 16 h and harvested with GM buffer (150mM Tris-HCl, PH7.4). Half of total protein extracts were collected as input, and
the other half were used to perform a Co-IP assay according to standard procedure (Catch and Release v2.0 Reversible Immunoprecipitation System; Millipore; 17-500). HSFA6b-3XFLAG protein was Co-IP from total protein extracts with anti-GFP antibody (Abcom; ab290) and detected by SDS-PAGE followed by immunoblotting using α-FLAG antibody (Santa Cruz Biotechnology; sc-807).

**Chromatin Immunoprecipitation (ChIP) Assay**

1.3 g of mature leaves were collected from 4-week-old seedlings harboring HA-tagged HSFA6b (OE5-6) and incubated with 150 mM NaCl for 6 h or air-dried for 2.5 h (dehydration). ChIP involved use of the EpiQuik Plant ChIP Kit (Epigentek; P-2014-24). An HA-specific antibody (Sigma-Aldrich; H3663) was used to precipitate the complexes of HSFA6b-HA with DNA from chromatin. Immunoprecipitated DNA underwent PCR and qRT-PCR, and 18S rDNA was an input control (Kim et al., 2011).

**Microarray Assay**

Seven-d-old seedlings were treated with 150 mM NaCl for 6 h or 37°C HS for 1 h, with 22°C treatment as a control, then collected for total RNA purification by use of the RNeasy Kit (Qiagen). RNA was used for cDNA synthesis, labeling, and hybridization of Affymetrix ATH1 arrays according to the Affymetrix Gene Chip Expression Analysis manual (http://www.affymetrix.com). The results were representative of 2 independent biological replicates, with Microarray Suite 5.0 (Affymetrix) and GeneSpring 7.3 (Silicon Genetics) used for data analysis. The ATH1 GeneChip array probesets with expression intensity < 100 were filtered out in all samples. Genes with differential expression (DEGs), or expression change > 6-fold at \( p < 0.10 \) compared with the Col control treatment, were collected. Gene Ontology (GO) enrichment analysis involved with the Cytoscape 3.0.2 (http://www.cytoscape.org) plugin BiNGO categories using the GO_Full categories (Maere...
et al., 2005) identified by a hypergeometric test with $p \leq 5.00E-04$ after Benjamini and Hochberg false discovery rate correction. The expression data from these experiments are available at Gene Expression Omnibus (GEO; accession no. GSE63372).

Statistical analysis

Data are expressed as the mean ± SD from at least 3 independent biological experiments. Statistical analysis involved Student’s $t$ test (two-tailed, unpaired). $P < 0.05$ was considered statistically significant.

Primers and Accession Numbers

Primers used and accession numbers are in Supplemental Table S1.
The following materials are available in the online version of this article.

**Supplemental Figure S1.** The expression profiles of Arabidopsis 21 heat shock factor (HSF) genes under abiotic stresses and during development.

**Supplemental Figure S2.** Modular structures of Arabidopsis HSFs HSFA6a and HSFA6b.

**Supplemental Figure S3.** Interaction of HSFA6b and different HSFs.

**Supplemental Figure S4.** Characterization of HSFA6b and HSFA6b T-DNA insertion, overexpression, and dominant-negative mutant lines.

**Supplemental Figure S5.** The expression levels of HSFA6b and ABA sensitivity in HSFA6b mutant lines.

**Supplemental Figure S6.** The expression levels of ABA biosynthesis and responsive genes in response to salt and ABA treatments in HSFA6b mutants.

**Supplemental Figure S7.** The expression profiles of Arabidopsis 18 small heat shock protein (sHSP) genes under various abiotic stresses and during development.

**Supplemental Figure S8.** The expression of HS-related genes in response to HS, salt, and ABA treatments in HSFA6b mutants.

**Supplemental Figure S9.** The expression levels of HS and oxidative related genes, superoxide dismutase (SOD) activity, and H$_2$O$_2$ detection in response to HS, NaCl, and ABA treatments in HSFA6b mutants.

**Supplemental Figure S10.** The expression level of APX2 in response to HS, NaCl, and ABA treatment in HSFA6b mutants, as well as HSFA6b mediated HSFA3 activation of the APX2 promoter.

**Supplemental Figure S11.** Characterization of hsfa3 and hsfa6a hsfa6b double mutant lines, as well as the HSFA6a and HSP18.1-CI transcription levels in response to NaCl treatment in HSFA6b mutants.

**Supplemental Figure S12.** Thermotolerance test in HSFA6b mutants.
Supplemental Figure S13. Thermotolerance test in HSFA6a and HSFA6b mutants.

Supplemental Figure S14. Venn diagram of gene transcripts in response to salt or HS treatment in HSFA6b mutants.

Supplemental Figure S15. GO enrichment analysis of gene transcripts in response to salt treatment in HSFA6b mutants.

Supplemental Figure S16. GO enrichment analysis of gene transcripts in response to HS treatment in HSFA6b mutants.

Supplemental Figure S17. The expression levels of HSFA6a and HSFA6b in response to HS treatment in hsfa1a/b/d/e quadruple-KO (QK) and hsfa2 mutant lines.

Supplemental Table S1. Primers for genotyping, cloning, RT-PCR and real-time quantitative PCR, and accession numbers.

Supplementary Table S2. The differentially expressed genes (DEGs) with levels changed > 6-fold at $p < 0.10$ in HSFA6b mutants OE5-6 and RD3-5 after 150 mM NaCl for 6 h or 37°C HS for 1 h as compared with the Col control (CK) treatment.

Supplementary Table S3. Enriched gene ontology (GO) terms for DEGs with levels changed > 6-fold at $p < 0.10$ in HSFA6b mutants OE5-6 and RD3-5 after 150 mM NaCl for 6 h or 37°C HS for 1 h treatment as compared with the Col control (CK) treatment.

Supplementary Table S4. The top 39 DEGs with levels changed > 30-fold at $p < 0.05$ in HSFA6b mutants OE5-6 and RD3-5 after 150 mM NaCl for 6 h or 37°C HS for 1 h treatment as compared with the Col control (CK) treatment.
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Figure 1. The expression profiles of HSFA6a and HSFA6b in response to HS, salt, osmotic, and ABA stress. (A to D) Nine-d-old seedlings were treated with HS or incubated with H2O containing NaCl, mannitol, or ABA, at different concentrations and times. The transcription levels of HSFA6a and HSFA6b were analyzed by RT-PCR. The pictogram shows the HS regime (right of panel A): 37°C sublethal HS for 1 h (HS) → 22°C recovery for 2 h (R) → 44°C lethal HS for 190 min (LHS). 22°C treatment was a control (CK). HSP18.1-CI, used as a reference, is an HS-responsive marker gene. ACTIN2 (ACT2) was used as a loading control.

Figure 2. Transient expression profiles of HSFA6a and HSFA6b in mesophyll protoplasts. (A) Confocal microscopy of Arabidopsis Col protoplasts transfected with HSFA6b fused to the N- or C-terminal YFP reporter gene, for HSFA6b-YFP or YFP-HSFA6b. YFP-HSFA6b transfected cells were also treated with 10-μM ABA or 20-nM Lepomycin B for 2 h. (B) YFP-tagged HSFA6a transfection served as comparison. Blue shows nuclei stained with 4',6-diamino-phenylindole (DAPI), and red shows chlorophyll autofluorescence. Bars = 20 μm.

Figure 3. The expression levels of HSFA6b and AREB1 in ABA deficient and insensitive mutant lines. Nine-d-old seedlings of ABA biosynthesis mutants aba2-1 and aba3-1, and ABA insensitive mutants abi1-1 and abi2-1, incubated in H2O with 200 mM NaCl for 6 h or 20 μM ABA for 3 h. (A to C) The transcription levels of HSFA6b and AREB1 were analyzed by qRT-PCR. (D) NaCl and ABA responsive marker genes RD29A and RD29B were used as references. The fold change expression was normalized relative to the level of Col or Ler H2O treatment. Data are means ± SD of 3 biological replicates. *, Significant at P < 0.05 compared with the Col or Ler. PP2A was an internal control.
Figure 4. The transcriptional activation of the HSFA6b promoter in response to ABA and AREB1 treatments were analyzed in mesophyll protoplasts. (A) Gray boxes are predicted ABRE motifs (T/CACGTGGC) upstream of the HSFA6b promoter region at -1257 [reverse (-)]; -1299 [forward strand (+)]; -1602 (-); -1605 (+); -1776 (-); -1846 (-); -1849 (+); -1864 (+); -1869 (-) and -1872 bp (+). (B and C) The transcriptional activation assay of the HSFA6b promoter. \( P_{\text{GAL4-UAS}4::GUS} \) was a control, and 1- or 1.7-kb lengths of the HSFA6b promoter region, \( P_{\text{HSFA6b}1.0} \) or 1.7, respectively, were fused with \( GUS \) gene and used in the corresponding transient reporter assays. Transfected protoplasts were then treated with (+) or without (-) 10-\( \mu \)M ABA for 12 h and with or without effector AREB1-3XFLAG co-expression, as indicated. The AREB1-3XFLAG fusion protein, under the control of the CaMV 35S promoter, was detected by immunoblotting (\( \alpha \)-FLAG) and the Rubisco large subunit (Rbc L) shown for equal loading (insert). The amount of relative GUS activity was normalized by luciferase (LUC) luminescence. The fold expression was normalized relative to the amount of \( P_{\text{HSFA6b}1.0} \) without ABA (panel B), or \( P_{\text{HSFA6b}1.7} \) without ABA and ARER1 (panel C). Data are means ± SD of 3 biological replicates. *, Significant at \( P < 0.05 \).

Figure 5. Seed germination and post-germination growth in response to salt and ABA treatments in HSFA6 mutants. (A) Seed germination (%) with either 1-\( \mu \)M ABA or 200-mM NaCl treatment. H\(_2\)O treatment was used as a control. (B) Seedling growth with 0.5 to 1 \( \mu \)M ABA on plates was photographed at day 12 after planting. \( abi4-1 \), an ABA insensitive mutant (Col ecotype), was used as reference. (C) Cotyledon greening (%) with 150 mM NaCl on plates was measured at day 12 after planting. Data are means ± SD of 3 biological replicates. *, Significant at \( P < 0.05 \) compared with the Col.
Figure 6. Root growth in response to salt, osmotic, and ABA treatment in HSFA6s mutants. (A and B) Three-d-old seedlings grown on 1/2 MS medium were transferred to plates containing NaCl, mannitol, or ABA, as indicated. Root length (cm) and relative root elongation (%) were measured at day 9. (C) Seeds were planted in 1/2 MS medium (control), and medium containing 100 mM NaCl or 150 mM mannitol, and then photographed at day 9. Data are means ± SD of 3 biological replicates. *, Significant at $P < 0.05$ compared with the Col.

Figure 7. HSFA6b and AREB1 activated the transcriptional activity of the HSP18.1-CI and DREB2A promoters. (A and B) a6b-1 mutant protoplasts were used for a transcriptional activation assay, as indicated in Figure 4. The 1-kb length of the HSP18.1-CI or DREB2A promoter region, respectively, was fused with GUS reporter gene. Protoplasts were transfected with or without effectors HSFA6b-3XFLAG and AREB1-3XFLAG. The effectors were analyzed by immunoblotting (insets). The fold expression was normalized relative to the level of $P_{HSP18.1}$ and $P_{DREB2A}$ without effectors. (C) HSFA6b binding to the HSE sequence of the DREB2A promoter was analyzed by chromatin immunoprecipitation (ChIP) assay. Schematic map of the 314-bp upstream of the DREB2A promoter region (left); ABRE (GACACGTA; -86 to -93 bp) and HSE (AGAAGATTCG; -151 to -160 bp) are in gray boxes. Seedlings were treated with 6 h 150 mM NaCl or 2.5 h dehydration. The fold enrichment of the HSE-containing region (qPCR) after ChIP was analyzed by qRT-PCR and normalized to the Col H2O treatment (right). 18S rDNA was an input control. (D and E) Transcriptional activation assay in hsfA3 and a6a a6b-1 double mutant protoplasts with or without effectors HSFA6b-3XFLAG, HSFA3-3XFLAG, and HSFA6a-3XFLAG, are as described in Panels A and B. The fold expression was normalized relative to that of the $P_{HSP18.1}$ without effectors. Data are means ± SD of 3 biological replicates. *, Significant at $P < 0.05$. 

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**Figure 8. Thermotolerance test in HSFA6b mutants.** Basal thermotolerance (BT) and acquired thermotolerance (AT) analysis in 9-d-old seedlings. A heat sensitive mutant hsp101 was used as reference. Pictograms show the HS regime. *(A and B)* Plates were incubated without or with 2.5 ml 10-μM ABA solution, respectively, 12 h before HS treatment. Survival was measured at day 10 after HS treatment. Data are means ± SD of 3 biological replicates. *, Significant at $P < 0.05$ compared with the Col.

**Figure 9. Drought and salt tolerance test in HSFA6b mutants.** *(A)* 24-d-old plate-grown seedlings were photographed *(left)*, then the chlorophyll a/b and chlorophyll/anthocyanin ratios were measured *(right)*. Data are means ± SD of 3 biological replicates. *, Significant at $P < 0.05$ compared with Col. *(B and C)* Two-week-old seedlings were watered with 300 mM NaCl, then photographed at 2 and 5 weeks after treatment, respectively. The survival rates were analyzed after treatment with water containing 300 mM NaCl for 5 weeks. Arrows show surviving seedlings. One preventative data set is shown. Data are means ± SD of 3 biological replicates.

**Figure 10. Heat maps of the transcriptome analysis in HSFA6b mutants under salt and HS treatments.** *(A)* The top 39 DEGs with expression change > 30-fold at $p < 0.05$ in HSFA6b mutants under salt and HS treatments as compared with wild-type control treatment. Profiles of 39 genes in the wild type (Col), OE5-6 (OE), and RD3-5 (RD) mutants after treatment with 150 mM NaCl for 6 h or 37°C HS for 1 h; 22°C treatment was a control (CK). *(B)* The top 25 DEGs of HSFA6b HS response regulon in RD mutant had an expression change of < 1.5 fold when compared with Col. The gene locus, symbol, and potential function were downloaded from The Arabidopsis Information Resource (TAIR). Normalized and averaged signals (log2) were analyzed as “heat maps” *(left)* and the corresponding
genes are listed (right). As well, the HSFA6b HS-regulon overlap with the DREB2A and HSFA3 HS-regulon is indicated by a “V”, as shown in Supplemental Figure S14B. * At4g36990 and At4g36988 shared the same probeset number, 246214_at, on ATH1 GeneChip array. HS-related genes and transcription activators were highlighted in blue and underlined, respectively.

Figure 11. Model of Arabidopsis HSFA6b as a hub connecting the ABA signaling pathway and ABA mediated thermoprotection. HS, heat shock; CaM, calmodulin; HSR, HS response; OxR, oxidative stress response. Under HS, activation of the HSFA1 (a, b, d, e) induces the expression of HSR genes including HSFA2 (the dominant factor), HSFA7 (a, b), as well as DREB2A and its downstream regulator HSFA3. HSFB (1, 2a, 2b) have roles in a negative feedback loop of HSR. HSFA4a is an H$_2$O$_2$ sensor, activating HSR and OxR genes expression. HSFA5 is the selective repressor of HSFA4a. HSFA1 (a, b, d) are negative regulators of HSFA6b. AREB1, HSFA6a, HSFA6b, and seed-specific HSFA9 are downstream regulators of the ABA dependent pathway. HSFA6a is an activator of drought and salt response. HSFA6b acts synergistically with AREB1 for DREB2A expression, and then HSFA3 in turn is upregulated, thus playing a role in HSR and OxR and mediating a cross talk between ABA dependent and HSR regulons. The black and orange arrows show pathways that have been studied. The induction pathways highlighted in the circle and blue arrows emphasize the new ABA signaling pathway merging into the complex HSR network in Arabidopsis.
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