The Heat Stress Factor HSFA6b Connects ABA Signaling and ABA-Mediated Heat Responses[1[OPEN]]

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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 (*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 down-regulated 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 heat stress 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; nGAAnnTTCn) 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 (*Arabidopsis thaliana*), 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 four *HSFs*, and *Drosophila* and *Caenorhabditis elegans* contain one each (Nover et al., 2001). Plants have predicted *HSF* families that are more diverse compared to animals: 24 *HSFs* and three *HSF*-like genes were identified in tomato, 25 in rice (*Oryza sativa*), 30 in maize (*Zea mays*), and 52 in soybean (*Glycine max*; 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, five in class B, and one in class C. The class A members, HSFAs, contain the conserved oligomerization, DNA-binding, and aromatic/hydrophobic/acidic-type activation domains, as well as nuclear localization and

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Y.-C.H., C.-Y.N., and C.-R.Y. designed, performed, and wrote the article; Y.-C.H. performed the microarray assay; T.-L.J. conceived the project and cowrote and revised the article.

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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 coregulators or repressors of the HSFAs, while class C HSF functions remain unclear (Boscheinen et al., 1997; Czarnecka-Verner et al., 2000, 2004; Kotak et al., 2007a; von Koskull-Döring 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., 2007a).

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 H_2O_2 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* gene expression (Sakuma et al., 2006; Schramm et al., 2006, 2008; Lim et al., 2007; 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 HSFAs, 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., 2007b). *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 cross talk exists between HS and other abiotic stress-signaling cascades mediated by HSFs (Kotak et al., 2007a). *HSFA6a* and *HSFA6b* transcript levels are particularly expressed in response to salt, osmotic, and cold stress (von Koskull-Döring et al., 2007; Hwang et al., 2014). Overexpression of *HSFA6b*, but not *HSFA6a*, could regulate the transcription of *DREB2A*, which suggests nonredundant 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 Pagès, 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 up-regulated 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-motif repression domain SRDX (Hiratsu et al.,

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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 HS

Analysis of the AtGenExpress database revealed the expression of 21 Arabidopsis *HSF*s responding to heat, salt, osmotic, drought, and cold stress (Supplemental Fig. S1, A 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 reverse transcription PCR (RT-PCR; Fig. 1) or real-time quantitative PCR (qRT-PCR; Supplemental Fig. 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 Fig. 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 (Fig. 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 (Fig. 2B).

HSFs form homo- or heterotrimers, resulting in altered nuclear localization, and then bind to their own promoters and/or to promoters of other HSF genes, enhancing or suppressing their promoter transcription (Miller and Mittler, 2006). We used a coimmunoprecipitation (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 Fig. S3, A and B). HSFA6b neither interacts with itself, even with the 10 μM ABA treatment, nor with HSFA6a. It interacts with the well-known HSFA1a, HSFA1b, and HSFA2 in the nucleus.

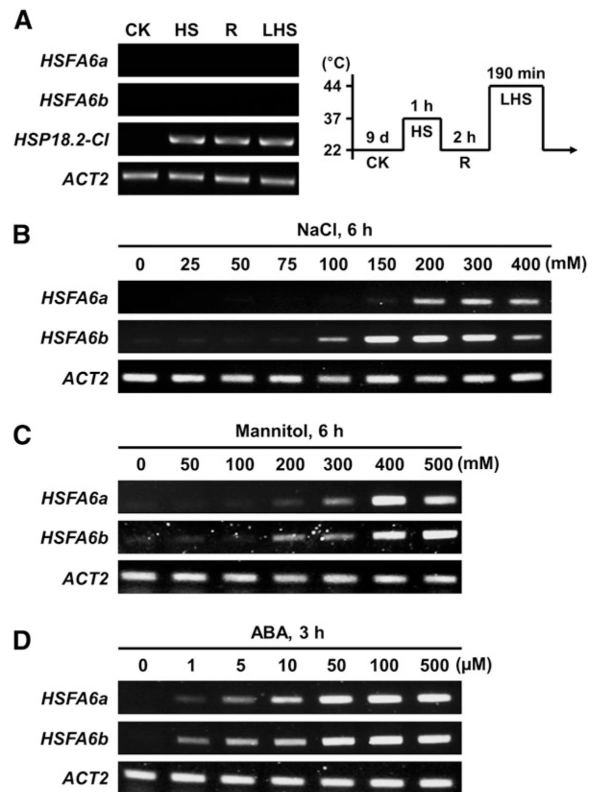

Figure 1. The expression profiles of *HSFA6a* and *HSFA6b* in response to HS, salt, osmotic, and ABA stress. A to D, Nine-day-old seedlings were treated with HS or incubated with water containing NaCl, mannitol, or ABA at different concentrations and times. The transcription levels of *HSFA6a* and *HSFA6b* were analyzed by RT-PCR. The pictogram shows the HS regime (right of 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.

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 Fig. 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 (Fig. 3). *HSFA6b* expression was greatly impaired in ABA-deficient mutants under NaCl treatment; however, ABA treatment notably restored expression (Fig. 3A). *HSFA6b* expression was likewise equally impaired in ABA-insensitive mutants under both NaCl and ABA treatment (Fig. 3B). The *AREB1* expression profile was similar to that of *HSFA6b* (Fig. 3C), and NaCl and drought-responsive marker genes *RESPONSIVE TO DEHYDRATION29A* (*RD29A*) and *RD29B* were used as references (Fig. 3D). Therefore, we suggest that NaCl-induced *HSFA6b* gene expression could be downstream of ABA perception and regulated by the ABA signal.

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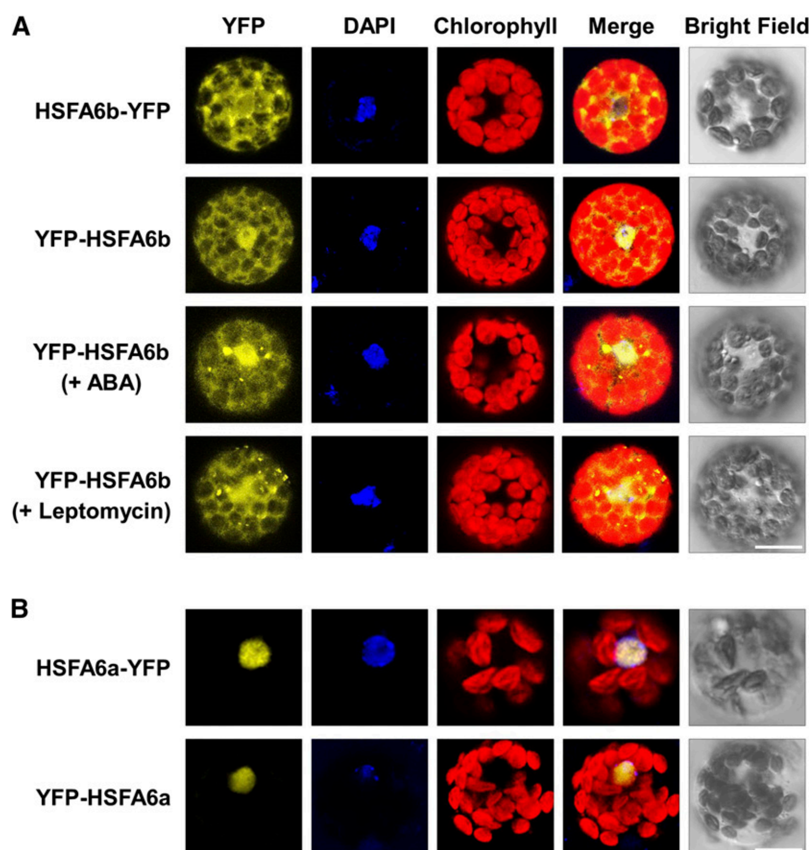

Figure 2. Transient expression profiles of *HSFA6a* and *HSFA6b* in mesophyll protoplasts. A, Confocal microscopy of Arabidopsis Col protoplasts transfected with *HSFA6b* fused to the N- or C-terminal *YFP* reporter gene, for *HSFA6b-YFP* or *YFP-HSFA6b*. *YFP-HSFA6b* transfected cells were also treated with 10 μM ABA or 20 nM Leptomycin 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.

ABA Treatment and AREB1/ABF2 Coexpression 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 (Fig. 4A). After fusing the 1.7-kb promoter region to the reporter gene *GUS*, the protoplast transactivation assay confirmed that a 10 μM ABA treatment significantly enhanced the transcriptional activity of *HSFA6b* (Fig. 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 (Fig. 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* (Columbia [Col] ecotype background) and *a6b-2* (Landsberg *erecta* [L*er*] ecotype background) were screened and confirmed by RT-PCR (Supplemental Fig. 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 Fig. 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 Fig. S4C). For *HSFA6b* dominant-negative effect (*HSFA6b*-RD) lines, an ETHYLENE-RESPONSIVE ELEMENT-BINDING FACTOR-associated amphiphilic repression-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 Fig. 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 Figs. S4, C and 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 (Supplemental Fig. S5, A and B). 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*,

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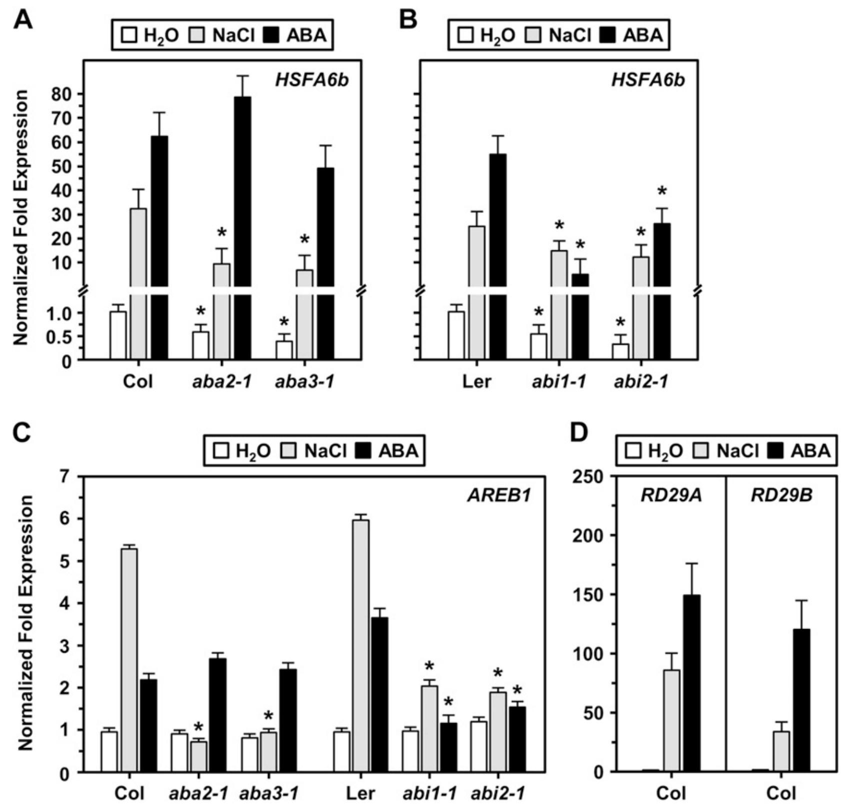

Figure 3. The expression levels of *HSFA6b* and *AREB1* in ABA-deficient and -insensitive mutant lines. Nine-day-old seedlings of ABA biosynthesis mutants *aba2-1* and *aba3-1*, and ABA-insensitive mutants *abi1-1* and *abi2-1*, incubated in H_2O with 200 mM NaCl for 6 h or 20 μM ABA for 3 h. A to C, 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 L*er* H_2O treatment. Data are means ± SD of three biological replicates. *Significant at $P < 0.05$ compared with the Col or L*er*. *PP2A* was an internal control.

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*, and *a6a* mutants and the wild-type Col and L*er* for 1 μM ABA and 200 mM NaCl treatments (Fig. 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; postgermination 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 (Fig. 5B; Supplemental Fig. S5B). 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 L*er*, 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* (Fig. 5C), which agreed with the ABA treatment findings (Fig. 5B). The cotyledon greening ratio for *a6a* and the Col was similar.

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 (Fig. 6A). However, root elongation was significantly affected in *RD* lines (Fig. 6, B 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

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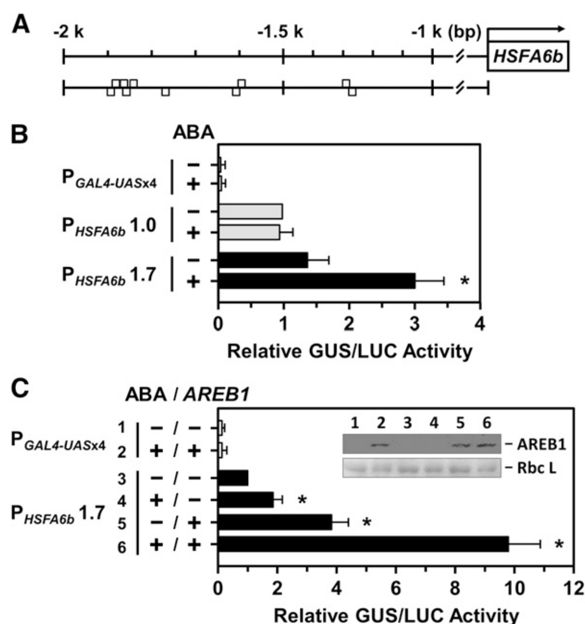

Figure 4. The transcriptional activation of the *HSFA6b* promoter in response to ABA and AREB1 treatments were analyzed in mesophyll protoplasts. A, Gray boxes are predicted ABRE motifs (T/CACGTGGC) upstream of the *HSFA6b* promoter region at −1257 (reverse strand [−]); −1299 (forward strand [+]); −1602 (−); −1605 (+); −1776 (−); −1846 (−); −1849 (+); −1864 (+); −1869 (−); and −1872 bp (+). B and C, The transcriptional activation assay of the *HSFA6b* promoter. $P_{GAL4-UASx4}$::*GUS* was a control, and 1- or 1.7-kb lengths of the *HSFA6b* promoter region, P_{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 μM ABA for 12 h and with or without effector AREB1-3XFLAG coexpression, as indicated. The AREB1-3XFLAG fusion protein, under the control of the CaMV *35S* promoter, was detected by immunoblotting (α-FLAG) and the Rubisco large subunit (Rbc L) shown for equal loading (inset). The amount of relative GUS activity was normalized by luciferase (LUC) luminescence. The fold expression was normalized relative to the amount of P_{HSFA6b} 1.0 without ABA (B), or P_{HSFA6b} 1.7 without ABA and ARER1 (C). Data are means ± SD of three biological replicates. *Significant at $P < 0.05$.

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., 2012b; Supplemental Fig. S6).

With NaCl treatment, the expression of *NCED3* was higher than that of *AAO3* in Col plants (Supplemental Fig. 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 Fig. 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 Fig. 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* Gene Expression

The induction and accumulation of HSPs are essential for establishing thermotolerance, and *HSP* gene transcription can also be induced by other abiotic stresses such as salt and drought (Supplemental Fig. 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 Fig. S8A), the expression of *HSPs* was up-regulated, 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 Fig. S9A). The expression of *HSFs* was similar to that of *HSPs* in response to HS (Supplemental Fig. S8A).

Under 150 mM NaCl and 20 μM ABA treatments (Supplemental Fig. S8, B and C), the expression of *HSPs* was markedly suppressed in *RD3-5* but effectively up-regulated 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 *HSF* expression in *OE5-6*, whereas those of *HSFA6a* and class B *HSF* genes *HSFB1*, *HSFB2a*, *HSFB2b*, and *HSFB4* were down-regulated 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 Fig. S9B). Notably, the expression of *APX2* was significantly up-regulated and suppressed in *OE5-6* and *RD3-5*, respectively, in response to HS (Supplemental Fig. S9B), NaCl, and ABA (Supplemental Fig. S10A). As well, superoxide dismutase (SOD) activity and H_2O_2 accumulation were not affected in *HSFA6b* mutants under the treatments (Supplemental Fig. S9, C 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*

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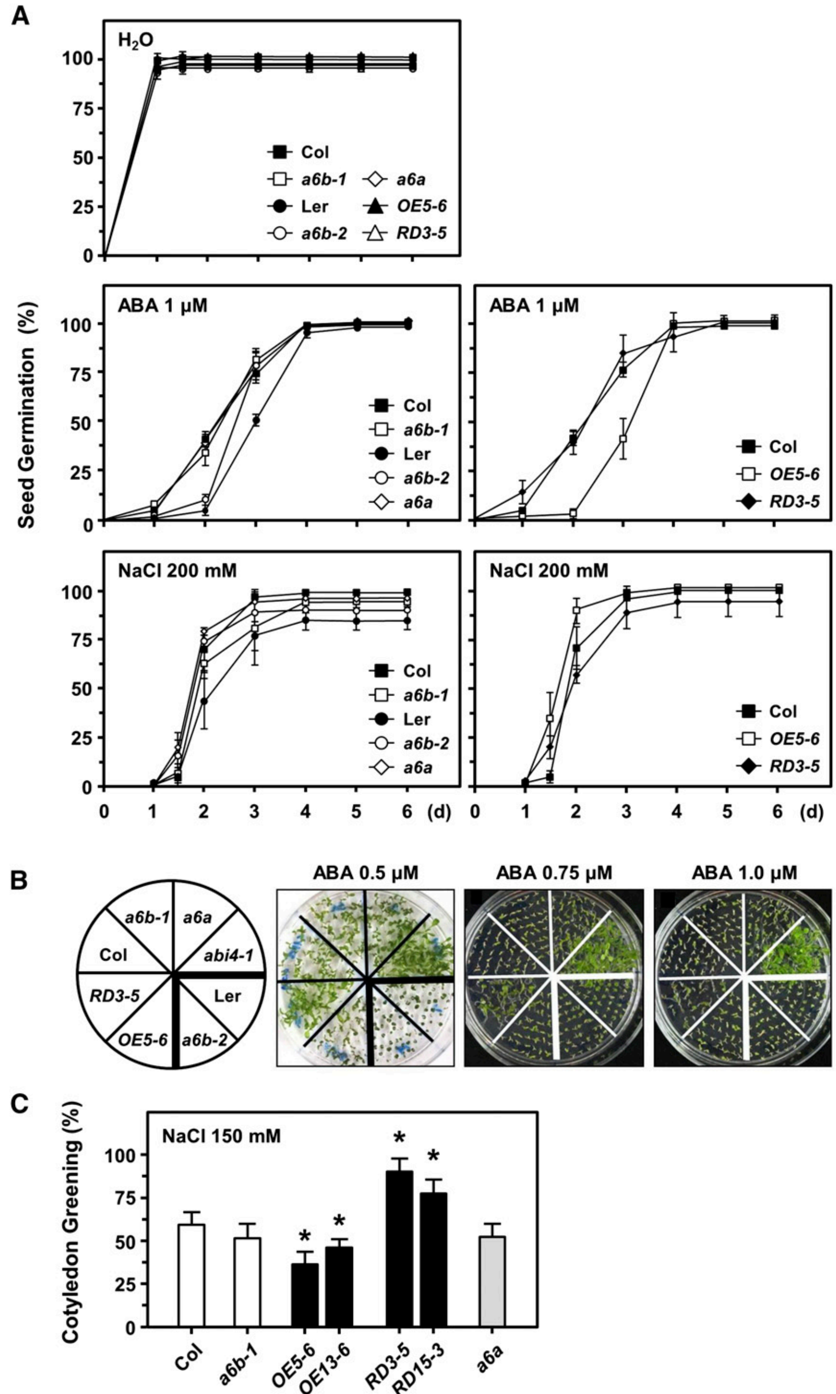

Figure 5. Seed germination and postgermination growth in response to salt and ABA treatments in *HSFA6* mutants. A, Seed germination (%) with either 1 μM ABA or 200 mM NaCl treatment. Water treatment was used as a control. B, Seedling growth with 0.5 to 1 μM ABA on plates was photographed at day 12 after 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 three biological replicates. *Significant at $P < 0.05$ compared with the Col.

and *DREB2A* promoters (Fig. 7, A and B), and coexpression of *AREB1* had an additive effect on two promoters' expressions. The interaction of HSFA6b with the 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 (Fig. 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 Fig. S11A), *HSFA6b* overexpression significantly activated the transcription of *HSP18.1-CI* and *APX2* promoters (Fig. 7D; Supplemental Fig. S10B), and *HSFA3* coexpression had an additive effect on their expression. In *HSFA6a* and *HSFA6b* double-KO protoplasts (Supplemental Fig. S11B), we confirmed that

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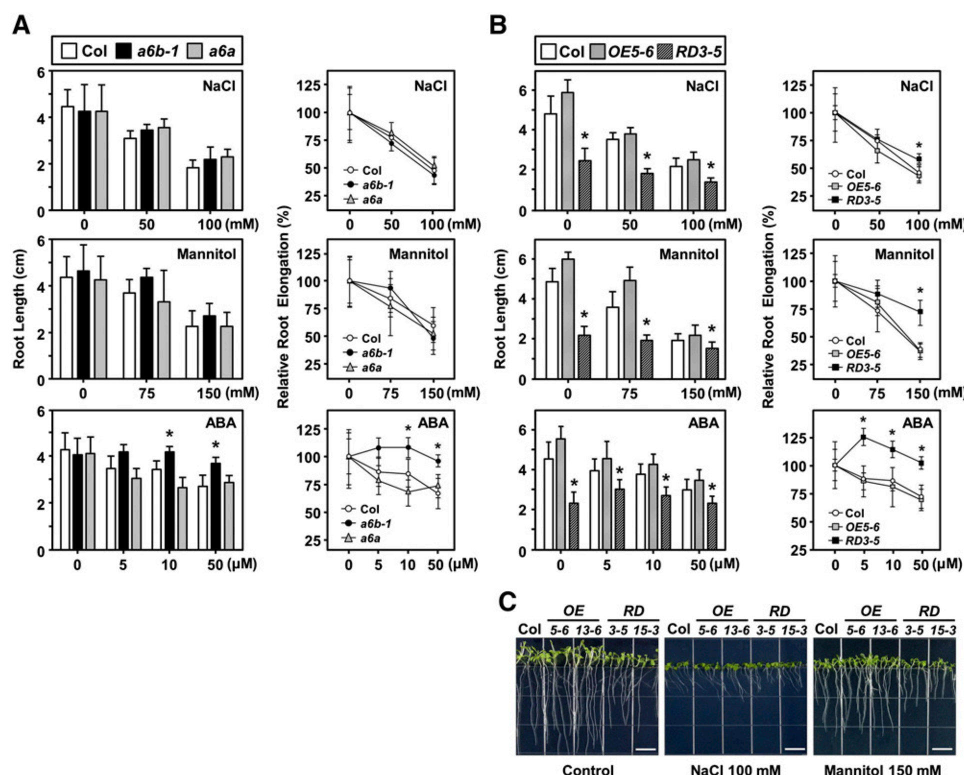

Figure 6. Root growth in response to salt, osmotic, and ABA treatment in *HSFA6s* mutants. A and B, Three-day-old seedlings grown on half-strength Murashige and Skoog medium were transferred to plates containing NaCl, mannitol, or ABA, as indicated. Root length (cm) and relative root elongation (%) were measured at day 9. C, Seeds were planted in half-strength Murashige and Skoog medium (control), and medium containing 100 mM NaCl or 150 mM mannitol, and then photographed at day 9. Data are means ± SD of three biological replicates. *Significant at $P < 0.05$ compared with the Col.

HSFA6b functions independently of *HSFA6a* on *HSP18.1-CI* and *APX2* promoter activation (Fig. 7E; Supplemental Fig. S10C).

HSFA6b Positively Regulated Thermotolerance

BT and 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 (Fig. 8A). The transgenic lines of *OE12-6* and *RD23-1* (transgene expression similar to that of the Col; Supplemental Fig. S5A), used as references, showed similar thermotolerant phenotypes as the Col. *RD3-5* also showed an HS-sensitive phenotype to long-term AT (Supplemental Fig. S12), with 37°C HS for 1 h, then 22°C 2-d 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 (Fig. 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 Fig. S13).

HSFA6b Overexpression Conferred Drought and Salt Tolerance

We grew 200 seedlings in plates (9-cm diameter with 20 mL medium) for 24 d, then the growth medium was kept dehydrated (Fig. 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 (Fig. 9, B 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 (Fig. 9A, right). Although no phenotype appeared at 2 weeks (Fig. 9B), the salt-responsive phenotype was observed at 5 weeks (Fig. 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

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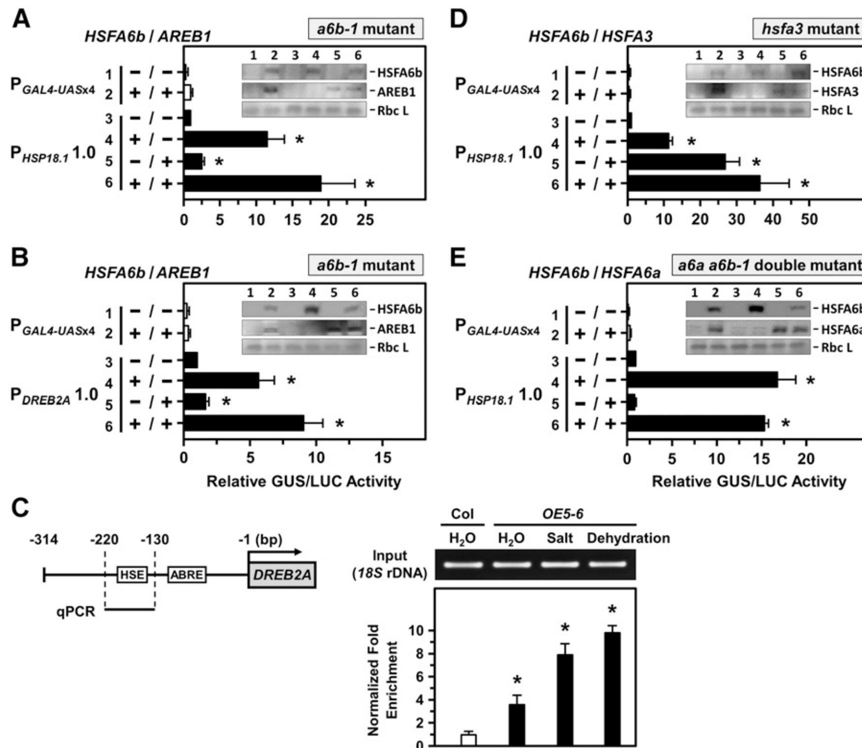

Figure 7. HSFA6b and AREB1 activated the transcriptional activity of the *HSP18.1-CI* and *DREB2A* promoters. A and B, *a6b-1* mutant protoplasts were used for a transcriptional activation assay, as indicated in Figure 4. The 1-kb length of the *HSP18.1-CI* or *DREB2A* promoter region, respectively, 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 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 H_2O 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 A and B. The fold expression was normalized relative to that of the $P_{HSP18.1}$ without effectors. Data are means ± SD of three biological replicates. *Significant at $P < 0.05$.

or 37°C HS treatment, as compared with the Col control treatment (Supplemental Table S2). The Venn diagram showed 246 NaCl- and 197 HS-induced DEGs (Supplemental Fig. 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 (Supplemental Fig. S15; Supplemental 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* (Supplemental Fig. S16; Supplemental 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 (Fig. 10A; Supplemental Table S4). We also highlighted the top 25 DEGs of *HSFA6b* HSR regulon in *RD3-5* with an expression change of <1.5-fold compared with the Col HS (Fig. 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 (Supplemental

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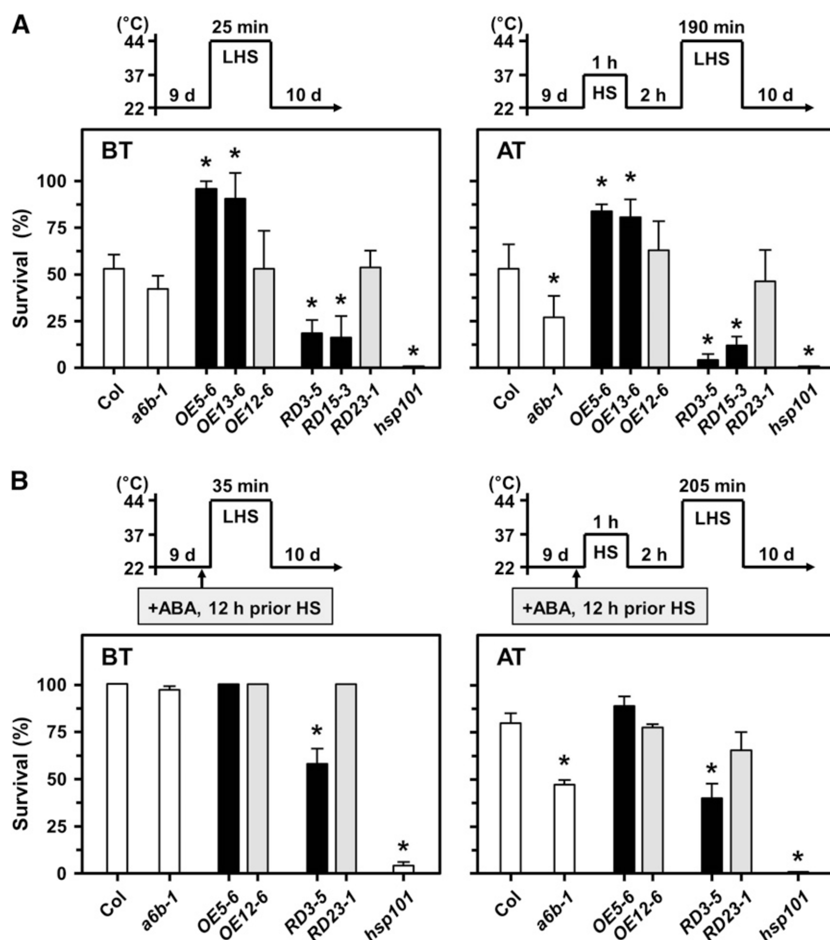

Figure 8. Thermotolerance test in *HSFA6b* mutants. BT and acquired AT analysis in 9-day-old seedlings. A heat-sensitive mutant *hsp101* was used as reference. Pictograms show the HS regime. A and B, Plates were incubated without or with 2.5 mL 10 μM ABA solution, respectively, 12 h before HS treatment.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

Fig. S14B) showed overlap coregulation in part with *DREB2A* and *HSFA3* HS regulons (Sakuma et al., 2006; Yoshida et al., 2008).

These data support 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., 2012b). 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 *aba1* and *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 *HSP18.1-CI* and *HSFA2*, which contain the perfect/active HSE motif (nTTCnnGAAnnTTCn/nGAAnnTTCn), *HSFA6a* and *HSFA6b* potential promoter regions only contain one aaAAtcTTCt and aGAAggaTCt sequence (Nover et al., 2001), respectively. This supports the expression data showing that neither *HSFA6a* nor *HSFA6b* respond to HS (Fig. 1; Supplemental Fig. S1). HSFA6b is a nuclear-localized factor (Fig. 2), and during ABA or Leptomycin B treatment, the nuclear localization of the YFP-tagged HSFA6b showed partial but not completed translocation to the nucleus, which implies that a limited amount of nuclear-localized HSFA6b is required for its function.

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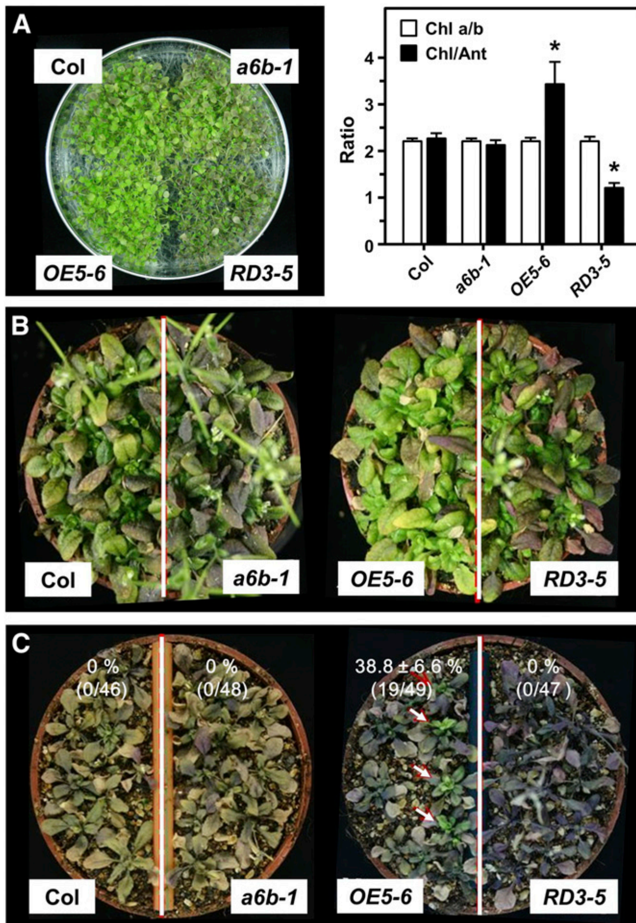

Figure 9. Drought and salt tolerance test in *HSFA6b* mutants. A, Twenty-four-day-old plate-grown seedlings were photographed (left), then the chlorophyll *a/b* and chlorophyll/anthocyanin ratios were measured (right). Data are means ± SD of three biological replicates. *Significant at $P < 0.05$ compared 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 three biological replicates.

As promoters of ABA-responsive genes, ABREs serve as binding motifs for AREBs/ABFs. Reduced expression of *HSFA6b* in the *areb1/areb2/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 (Fig. 3), and the promoter translational activity assay revealed significantly induced transcription of *HSFA6b* by ABA and the coexpressed *AREB1* (Fig. 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 (Fig. 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 (Fig. 1; Supplemental Fig. S1), as well as that *HSFA6b* mutants affect *HSFA6a* expression (Supplemental Figs. S8 and S11). In addition, *HSFA6a* and *HSFA6b* expression showed a differential expression pattern in the *hsfa1a/b/d/e* quadruple mutant under HS stress (Supplemental Fig. 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* (Fig. 6A; Supplemental Fig. S13B), and *HSFA6b* functioned independently of *HSFA6a* on *HSP18.1-CI* and *APX2* promoter activation (Fig. 7E; Supplemental Fig. S10C). Therefore, *HSFA6b* has an independent specific function in HS and oxidative stresses from *HSFA6a*.

HSFA6b, a Positive Regulator, Is Involved in ABA-Mediated Drought, Salt, and Thermotolerance

HSFA6b expression was highly induced by NaCl, mannitol, and ABA treatments (Fig. 1). The *a6b-1* was insensitive to ABA in terms of primary root growth as compared with the wild type but displayed no significant differences under salt or drought/osmotic stress (Fig. 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 (Figs. 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

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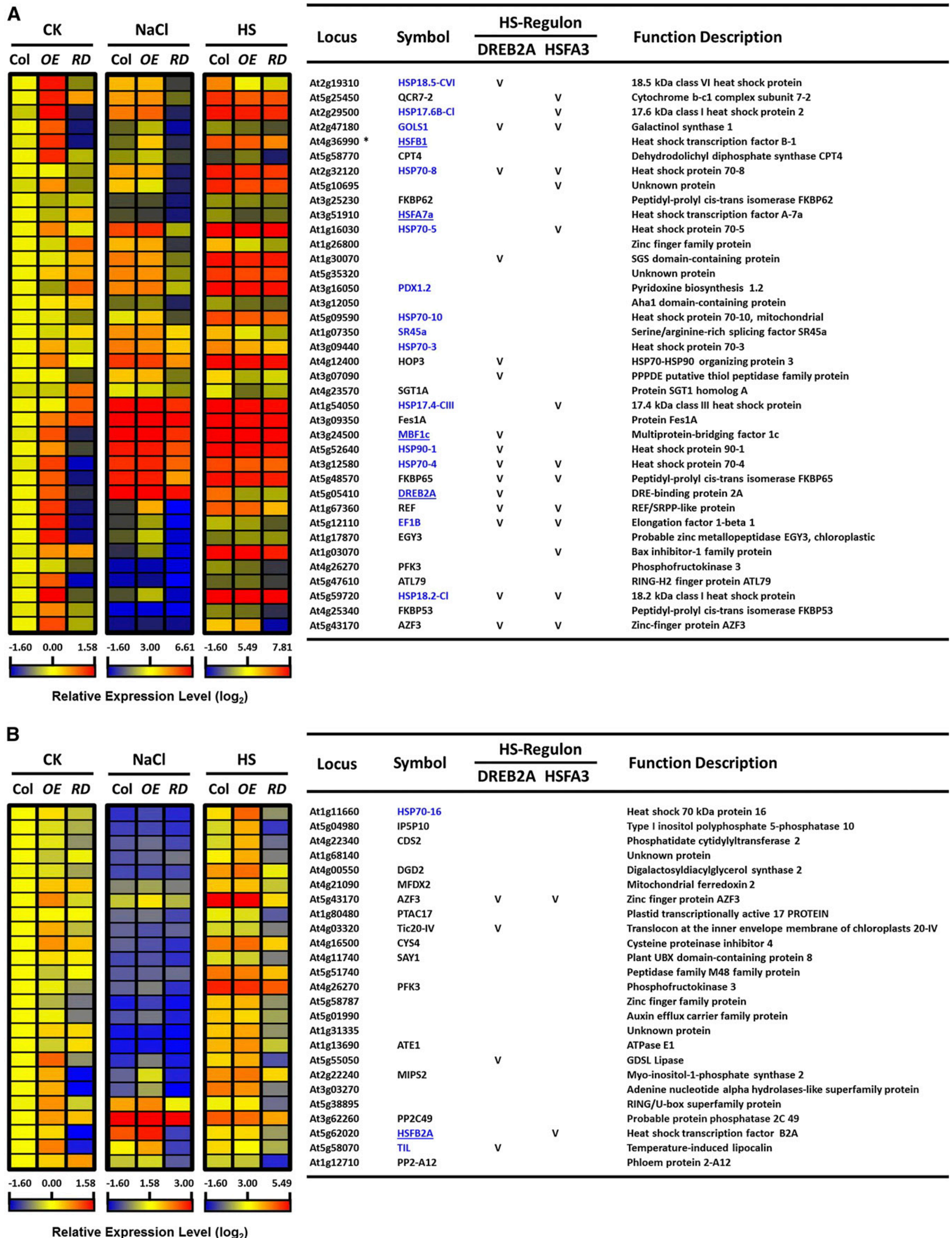

Figure 10. Heat maps of the transcriptome analysis in *HSFA6b* mutants under salt and HS treatments. A, The top 39 DEGs with expression change >30-fold at $P < 0.05$ in *HSFA6b* mutants under salt and HS treatments as compared with wild-type control treatment. Profiles of 39 genes in the wild type (Col), *OE5-6* (*OE*), and *RD3-5* (*RD*) mutants after treatment with 150 mM NaCl for

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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 cross talk among stress-signaling pathways. We also confirmed that the BT, AT, and long-term AT 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 (Fig. 8; Supplemental Fig. 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 Figs. 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 Fig. S9, B 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 up-regulation of *HSP18.1-CI* and *HSP26.5-MII* expression, in addition to *APX2* (Supplemental Figs. S8, B and 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 (Fig. 9). Thus, *HSFA6b* is required for drought and salt stress response.

Cross Talk of HSFA6 and DREB2A in HSR Genes Expression and Regulatory Networks

The physiological drought/osmotic stress responsive regulons largely depend on two 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 down-regulated after 1-h HS treatment in the Arabidopsis *hsfa1a/b/d/e* quadruple mutant, with 658 genes HS-up-regulated in the wild type and 81 genes HS-up-regulated in transgenic plants overexpressing a constitutive active form of *DREB2A* (*DREB2A*-CA). In the top 100 down-regulated 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-up-regulated genes in the wild type, 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 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 up-regulates *HSFA3* but has no effect on *HSFA6b* expression (Yoshida et al., 2011). HSFA3 in turn up-regulates the expression of *HSP18.1-CI* (one DRE and two HSEs), *HSP26.5-MII* (one DRE and no HSE), and *HSP70* (one DRE and one 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* gene expression was up-regulated in 15 min, 30 to 60 min, and 1 h after the HS, respectively (Supplemental Fig. 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 (Fig. 7, B 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 (Fig. 7, A and D; Supplemental Fig. S10B). This result appears to

Figure 10. (*Continued.*)
6 h or 37°C HS for 1 h; 22°C treatment was a control (CK). B, The top 25 DEGs of HSFA6b HSR 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 (log_2) 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.

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support an HSFA6b and AREB/ABF cross talk 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 Fig. 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 downstream gene expression (Fig. 10) plays a role in ABA-mediated thermotolerance.

The drought inducible genes were up-regulated by salt stress, but a few were cold inducible. The activators DREB1/CBF and DREB2 function separately in the cold, drought, and salt signaling pathways. Transcription of the cold-induced marker gene *RD29A* (one ABRE and three DREs) was through the DREB1/CBF-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 Fig. S6), so HSFA6b might not contribute to the DREB1/CBF-DRE regulon. Induced expression of *RD29B* (four ABREs and one DRE), which mainly depends on an ABA pathway, was largely affected in the *HSFA6b* mutant (Supplemental Fig. S6B). 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 Fig. S6B). *MYB2* and *MYC2* expression was also enhanced by JA, which further indicates that abiotic and biotic stress transduction pathways are interconnected. In summary, cross talk 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., 2012b).

The top 39 DEGs of 197 DEGs (Supplemental Fig. 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 (Fig. 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* HSR regulon in *RD3-5* showed an expression change of <1.5-fold when compared with Col HS treatment (Fig. 10B), including HSP70, HSFB2A, temperature-induced lipocalins, and enzymatic genes. Temperature-induced lipocalins 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., 2014).

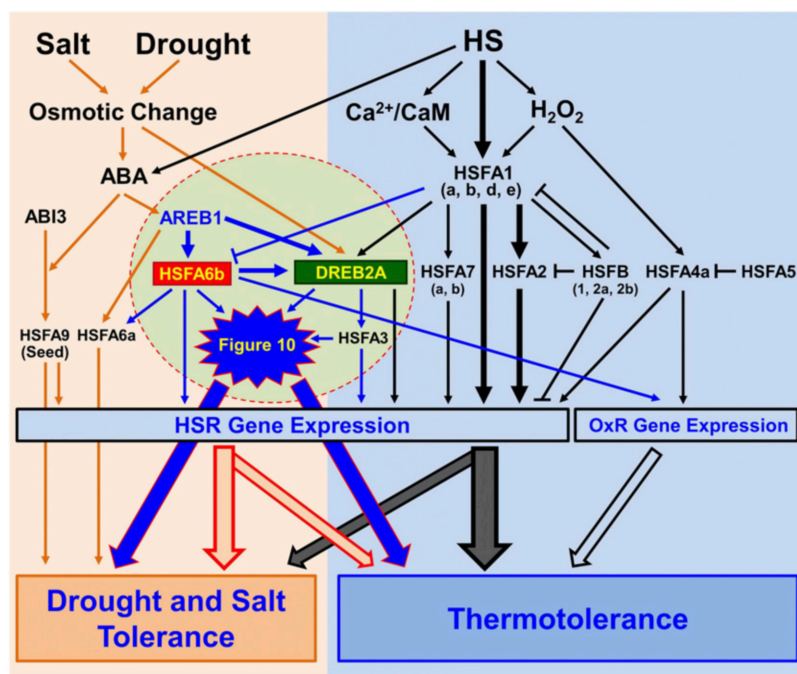

Figure 11. Model of Arabidopsis HSFA6b as a hub connecting the ABA signaling pathway and ABA-mediated thermoprotection. CaM, Calmodulin; OxR, oxidative stress response. Under HS, activation of the HSFA1 (a, b, d, e) induces the expression of HSR genes including HSFA2 (the dominant factor), HSFA7 (a, 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_2O_2 sensor, activating HSR and OxR gene 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 up-regulated, 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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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* (Figs. 1, 3, 4, and 7, B and C) 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 (*Arabidopsis thaliana*) *HSFA6a*-, *HSFA6b*-, and *HSFA3*-KO lines, *a6a* (SALK_045608; Col ecotype), *a6b-1* (GK-513A02; Col ecotype), *a6b-2* (GT_3_7561; L*er* ecotype), and *hsfa3* (SALK_01117; Col ecotype) were obtained from the Arabidopsis Biological Research Center or the Nottingham Arabidopsis Stock Center. Transgenic plants were created in the 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°C to 24°C with 16-h light at 60 to 100 μmol m^{-2} s^{-1}.

RNA Preparation, cDNA Synthesis, and 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 MyiQ 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 *Sal*I and *Pst*I to confer a HA tag at the N terminus, then subcloned into pCambia3300 (CAMBIA) with *Sac*I 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 *Sma*I 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 previously (Yoo et al., 2007). *HSFA6a* and *HSFA6b* were cloned into p*35S*::EYFP (Kuo et al., 2013) to insert 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 2×10^4 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 (TCS SP5; Leica) as described previously (Hsu et al., 2010).

Protoplast Transactivation Assay

The potential promoters for *HSFA6b* (1.0 or 1.7 kb) as well as *DREB2A*, *HSP18.1-CI*, or *APX2* (1.0 or 0.865 kb) were amplified and cloned into the $P_{GAL4\text{-}UASx4}$::*GUS* vector. A CaMV *35S* promoter was cloned into the $P_{GAL4\text{-}UASx4}$::*GUS* vector, which resulted in P_{35S}::*GUS* construct, to be used as a positive control. The transactivation assay involved cotransfection with a mixture of 15 μg effector (P_{35S}::*AREB1-3XFLAG*, P_{35S}::*HSFA3-3XFLAG*, P_{35S}::*HSFA6a-3XFLAG*, or P_{35S}::*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 half-strength Murashige and Skoog 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 d, when the radicle had emerged from the testa, and cotyledon greening percentage was quantified at day 10. Three-day-old seedlings were transferred to NaCl-, mannitol-, or ABA-containing plates, and primary root growth was measured at days 7 to 9 by use of ImageJ (http://imagej.nih.gov/ij).

Thermotolerance, SOD Activity, H_2O_2, and Pigment Content Determination

Thermotolerance testing was performed as described (Charng et al., 2006; Hsu et al., 2010). Seedlings were grown at 22°C to 24°C with 16-h light for 6 to 9 d 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 d after the end of HS treatment. In-gel SOD activity assay was as described (Chu et al., 2005; Huang et al., 2012a). The detection of H_2O_2 involved 3,3′-diaminobenzidine staining as described (Thordal-Christensen et al., 1997). Chlorophyll and anthocyanin measurement was as described (Porra et al., 1989; Neff and Chory, 1998).

Co-IP Assay

An amount of 4×10^5 protoplasts was transfected with 20 μg each of HSFA6b-3XFLAG and tester (HSFs)-YFP^N plasmids, then incubated at 22°C for 16 h and harvested with GM buffer (150 mM Tris-HCl, pH 7.4). 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 coimmunoprecipitated from total protein extracts with anti-GFP antibody (ab290; Abcom) and detected by SDS-PAGE followed by immunoblotting using α-FLAG antibody (sc-807; Santa Cruz Biotechnology).

ChIP Assay

Mature leaves (1.3 g) 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 (P-2014-24; Epigentek). An HA-specific antibody (H3663; Sigma-Aldrich) 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-day-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 two 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. DEGs, or genes with expression change >6-fold at $P < 0.10$ compared with the Col control treatment, were collected. GO enrichment analysis involved with the Cytoscape 3.0.2 (http://www.cytoscape.org) plugin BiNGO categories using the GO_Full categories

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(Maere et al., 2005) identified by a hypergeometric test with $P \leq 5.00E\text{-}04$ 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 three 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.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. The expression profiles of 21 Arabidopsis 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, SOD activity, and H_2O_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 qRT-PCR, and accession numbers.

Supplemental Table S2. The 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.

Supplemental Table S3. Enriched 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.

Supplemental 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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