The roles of *Arabidopsis HSFA2*,HSFA4a,
and HSFA7a in the heat shock response
and cytosolic protein response

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Abstract

Previously, we found that *Arabidopsis* plants transformed with a construct containing the promoter of Oshsp17.3 from rice fused to the β-glucuronidase gene (GUS), Oshsp17.3Pro::GUS (Oshsp17.3p), showed a GUS signal after heat shock (HS) or azetidine-2-carboxylic acid (AZC) treatment. HS and AZC trigger the heat shock response (HSR) and cytosolic protein response (CPR), respectively, in the cytosol by modulating specific heat shock factor (HSF) activity. Here we further identified that AtHSFA2 (At2g26150), AtHSFA7a (At3g51910), AtHSFB2a (At5g62020), and AtHSFB2b (At4g11660) are HS− and AZC-inducible; AtHSFA4a (At4g18880) is AZC-inducible; and AtHSFA5 (At4g13980) is less AZC− and HS-inducible. To investigate the roles of these 6 AtHSF in the HSR or CPR, we crossed two independent Oshsp17.3p transgenic *Arabidopsis* plants with the AtHSF-knockout mutants athsfA2 (SALK_008978), athsfA4a (GABI_181H12), athsfA7a (SALK_004385), athsfA2a (SALK_080138), athsfB2a (SALK_137766), and athsfB2b (SALK_047291), respectively. As compared with the wild type, loss-of-function mutation of AtHSFA2, AtHSFA4a, and AtHSFA7a decreased HS and AZC responsiveness, so these 3 AtHSFs are essential for the HSR and CPR. In addition, loss-of-function results indicated that AthsfB2b is involved in regulating the HSR in *Arabidopsis*. Furthermore, analysis of the relative GUS activity of two double knockout mutants, athsfA2/athsfA4a and athsfA2/athsfA7a, revealed that AtHSFA2, AtHSFA4a, and AtHSFA7a function differentially in the HSR and CPR. Transcription profiling in athsf mutants revealed positive or negative transcriptional regulation among the 6 AtHSFs in *Arabidopsis* plants under HS and AZC conditions. Tunicamycin treatment demonstrated that these 6 AtHSFs are not involved in the unfolded protein response.

Keywords: Azetidine−2-carboxylic acid, Cytosolic protein response, Heat shock factor, Heat shock protein, Heat shock response, Unfolded protein response

Background

Protein homeostasis is crucial for maintaining normal cellular function. Plants, being sessile organisms, cannot escape from their growing environments. Extremes in environmental factors can result in stressful conditions that inevitably damage proteins directly or cause cells to synthesize misfolded proteins, which can lead to perturbed cell function and stress-induced cell death. Plants have evolved an extensive network of chaperone systems to restore protein folding or to remove irreversibly unfolded proteins (Mehdy 1994; Shinozaki and Yamaguchi-Shinozaki 1996; Bukau et al. 2006; Cramer et al. 2011; Redondo-Gómez 2013).

Accumulation of unfolded proteins within cells, eliciting compartment-specific chaperones and pathways, is termed the unfolded protein response (UPR). The UPR initiates the dissociation of the endoplasmic reticulum (ER) chaperone, immunoglobulin binding protein, and ER master sensors, such as inositol-requiring 1 and protein kinase R-like ER kinase, to activate downstream effectors to restore protein homeostasis in the lumen of the ER. A cytosolic process, the cytoplasmic protein response (CPR), increases the synthesis of molecular chaperones such as heat shock proteins (HSPs). In
contrast to the better-understood UPR of the ER, the regulatory molecules in the CPR are not well elucidated.

The heat-shock response (HSR), predominantly a response to maintain protein-folding homeostasis in the cytosol, causes transcriptional activation of HSPs under thermal stress (Aparicio et al. 2005; Jungkunz et al. 2011). The expression of HSP genes is mainly under the control of heat shock transcription factors (HSFs) (Schöffl et al. 1998; Nover et al. 2001). The number of HSFs is characteristically higher in plants than in other organisms. For example, Arabidopsis and rice have 21 and 25 HSFs, respectively, but Drosophila, C. elegans and yeast have only one HSF (Nover et al. 2001; Guo et al. 2008; Scharf et al. 2012). The multiplicity of members of the HSF family in plants may contribute to their fitness to face varied environmental challenges such as extreme temperatures, drought, and salinity (Busch et al. 2005).

Plant HSFs are classified into three classes (A, B, and C) on the basis of structural characteristics and phylogenetic comparison. Class A HSFs contain a DNA binding domain, an oligomerization domain, nuclear localization domains, and transcriptional activation domains. Classes B and C lack a transcriptional activation domain (Nover et al. 2001). Recent studies of tomato HSF1a mutants and an Arabidopsis HSF1a/1b/1d/1e quadruple mutant revealed that members of HSF1 genes can function as master regulators for the HSR and play important roles in cross-regulation for abiotic stress responses (Mishra et al. 2002; Liu et al. 2011). Increasing evidence shows functional diversification among different HSF members.

In addition to heat shock (HS), a proline analog, azetidine-2-carboxylic acid (AZC), can induce accumulation of abnormal-misfolded proteins in the cytosol to trigger the CPR by modulating HSF2 activity (Yeh et al. 2007; Sugio et al. 2009; Nishizawa-Yokoi et al. 2011). In the current study, we fused the promoter of AZC-inducible small HSP (sHSP), Oshsp17.3, with the β-glucuronidase (GUS) (Oshsp17.3pro::GUS) and transformed into Arabidopsis AtHSF mutants, and detected GUS activity in response to AZC and HS (Guan et al. 2010). Our results allowed us to characterize the roles of Arabidopsis HSFs in the HSR and CPR.

**Methods**

**Plant materials**

The Arabidopsis thaliana ecotype Col-0 was used in this study as the wild type (WT). Seeds were surface-sterilized in commercial bleach that contained 5% (v/v) sodium hypochlorite and 0.1% (v/v) Triton X-100 solution for 10 min, rinsed in sterilized water, and stratified at 4 °C for 2 days in the dark. Seeds were germinated on growth agar plates [1/2 Murashige and Skoog medium (MS; Duchefa), 1% sucrose (w/v), 0.8% agar (w/v)]. The T-DNA insertion lines SALK_008978 (athsfa2), GABI_181H12 (athsfa4a), SALK_004385 (athsfa5), SALK_080138 (athsfa7a), SALK_137766 (athsfb2a), and SALK_047291 (athsfb2b) mutants were obtained from the Arabidopsis Biological Resources Center (ABRC, Columbus, OH, USA) (Liu et al. 2011; Kleinboelting et al. 2012). The athsf2a/athsfa4a and the athsf2a/athsfa7a double mutants were generated by crossing athsf2a with athsf4a and athsf7a mutants. Mutant seeds were germinated and selected on selection agar plates [1/2 MS, 1% sucrose (w/v), 25 μg/ml hygromycin, 0.8% agar (w/v)]. All seedlings were grown at 23 °C in a 16-h light/8-h dark cycle in a growth chamber with 60% relative humidity.

**RNA isolation and RT-PCR**

Total RNA was extracted from 10-day-old Arabidopsis seedlings as described (Guan et al. 2010). The first-strand cDNA was synthesized with 1 μg total RNA by using the SuperScript III First-Step Synthesis System (Invitrogen). PCR amplification corresponding to different AtHSFs shown in Fig. 3 were 30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C, then 5 min at 72 °C. Primers used for analysis of gene expression were designed by use of NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are in Table 1. DNA from 15 μl of each PCR reaction was fractionated by electrophoresis on 1.2% (w/v) agarose gel with 0.01% (w/v) ethidium bromide in 1× Tris–Acetate EDTA buffer. The gel was

**Table 1 Oligonucleotides used in RT-PCR**

| Gene | Primer name | Sequence |
|------|-------------|----------|
| AtHSFA2 | AtHSFA2-Fw | 5′-CCATGGGACAAGTCAGCTGGGAAATGG AGG-3′ |
| AtHSFA2 | AtHSFA2-Rv | 5′-GGCAGAGCCTTGGCAAGACTGAG-3′ |
| AtHSFA4a | AtHSFA4a-Fw | 5′-CATCAAAGCTGGAGGAAATGCTAG‑3′ |
| AtHSFA4a | AtHSFA4a-Rv | 5′-GCTCTGCACTATTTTCACGCA-3′ |
| AtHSF5 | AtHSF5-Fw | 5′-GAGAAAGCTGGTGCTGTTGATG‑3′ |
| AtHSF5 | AtHSF5-Rv | 5′-GATCTGGCTGGTTCATATGAA-3′ |
| AtHSFA7a | AtHSFA7a-Fw | 5′-GAGAAAGCTGGTGCTGTTGATG‑3′ |
| AtHSFA7a | AtHSFA7a-Rv | 5′-ATCTGACCAATATATGCGAAT‑3′ |
| AtHSFB2a | AtHSFB2a-Fw | 5′-GGCCTGTTGATGCTGTTGATG‑3′ |
| AtHSFB2a | AtHSFB2a-Rv | 5′-GAGAAAGCTGGTGCTGTTGATG‑3′ |
| AtTubulin | AtTubulin-Fw | 5′-GAAATCGGTGCTGCTTATCC‑3′ |
| AtTubulin | AtTubulin-Rv | 5′-GAAATCGGTGCTGCTTATCC‑3′ |
| AtbZIP60 | AtbZIP60-Fw | 5′-GGGAAAGCTGGTGCTGTTGATG‑3′ |
| AtbZIP60 | AtbZIP60-Rv | 5′-GGGAAAGCTGGTGCTGTTGATG‑3′ |
digitally photographed and the corresponding DNA signal was quantified by using ImageJ (http://rsbweb.nih.gov/ij/) (Schneider et al. 2012) and normalized to 18S rRNA expression.

**Preparation of DNA constructs and transformation**

Oshsp17.3pΔAZRE::GUS (Oshsp17.3pΔAZRE::GUS) were constructed and transformed Arabidopsis plants as described (Guan et al. 2010). Transgenic plants #5 and #11 of Oshsp17.3Pro::GUS (ΔAZRE::GUS) were constructed and showed weak GUS expression induced by HS and AZC (Guan et al. 2010), were selected to cross with AtHSF mutants athsfA2, athsfA4a, athsfA5, athsfA7a, athsfB2a, athsfB2b, athsfA2/athsfA4a, and athsfA2/athsfA7a mutants, respectively. F2 lines Oshsp17.3p5/athsfA2, Oshsp17.3p5/athsfA4a, Oshsp17.3p5/athsfA5, Oshsp17.3p5/athsfA7a, Oshsp17.3p5/athsfB2a, Oshsp17.3p5/athsfB2b, Oshsp17.3p5/athsfA2/athsfA4a, Oshsp17.3p5/athsfA2/athsfA7a, Oshsp17.3p11/athsfA2, Oshsp17.3p11/athsfA4a, Oshsp17.3p11/athsfA5, Oshsp17.3p11/athsfA7a, Oshsp17.3p11/athsfB2a, Oshsp17.3p11/athsfB2b, Oshsp17.3p11/athsfA2/athsfA4a, and Oshsp17.3p11/athsfA2/athsfA7a were obtained and then self-pollinated to produce the F3 generation, which was used for analysis of HS and AZC responsiveness in this study. In addition, transgenic plants #2 and #7 of Oshsp17.3Pro::ΔAZRE::GUS (Oshsp17.3pΔAZRE), which showed weak GUS expression with HS and AZC treatment (Guan et al. 2010), were used as the negative control.

**Stress treatment of transgenic Arabidopsis mutants**

For HS treatment, 10-day-old F3-generation Arabidopsis seedlings were incubated in shaking buffer [1% sucrose (w/v), 5 mM potassium phosphate buffer, pH 6.8] at 39 °C for 1 h, then 23 °C for 20 h of recovery. For AZC treatment, 10-day-old F3-generation Arabidopsis seedlings were incubated in shaking buffer with or without 5 mM AZC at 23 °C for 4 h, rinsed in sterilized water, then incubated in shaking buffer at 23 °C for 15 h of recovery. For tunicamycin (Tm) treatment, 10-day-old F3-generation Arabidopsis seedlings were incubated in shaking buffer with or without 5 μg/ml Tm at 23 °C for 4 h, rinsed in sterilized water, then incubated in shaking buffer at 23 °C for 15 h of recovery. All samples were frozen by liquid nitrogen and stored at −80 °C.

**GUS staining**

GUS staining was described previously (Guan et al. 2010). In brief, 10-day-old seedlings were treated and incubated in the fixation solution (0.3% formaldehyde, 0.1% Triton X-100, 0.1% β-mercaptoethanol, 100 mM sodium phosphate buffer, pH 7.0) for 60 min. Then the fixation solution was replaced with washing solution (100 mM sodium phosphate buffer, 1 mM EDTA, pH 7.0) twice for 15 min. Washed seedlings were vacuum-infiltrated for 5 min in GUS staining buffer (1 mM X-Gluc, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 0.1% Triton X-100, 10 mM EDTA, 100 mM sodium phosphate buffer, pH 7.0), then incubated at 37 °C for 24 h. The staining reaction was stopped by adding distilled water, the color of chlorophyll was removed with 70% ethanol (v/v) several times, and seedlings were soaked in 95% ethanol (v/v) for 1 h. Plants were photographed to record deposition of the GUS.

**Analysis of GUS activity**

Seedlings after HS or AZC treatment were powdered in liquid nitrogen and extracted with GUS extraction buffer (50 mM sodium phosphate buffer, 10 mM EDTA, 0.1% SDS, 0.1% triton X-100, 0.1% β-mercaptoethanol, 1 mM PMSE, pH 7.0). After centrifugation, 10-μl protein extract was mixed with 990-μl GUS assay solution [2.5 mM MUG, 50 mM NaPO4, 10 mM EDTA, 10 mM DTT, 2% Leupeptin (w/v), 20% methanol (v/v), pH 7.0], which was preheated in 37 °C for 5 min, and extract was incubated in 37 °C for 1 h. For GUS activity assay, the fluorescence was measured in a Fluoroskan Ascent FL fluorometer (Labsystems, Helsinki, Finland).

**Statistical analysis**

Data are shown as mean±SE from three independent experiments. Statistical differences were analyzed by Student t test or Duncan multiple range test. P<0.05 was considered statistically significant.

**Results**

**Transcript levels of AtHSFs under heat and AZC stress**

HSF2A, HSF2a7a, HSF2B1, HSF2B2a, and HSF2B2b were previously found as AZC- and HS-inducible HSFs in Arabidopsis seedlings (Sugio et al. 2009). To further confirm the responsiveness of Arabidopsis HSFs to AZC and HS under our test conditions, we analyzed transcript levels of Arabidopsis HSFs under AZC and heat treatments (data not shown). We selected highly AZC- and HS-inducible AtHSF2a (At2g26150; 41.8–21.8-fold and 31.3–5-fold induction, respectively), AtHSF2a7a (At3g51910; 4.1–2.9-fold and 8.8–2.2-fold, respectively), AtHSF2b2a (At5g62020; 26.8–18.6-fold and 8.7–6.7-fold, respectively), and AtHSF2b2b (At4g11660; 4.5–2.9-fold and 8.7–3.5-fold, respectively) as candidate HSFs for further study (Fig. 1). In addition, AtHSE4a (At4g18880), which showed AZC responsiveness (3.9–3.2-fold), and AtHSE5 (At4g13980), which showed less AZC and HS responsiveness, were included in the test.
**AtHSFA2, AtHSFA4a, and AtHSFA7a genes function differentially in response to heat and AZC stress**

To investigate whether the AtHSFs examined are involved in the HS or AZC responsiveness, two independent OsHS1.3p5GUS transgenic plants, OsHS1.3p5 and OsHS1.3p11, were separately crossed with athsfA2, athsfA4a, athsfA5, athsfA7a, athsfB2a, and athsfB2b mutants. Lines OsHS1.3p5/athsfA2, OsHS1.3p5/athsfA4a, OsHS1.3p5/athsfA5, OsHS1.3p5/athsfA7a, OsHS1.3p5/athsfB2a, OsHS1.3p5/athsfB2b, OsHS1.3p5/athsfA7a/athsfA4a, OsHS1.3p5/athsfA7a/athsfA2/athsfA4a, OsHS1.3p5/athsfA7a/athsfA2/athsfA4a, OsHS1.3p5/athsfB2a/athsfB2b, OsHS1.3p5/athsfB2b/athsfB2a, OsHS1.3p5/athsfA7a/athsfA2/athsfA4a, OsHS1.3p5/athsfA7a/athsfA2/athsfA4a, OsHS1.3p5/athsfA5/athsfB2a, OsHS1.3p5/athsfA5/athsfB2b, OsHS1.3p5/athsfB2a/athsfA7a, OsHS1.3p5/athsfB2b/athsfA7a, Oshsp17.3p5/athsfA2, Oshsp17.3p5/athsfA4a, Oshsp17.3p5/athsfA5, Oshsp17.3p5/athsfA7a, Oshsp17.3p5/athsfA2/athsfA4a, Oshsp17.3p5/athsfA2/athsfA7a, Oshsp17.3p5/athsfA2/athsfB2a, Oshsp17.3p5/athsfA2/athsfB2b, Oshsp17.3p5/athsfA4a/athsfA7a, Oshsp17.3p5/athsfA4a/athsfA5, Oshsp17.3p5/athsfA7a/athsfB2a, Oshsp17.3p5/athsfA7a/athsfB2b, Oshsp17.3p5/athsfB2b/athsfA7a, Oshsp17.3p5/athsfB2a/athsfA7a, and Oshsp17.3p5/athsfB2a/athsfB2b were obtained for analyzing HS and AZC responsiveness.

Under the HS condition (39°C for 1 h), OsHS1.3p5 plants showed GUS staining; OsHS1.3p5/athsfA2, OsHS1.3p5/athsfA4a, OsHS1.3p5/athsfA7a, and OsHS1.3p5/athsfB2b plants showed reduced GUS expression; and GUS staining was similar in OsHS1.3p5/athsfA5 and OsHS1.3p5/athsfB2a plants (Fig. 2a). Under AZC treatment (5 mM AZC for 4 h), both cotyledons and true leaves of OsHS1.3p5/athsfA2 and OsHS1.3p5/athsfA7a plants did not show any GUS signal (Fig. 2a), and true leaves of OsHS1.3p5/athsfA4a, OsHS1.3p5/athsfB2a, and OsHS1.3p5/athsfB2b plants showed little or no GUS signal; the profile of GUS staining was similar in OsHS1.3p5 and OsHS1.3p5/athsfA5 plants. Similar HS- and AZC-induced profile of GUS staining was found in OsHS1.3p11, OsHS1.3p11/athsfA2, OsHS1.3p11/athsfA4a, OsHS1.3p11/athsfA5, OsHS1.3p11/athsfA7a, OsHS1.3p11/athsfB2a, OsHS1.3p11/athsfB2b, OsHS1.3p11/athsfA2/athsfA4a, and OsHS1.3p11/athsfA2/athsfA7a (data not shown).

The reduction in HS and AZC responsiveness measured by GUS activity was further confirmed quantitatively. With HS treatment, GUS activity was about 55% lower for OsHS1.3p5/athsfA2 than OsHS1.3p5 plants (Fig. 2b). Also, GUS activity was lower for OsHS1.3p5/athsfA4a, OsHS1.3p5/athsfA7a, and OsHS1.3p5/athsfB2b than OsHS1.3p5 plants (36, 24–34, and 36% reduction, respectively). Similar reduction of GUS activity was further confirmed in OsHS1.3p5/11/athsfA2, OsHS1.3p5/11/athsfA4a, OsHS1.3p5/11/athsfA7a, and OsHS1.3p5/11/athsfB2b compared with OsHS1.3p5 plants (Fig. 2c).

We did not find a significant difference in GUS activity among OsHS1.3p5, OsHS1.3p5/11/athsfA5, OsHS1.3p5/11/athsfA7a, OsHS1.3p5/11/athsfA5/11/athsfA7a, OsHS1.3p5/11/athsfB2a, and OsHS1.3p5/11/athsfB2b plants. These loss-of-function results indicate that mutation of AthsfA2, AthsfA4a, AthsfA7a, and AthsfB2b may alter HS responsiveness in Arabidopsis plants.

Furthermore, we crossed athsfA2/athsfA4a and athsfA2/athsfA7a plants with OsHS1.3p5 and OsHS1.3p11 transgenic Arabidopsis, respectively and obtained OsHS1.3p5/athsfA2/athsfA4a, OsHS1.3p5/11/athsfA2/athsfA4a, OsHS1.3p5/11/athsfA2/athsfA7a, and OsHS1.3p5/11/athsfA2/athsfA7a Arabidopsis plants for testing HS and AZC responsiveness. With HS treatment, GUS signal was absent in true leaves of OsHS1.3p5/athsfA2/athsfA4a and cotyledons of OsHS1.3p5/athsfA2/athsfA7a, and AZC-induced GUS signal was not significant in OsHS1.3p5/athsfA2/athsfA4a or OsHS1.3p5/athsfA2/athsfA7a Arabidopsis plants, which was similar to OsHS1.3p5/athsfA2 and OsHS1.3p5/AAZRE plants (Fig. 2a). Similar HS- and AZC-induced profile of GUS staining was found in OsHS1.3p11/athsfA2/athsfA4a and OsHS1.3p11/
Fig. 2 Analysis of HS and AZC responsiveness of AtHSF mutants by GUS staining. AtHSF mutant plants were transformed with a chimeric Oshsp17.3-Pro::GUS gene as described. a Seedlings from independent transgenic lines underwent HS or AZC treatment as indicated and GUS histochemical staining. Non-stress control condition (Ctrl). Relative GUS activity of seedlings treated with b, c HS and d, e AZC. Data are mean ± SE GUS activity relative to that of the Ctrl from three independent experiments and the fold expression is indicated. Bars with the same letter are not significantly different at $P < 0.05$. 
of Arabidopsis plants. These results suggest that athsfA7a, Oshsp17.3p5/athsfA2, and Oshsp17.3p5/athsfA7a level was increased in athsfB2a plants and Oshsp17.3p5/Oshsp17.3p5/athsfA5 plants, significantly elevated in Oshsp17.3p5/Oshsp17.3p5/athsfA2 and Oshsp17.3p5/Oshsp17.3p5/athsfA4a and, Oshsp17.3p5/Oshsp17.3p7a plants (Fig. 2b). Also, GUS activity was lower for Oshsp17.3p5/athsfA2/athsfA4a than Oshsp17.3p5/athsfA2, 30 cycles. In addition, GUS activity did not significantly differ among Oshsp17.3p5/athsfA2/Oshsp17.3p5/athsfA4a, Oshsp17.3p5/athsfA2/Oshsp17.3p11 and Oshsp17.3p5/athsfA7a plants (Fig. 2c). With AZC treatment, the GUS activity for Oshsp17.3p5/athsfA2/Oshsp17.3p5/athsfA4a and Oshsp17.3p5/athsfA2/Oshsp17.3p5/athsfA7a plants dropped to a level (38 and 40% of GUS activity, respectively, versus Oshsp17.3p5/athsfA2 plants) comparable to that for Oshsp17.3p5/athsfA2 and Oshsp17.3p5/AZRE plants (Fig. 2d). In addition, GUS activity did not significantly differ among Oshsp17.3p5/athsfA2/Oshsp17.3p5/athsfA4a, Oshsp17.3p5/athsfA2/Oshsp17.3p11/athsfA4a, and Oshsp17.3p5/athsfA2 plants (Fig. 2e). These results suggest that AthSF2a, AthSF4a, and AthSF7a genes function independently in the HSR of Arabidopsis plants.

Positive and negative regulation among the AthSFS

Data in Fig. 1 revealed that AthSF2a, AthSF7a, AthSF2b, and AthSF2b were HS- and AZC-inducible and AthSF4a was AZC-inducible. We examined the expression profiles of the 6 AthSFs in the mutants under stress. After 1 h of heat treatment, compared with the WT and Oshsp17.3p5 plants, AthSF4a transcript level was significantly elevated in Oshsp17.3p5/athsfA5 and Oshsp17.3p5/athsfB2a plants and AthSF7a level was increased in Oshsp17.3p5/athsfA2, Oshsp17.3p5/athsfA5, Oshsp17.3p5/athsfB2a, and Oshsp17.3p5/athsfA2/athsfA4a plants, with no significant change in AthSF2a, AthSF5, AthSF2b, and AthSF2b levels in the mutant plants tested (Fig. 3a). With AZC treatment, AthSF2a and AthSF4a levels were reduced in Oshsp17.3p5/athsfB2a and Oshsp17.3p5/athsfA2 plants, respectively, and AthSF7a level was increased in Oshsp17.3p5/athsfA4a and Oshsp17.3p5/athsfB2b plants (Fig. 3b). Similar expression profiles of the 6 AthSFs were also found in Oshsp17.3p11, Oshsp17.3p11/athsfA2, Oshsp17.3p11/athsfA4a, Oshsp17.3p11/athsfA5, Oshsp17.3p11/athsfA7a, Oshsp17.3p11/athsfB2a, Oshsp17.3p11/athsfB2b, Oshsp17.3p11/athsfA2/athsfA4a, and Oshsp17.3p11/athsfA2/athsfA7a plants under HS and AZC conditions (data not shown). These results suggest a finely tuned activation and repression of the expression of HSFs under HS and AZC stress.

AthSF2a, AthSF4a, and AthSF7a are not responsive to Tm

AZC typically induces the UPR and CPR. The data in Fig. 2 indicated that AthSF2a, AthSF4a, and AthSF7a are essential for the HSR and AZC response in Arabidopsis. Studies have shown AthSF2a as a crucial regulatory component of the CPR (Sugio et al. 2009). To understand whether these AthSFs are involved in the UPR, we examined the effect of Tm treatment (UPR induction) in the AthSF mutants tested. Tm did not activate the expression of the 6 AthSF genes (Fig. 4a). On GUS analysis, no Tm responsiveness was detected in the mutant plants tested (Fig. 4b, c). These results confirmed that AthSF2a, AthSF4a, and AthSF7a function in the CPR.

Discussion

To adapt to biotic and abiotic stresses, plants have evolved a complex set of molecular responses, which often exhibit features sharing substantial overlap...
pathways and components. HSF/HSP responses are recognized as central chaperone components against unfolded protein accumulation, a signal for triggering HSR, UPR, or CPR based on distinct subcellular localization (Aparicio et al. 2005; Swindell et al. 2007; Yeh et al. 2007). Many reports have shown that HSFs are important for resistance to heat and other environmental stresses (Mishra et al. 2002; Charng et al. 2007; Banti et al. 2010; Liu et al. 2011). Using an HS- and AZC-sensitive promoter-GUS fusion system (Guan et al. 2010) together
with knockout plants, we aimed to identify the contribution of AtHSFA2, AtHSFA4a, AtHSFA5, AtHSFA7a, AtHSFB2a, and AtHSFB2b to the responses induced by HS, AZC, and Tm.

Plant HSFs are regulated by HS and AZC, including up- and downregulation. We found the expression of AtHSFA2, AtHSFA4a, AtHSFA7a, AtHSFB2a, and AtHSFB2b induced > twofold with 1-h HS treatment and then reduced after prolonged heat incubation (Fig. 1). As well, AZC upregulated AtHSFA2, AtHSFA4a, AtHSFA7a, AtHSFB2a, and AtHSFB2b expression > 2.9-fold during treatment. However, Tm did not affect the expression of the 6 AtHSFs (Fig. 4a). Despite a slight difference in plant material and treatment time, the results are similar to published microarray data (Busch et al. 2005; Schramm et al. 2008; Sugio et al. 2009), finding that AtHSFA2, AtHSFA4a, AtHSFA7a, AtHSFB2a, and AtHSFB2b are important for stress response networks.

Studies have shown that AtHSFA2 and AtHSFA7a knockout mutants lose acquired thermotolerance, and AtHSFA2 mutants also show reduced tolerance to AZC (Chang et al. 2007; Siddique et al. 2008; Sugio et al. 2009). In this study, loss-of-function mutation of AtHSFA2 significantly repressed relative GUS activity under HS and AZC treatment (Fig. 2b–e). By contrast, null mutation of AtHSFA4a and AtHSFA7a only slightly repressed relative GUS activity under HS and AZC stress. These results agree with others showing that AtHSFA2 is closely related to the regulation of HSR as well as CPR (Busch et al. 2005; Nishizawa et al. 2006; Ogawa et al. 2007; Sugio et al. 2009; Jung et al. 2010), whereas AtHSFA4a and AtHSFA7a have a lesser effect on HSR and CPR. Furthermore, as compared with AtHSFA2 knockout alone, double knockout with AtHSFA2 and AtHSFA4a or AtHSFA7a showed more significant repression of HS-induced GUS activity (Fig. 2b–e). Thus, AtHSFA2, AtHSFA4a, and AtHSFA7a may be linked to activation of different target genes/pathways in the HSR. However, AtHSFA2 appears to be a functionally redundant factor to AtHSFA4a and AtHSFA7a for AZC-induced CPR because the GUS activity of AtHSFA2-knockout plants was similar to that with double knockout of AtHSFA2 and AtHSFA4a or AtHSFA7a under AZC treatment (Fig. 2b–e).

Ikeda et al. (2011) reported that AtHsfB1 and AtHsfB2b, sharing functional redundancy in repressive activities, were able to suppress the accumulation of AtHSFA2 and AtHSFA7a transcripts and were indispensable for acquired thermotolerance. As compared with AtHSFA2 knockout, AtHsfB2b knockout slightly repressed GUS activity in response to HS treatment (Fig. 2b, c). We also revealed no significant change in HS-induced AtHSFA2 and AtHSFA7a transcript levels with AtHsfB2b knockout (Fig. 3a). These results suggest that AtHSFB2b may mediate the HSR but not CPR. Of note, AtHSFB2a is highly AZC- and HS-inducible, but we did not find a significant reduction in GUS activity with AtHsfB2a knockout during AZC treatment. However, we cannot absolutely exclude the role of AtHsfB2a in AZC-induced CPR because of its high expression under AZC and HS treatment.

In conclusion, we confirmed and characterized the roles of AtHSFA2, AtHSFA4a, AtHSFA5, AtHSFA7a, AtHSFB2a, and AtHSFB2b in the HSR and CPR. For simplifying our result, we propose a working model to show the roles of following AtHSFs in CPR and HSR (Fig. 5). AtHSFA2, AtHSFA4a, and AtHSFA7a function independently in the HSR, but AtHSFA2 may function redundantly with AtHSFA4a and AtHSFA7a in the CPR. AtHSFB2b has some role in mediating the HSR, and AtHSFA5 and AtHSFB2a cannot mediate the HSR and CPR. These 6 AtHSFs are not involved in the UPR.

Abbreviations
AZC, azetidine-2-carboxylic acid; AZRE, azetidine-2-carboxylic acid response element; CPR, cytosolic protein response; HSF, heat shock factor; HSP, heat shock protein; HSR, heat shock response; Tm: tunicamycin.

Authors’ contributions
C-AL, S-JW, and C-HY conceived the concept and designed the experiment. K-FL and M-YT have equally contributed towards this manuscript. K-FL and M-YT performed the experiments and analyzed the data. C-HY wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests. And there have neither financial competing interests nor other competing interests.
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