Common and Distinct Functions of Arabidopsis Class A1 and A2 Heat Shock Factors in Diverse Abiotic Stress Responses and Development[1][W][OPEN]

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There are 21 heat shock factor (HSF) homologs in Arabidopsis (*Arabidopsis thaliana*), of which members of class A1 (HSFA1a/HSFA1b/HSFA1d/HSFA1e) play the major role in activating the transcription of heat-induced genes, including *HSFA2*. Once induced, HSFA2 becomes the dominant HSF and is able to form heterooligomeric complexes with HSFA1. However, whether HSFA2 could function independently as a transcription regulator in the absence of the HSFA1s was undetermined. To address this question, we introduced a *Cauliflower mosaic virus* 35S promoter:*HSFA2* construct into *hsfa1a/hsfa1b/hsfa1d/hsfa1e* quadruple knockout (*QK*) and wild-type (*Wt*) backgrounds to yield transgenic lines *A2QK* and *A2Wt*, respectively. Constitutive expression of HSFA2 rescued the developmental defects of the *QK* mutant and promoted callus formation in *A2QK*, but not in *A2Wt*, after heat treatment. Transcriptome analysis showed that heat stress response genes are differentially regulated by the HSFA1s and HSFA2; the genes involved in metabolism and redox homeostasis are preferentially regulated by HSFA2, while HSFA1-preferring genes are enriched in transcription function. Ectopic expression of HSFA2 complemented the defects of *QK* in tolerance to different heat stress regimes, and to hydrogen peroxide, but not to salt and osmotic stresses. Furthermore, we showed that HSFA1a/HSFA1b/HSFA1d are involved in thermotolerance to mild heat stress at temperatures as low as 27°C. We also noticed subfunctionalization of the four Arabidopsis A1-type HSFs in diverse abiotic stress responses. Overall, this study reveals the overlapping and distinct functions of class A1 and A2 HSFs and may enable more precise use of HSFs in engineering stress tolerance in the future.

Plants have evolved complex response systems to cope with environmental stresses. Dynamic reprogramming of transcriptional activities constitutes one of the major events that enhance stress tolerance. Several transcription factor families are involved in complex and overlapping responses under different stress conditions (Singh et al., 2002). WRKY, MYB, APETALA2/ethylene response factor, bZIP, NAC, zinc-finger proteins, and heat shock factors (HSFs) are encoded by large gene families and have been intensively studied for their roles in stress responses (Singh et al., 2002; Saibo et al., 2009; Hirayama and Shinozaki, 2010; Santos et al., 2011; Scharf et al., 2012). Among these transcription factor families, HSFs are of particular interest because their functions in heat stress response and thermotolerance are highly conserved across all eukaryotes.

HSFs are characterized by a DNA-binding domain and hydrophobic heptad repeat regions (Wu, 1995; Morimoto, 1998; Åkerfelt et al., 2010). Current models suggest that in higher eukaryotes, typical HSFs assemble into an active, trimeric conformation via the hydrophobic heptad repeat regions in response to stress factors (Åkerfelt et al., 2010; Anckar and Sistonen, 2011). The trimerized transcription factors bind to the conserved heat shock cis-elements (GAAnnTTC) in the promoters of target genes via the DNA-binding domains and further recruit transcription machineries for gene expression. During nonstress or poststress periods, HSF activity is negatively regulated by attenuators such as HEAT SHOCK PROTEIN70 (HSP70), HSP90, and HSF binding protein (HSBP) or modulated by posttranslational modifications such as phosphorylation, acetylation, and sumoylation (Morimoto, 1998; Satyal et al., 1998; Pirkkala et al., 2001; Åkerfelt et al., 2010; Xu et al., 2012). In contrast to yeast (*Saccharomyces cerevisiae*) and animals, which contain only one to four HSFs (Åkerfelt et al., 2010), between 19 and 52 HSF homologs have been identified in the sequenced genomes of flowering plants (Scharf et al., 2012). The multiplicity of HSFs in plant species has been attributed to gene duplication and functional divergence during evolution (Scharf et al., 2012).

The Arabidopsis (*Arabidopsis thaliana*) genome encodes 21 HSF homologs that can be categorized into three major classes (A, B, and C) and 14 groups (A1–A9, B1–B4, and C1; Nover et al., 2001; von Koskull-Döring et al., 2007). There are four *HSF* genes in the A1 group, *HSFA1a*, *HSFA1b*, *HSFA1d*, and *HSFA1e*. *HSFA1a* and *HSFA1d*, and *HSFA1b* and *HSFA1e* both constitute pairs of duplicated genes diverged after a recent whole genome duplication

[1] This work was supported by the National Science Council, Taiwan (grant no. 97–2311–B–001–007–MY3).

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[W] The online version of this article contains Web-only data.

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www.plantphysiol.org/cgi/doi/10.1104/pp.113.221168

event (Blanc et al., 2003). Studies on *HSFA1a/HSFA1b/HSFA1d/HSFA1e* quadruple knockout (KO) and four triple KO mutants showed that the four A1-type HSFs have overlapping functions in the development of seeds and cotyledons and HSFA1a, HSFA1b, or HSFA1d alone, but not HSFA1e, could trigger heat stress response and confer thermotolerance (Liu et al., 2011; Yoshida et al., 2011). The triple KO of *HSFA1a/HSFA1b/HSFA1d* and the quadruple KO (*QK*) mutants show dramatic defects in tolerance to different heat stress regimes, suggesting that *HSFA1a/HSFA1b/HSFA1d* are the master regulators of heat stress response in Arabidopsis. In addition, tolerance to salt, osmotic, and oxidative stresses were compromised in the *QK* mutant (Liu et al., 2011), suggesting that HSFA1s function under a broad range of adverse conditions that are not limited to heat stress. The roles of individual HSFA1s in response to the stress factors other than heat have not been determined.

In response to elevated temperature, HSFA1s trigger a transcriptional cascade by inducing the expression of diverse transcription regulators, including HSFs of other classes (class A2, A3, A7, B1, and B2), DREB2A, DREB2B, MBF1C, and bZIP28 (Liu et al., 2011; Yoshida et al., 2011; Liu and Charng, 2012). Several of these transcription regulators have been shown to be involved in heat stress response and thermotolerance (Sakuma et al., 2006; Charng et al., 2007; Gao et al., 2008; Larkindale and Vierling, 2008; Schramm et al., 2008; Suzuki et al., 2008; Yoshida et al., 2008; Ikeda et al., 2011). The heat inducibility of the HSFs is a feature unique to plants and is not found in yeast and animals (Nover et al., 2001; von Koskull-Döring et al., 2007), and of the HSFs, HSFA2 is the most highly induced upon heat stress (Busch et al., 2005). In Arabidopsis and tomato (*Solanum lycopersicum*), HSFA2 is structurally similar to the HSFA1s, in that they share conserved functional modules (Scharf et al., 2012). As a downstream gene of HSFA1, HSFA2 is involved in the late phase of heat stress response (Wunderlich et al., 2007), extending the effect of heat acclimation (Charng et al., 2007) and mediating the amplification of a subset of heat stress response genes (Schramm et al., 2006). In addition to heat stress, expression of HSFA2 is also positively regulated by HSFA1 under salt, osmotic, and oxidative stresses (Liu et al., 2011; Nishizawa-Yokoi et al., 2011), suggesting an epistatic relationship between these two types of HSFs under several stress conditions.

Transient expression of tomato HSFs in tobacco (*Nicotiana tabacum*) protoplasts indicated that the efficient nuclear import of HSFA2 requires interaction with HSFA1a (Scharf et al., 1998; Heerklotz et al., 2001). Interaction between tomato HSFA1a and HSFA2 leads to the formation of heterooligomeric complexes that can synergistically activate the expression of a small HSP (Chan-Schaminet et al., 2009). Interaction between Arabidopsis HSFA1a/HSFA1b and HSFA2 has also been demonstrated (Li et al., 2010). These observations raise the question of whether HSFA2 can function independently as a transcription regulator in the absence of the HSFA1s and, if so, to what extent. Moreover, the hetero- and homooligomers of HSFA1 and HSFA2 may preferentially activate different sets of genes. However, this notion is difficult to assess at the genomic level as the expression of HSFA2 strictly requires the presence of the HSFA1s (Mishra et al., 2002; Liu et al., 2011; Nishizawa-Yokoi et al., 2011; Yoshida et al., 2011).

To address the above questions, we produced transgenic plants constitutively expressing Arabidopsis HSFA2 in the background of the *HSFA1 QK* mutant and examined whether ectopic HSFA2 expression could rescue the defects of the mutant in development and stress tolerance under various conditions. Interestingly, complete or partial recovery from multiple defects was observed in the transgenic plants, suggesting that HSFA2 can at least partially replace the function of the HSFA1s. However, it could not rescue the defect of the *QK* mutant under salt or osmotic stresses. Genes preferentially activated by HSFA1 or HSFA2 upon heat stress were identified by microarray analysis of the transcriptomes of the wild type, *hsfa2* (the transfer DNA KO mutant of *HSFA2*), the *QK* mutant, and the *QK* mutant transformed with recombinant *35S:HSFA2* (*A2QK*). We also showed that HSFA1a/HSFA1b/HSFA1d are involved in thermotolerance to mild heat stress at temperatures as low as 27°C. Furthermore, results from the response of the triple KO mutants of HSFA1s to salt, osmotic, and oxidative stresses suggest that Arabidopsis HSFA1s went through subfunctionalization after gene duplication.

RESULTS

Ectopic Expression of HSFA2 Complements the Defects of *HSFA1 QK* Mutant in Growth and Development

To examine whether and to what extent HSFA2 functions in the absence of the HSFA1s, we generated transgenic lines containing a recombinant *HSFA2* complementary DNA fused to the *Cauliflower mosaic virus* 35S promoter in the *HSFA1a/HSFA1b/HSFA1d/HSFA1e QK* mutant background. Four transgenic lines, designated as *A2QK-7*, *A2QK-9*, *A2QK-10*, and *A2QK-12*, with a single insertion event were selected from 15 independent lines for further studies. As a comparison, the same transgene construct was introduced into the ecotype Columbia (Col-0) wild-type background to yield a transgenic line designated as *A2Wt*. Figure 1 shows the expression of *HSFA2* and the *HSFA1s* in different transgenic lines as determined by reverse transcription (RT)-PCR to confirm their genotypes. Under normal conditions, transcripts of *HSFA2* were detected in all the transgenic lines, but not in the wild type and *QK* mutant, indicating the constitutive expression of the transgene. *A2QK-7*/*A2QK-10* and *A2QK-9*/*A2QK-12* lines had high and low expression levels of *HSFA2*, respectively.

The growth and morphologies of the transgenic plants readily manifested the effect of constitutive expression of *HSFA2* in the *HSFA1 QK* mutant. Previously, we showed that the seedlings of the *QK* mutant had abnormal cotyledons, grew significantly slower

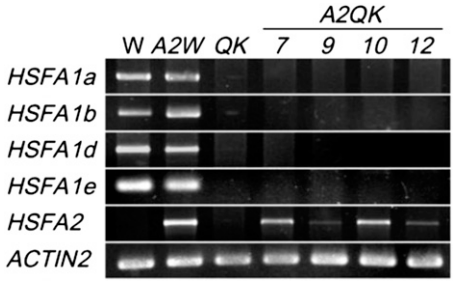

Figure 1. Transcript levels of the four *HSFA1*s and *HSFA2* in transgenic plants overexpressing HSFA2. RT-PCR analysis of the transcript levels of *HSFA1a*, *HSFA1b*, *HSFA1d*, *HSFA1e*, and *HSFA2* under normal conditions in 7-d-old seedlings of wild-type (W), *HSFA1*s *QK* mutant, and transgenic plants transformed with *35S:HSFA2* in wild-type (*A2W*) or *QK* backgrounds (*A2QK-7*, *A2QK-9*, *A2QK-10*, and *A2QK-12*). *ACTIN2* is shown as a loading control. For *HSFA2* and *ACTIN2*, 29 cycles of PCR were performed. For *HSFA1a*/*HSFA1b*/*HSFA1d*/*HSFA1e*, 34 cycles of PCR were performed. The amplification products were resolved electrophoretically on 1% to 3% agarose gels.

than the wild type, and produced smaller seeds (Liu et al., 2011). Expression of *HSFA2* in the *HSFA1 QK* mutant restored at least partially the seed size, seedling growth rate, cotyledon morphology, and root length (Fig. 2, A–D). In this study, we additionally noticed that the *QK* mutant produced aborted seeds at a rate about 10-fold higher than that of the Col-0 wild type (Fig. 2, E and F). When crossing Col-0 and the *QK* mutant with different gametophyte combinations, the higher abortion rate was associated with the female gametophyte of the *HSFA1 QK* mutant. Ectopic HSFA2 expression in the *QK* background significantly reduced the seed abortion rate (Fig. 2, E and F). These results suggest that HSFA2 could at least partially replace the role of the HSFA1s in growth and development.

Constitutive Expression of HSFA2 Induces Expression of HSPs in the Absence of HSFA1s

To investigate whether HSFA2 could activate the expression of HSPs in the absence of the HSFA1s, western-blot analysis was conducted to detect the levels of HSFA2, HSP101, HSP90, HEAT-STRESS-ASSOCIATED 32-kD PROTEIN (HSA32), and class I small HSPs (sHSP-CI) in *A2QK* lines. Under normal conditions, *A2QK-7*, *A2QK-10*, and *A2Wt* produced high levels of HSFA2 protein, while the *A2QK-9* and *A2QK-12* lines produced undetectable and low levels of HSFA2, respectively (Fig. 3). The wild type and *QK* mutant did not contain detectable HSFA2 under the same condition. The protein levels of HSFA2 in these plants were consistent with the transcript levels of *HSFA2* as determined by RT-PCR (Fig. 1). The levels of HSFA2 correlated with the levels of HSP101, HSA32, and sHSP-CI, but not HSP90, in the transgenic lines under nonstressed conditions, suggesting that HSFA2 could activate the expression of some HSPs in the absence of the HSFA1s.

Strong Induction of *HSFA2* in Response to Heat Stress Is Mainly Regulated by the HSFA1s Rather Than by Autoregulation

The strong induction of *HSFA2* in response to heat stress depends on the function of the HSFA1s (Liu et al., 2011). However, it is not clear whether HSFA1s trigger an initial low-level expression of HSFA2, which then amplifies its own expression via autoregulation. We noticed that heat treatment at 37°C for 1 h did not further increase the levels of HSFA2 protein in the *A2Wt* and *A2QK* transgenic lines (Fig. 3), suggesting that the recombinant HSFA2 does not activate the endogenous *HSFA2* gene in the transgenic lines. Because the antibody against HSFA2 could not distinguish the recombinant HSFA2 protein from the endogenous protein, RT-PCR analysis with discriminating primers was performed to address this possibility.

Two different primer pairs were designed for quantitative RT-PCR to reveal the transcript abundance of the endogenous *HSFA2* gene and total *HSFA2* transcripts derived from the combination of the endogenous and transgenic *HSFA2* (Fig. 4A). Figure 4B shows the quantitative analysis of the expression levels of endogenous *HSFA2* in the presence or absence of constitutively expressed *HSFA2* transgene. Without heat treatment, the recombinant HSFA2, regardless of its abundance, was unable to up-regulate the endogenous *HSFA2* in the presence or absence of the HSFA1s, as revealed by comparing the wild type to the *A2Wt* line and *QK* to the *A2QK* line. By contrast, the transcript levels of *HSP101* and *HSA32* were significantly higher in accordance with the higher level of total *HSFA2* transcripts, which mainly came from the expression of the transgene (Fig. 4B). After heat treatment, the transcripts of endogenous *HSFA2* were slightly increased in *A2QK-10* and *A2QK-12* but were much less abundant than in the wild type and *A2Wt*. No substantial difference was found between the total and endogenous transcripts of *HSFA2* in the *A2Wt* line after heat treatment, which was about 2-fold lower than that in the wild type (Fig. 4B). These results suggest that the strong induction of *HSFA2* in response to heat stress is mainly regulated by HSFA1s without substantial positive autoregulation by *HSFA2*.

Heat Stress Response Genes Are Differentially Regulated by the HSFA1s and HSFA2

The results shown in Figure 4B indicate that the HSFA1s and HSFA2 differentially regulate heat stress response genes. To further explore this phenomenon at the level of the genome, we compared the transcriptomic profiles of *hsfa2* (the KO mutant of *HSFA2*) and *A2QK-10* (overexpression of HSFA2 in the absence of the HSFA1s) seedlings with or without heat shock treatment using the Affymetrix ATH1 microarray chips. The difference in heat stress response between *hsfa2* and *A2QK-10* was attributed to differential regulation by the HSFs and was identified as follows. First, the microarray data were combined with those of the Col-0 wild type and the *QK*

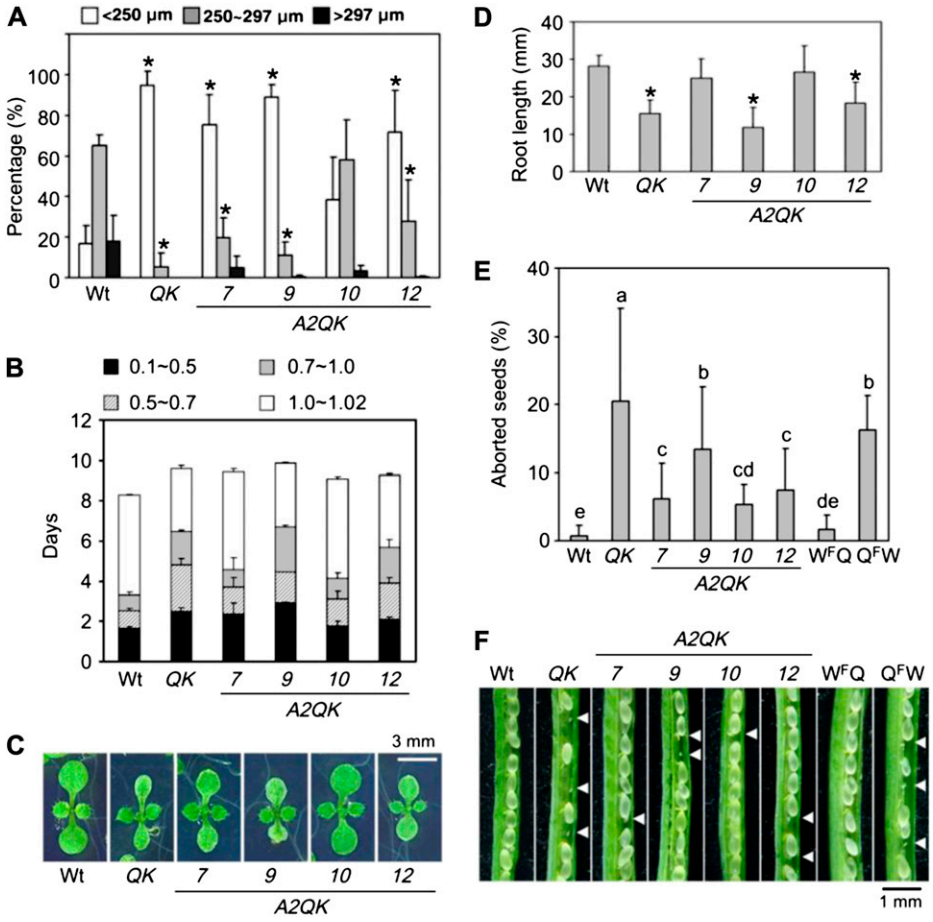

Figure 2. Ectopic HSFA2 expression complements the defects of *QK* mutant in development of seed and seedling. A, Distribution of seed size of Col-0 wild-type (Wt), *QK* mutant, and *A2QK* transgenic plants (*A2QK-7*, *A2QK-9*, *A2QK-10*, and *A2QK-12*). Results are presented as mean values of five replicates ± SD ($n \geq 100$ each). *$P < 0.01$ (versus the wild type, Student's *t* test). B, Growth rate from the imbibed seed to the two rosette leaf stage grown in 0.5× MS medium plates containing 1% Suc. Growth stages are defined as follows: 0.1, seed imbibition; 0.5, radicle emergence; 0.7, hypocotyl and cotyledon emergence; 1.0, cotyledons fully opened; and 1.02, two rosette leaves greater than 1 mm. The growth stages are presented as mean values of four replicates ± SD ($n = 45$ each). C, Morphologies of the representative plants at growth stage 1.02. D, Root length of the 7-d-old seedlings. Results are presented as mean values of six replicates ± SD ($n = 25$). *$P < 0.01$ (versus the wild type, Student's *t* test). E, The percentages of the aborted seeds in the immature siliques of wild-type, *QK* mutant, and *A2QK* transgenic plants and crosses with different gametophyte combinations of the wild type and *QK* mutant. W^FQ is the hybrid with the female gametophyte of the wild type and the male gametophyte of the *QK* mutant. Q^FW is the hybrid with the female gametophyte of the *QK* mutant and the male gametophyte of the wild type. The percentages were averaged from more than 15 siliques of five independent plants. Groups with different alphabets indicate there is a statistically significant difference among them (LSD, $\alpha = 0.05$). F, The development of seeds in immature siliques of wild-type, *QK* mutant, and *A2QK* transgenic plants and the hybrids of the wild type and *QK* mutant. The arrowheads indicate the aborted seeds.

mutant seedlings previously obtained under the same conditions (Liu et al., 2011). We found genes highly induced by heat by subjecting the signals of 22,810 probe sets on the microarray chip to two filters. Firstly, we eliminated 14,117 low-expressing probe sets whose normalized absolute expression values were lower than 100 in all samples. Secondly, 783 probe sets were selected that had at least a 5-fold change in any individual comparison with the Col-0 wild type under control conditions. Then, hierarchical clustering analysis was performed to group the coexpressed probe sets. We found 240 probe sets that were strongly heat induced in the wild type, *hsfa2*, or *A2QK*, but not in the *QK* mutant. The target preferences of the HSFA1s and HSFA2 were judged according to the ratio of expression level under heat stress conditions in the *hsfa2* and *A2QK-10* mutants (Supplemental Table S1). Genes with an expression level 2-fold higher in *hsfa2* than in *A2QK-10* were defined as HSFA1s preferring (Fig. 5A). HSFA2-preferring genes were defined as having a *hsfa2*/*A2QK-10* expression ratio of less than 0.5-fold (Fig. 5C), whereas genes shown in Figure 5B with an expression ratio between 0.5- and 2-fold were defined as having no substantial preference for HSFA1s or HSFA2.

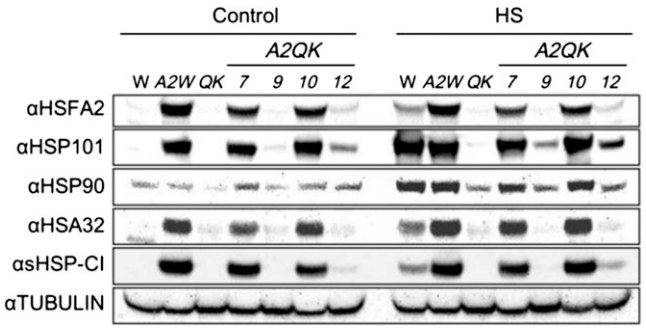

Figure 3. Constitutive expression of HSFA2 induces expression of HSPs in the absence of HSFA1s. Western-blot analysis of HSFA2 and HSPs in wild-type (W), *A2Wt* (*A2W*), *QK* mutant, and *A2QK* transgenic plants. Seven-day-old plants grown on medium plates were treated at 22°C (Control) or 37°C (Heat-shocked) for 1 h and recovered at 22°C for 2 h before protein extraction. In each lane, approximately 100 μg of total protein was loaded. Tubulin serves as a loading control.

Based on known or predicted functions, we sorted the 240 selected genes into functional categories (Table I and S1). For the HSFA1s-preferring genes, the most enriched categories were "chaperone and cochaperone" and "transcription," which contained 17 (23.6%) and 11 (15.3%) genes, respectively. For the genes with no preference for HSFA1s or HSFA2, the most enriched category was "chaperone and cochaperone" (27 genes; 35.1%). Among the genes with no preference, 14 genes were small HSPs. For the genes preferably regulated by HSFA2, 19 genes (20.9%) were found to be involved in various metabolic processes, eight genes (8.8%) were involved in redox homeostasis, and 13 genes (14.3%) had predicted enzyme functions. By contrast, only five genes (5.5%) were classified as chaperones and cochaperones.

To confirm the results of the microarray, we performed quantitative RT-PCR analysis of the transcript levels of *DREB2A* and *MITOCHONDRIAL HEAT SHOCK COGNATE 70-kD PROTEIN-2* (*mtHSC70-2*) (HSFA1s preferring), *HSP90.1* and *TEMPERATURE-INDUCED LIPOCALIN1* (*TIL1*; no preference), and *HSFA3* and *MYO-INOSITOL1-PHOSPHATE SYNTHASE2* (*MIPS2*; HSFA2 preferring). The expression ratios of heat-treated *hsfa2* versus *A2QK* plants for *DREB2A*, *mtHSC70-2*, *HSP90.1*, *TIL1*, *HSFA3*, and *MIPS2* were 3.34, 3.65, 0.5, 1.16, 0.07, and 0.016, respectively (Fig. 6). These data are consistent with those derived from the microarray.

Ectopic HSFA2 Expression Confers Tolerance to Diverse Heat Stress Regimes and Promotes Heat-Induced Callus Formation in the Absence of HSFA1s

Given the differential regulation of heat stress response genes by the HSFA1s and HSFA2, it was of interest to know whether HSFA2 could replace the role of the HSFA1s in conferring tolerance to four different heat stress regimes that are commonly used to reveal thermotolerance diversity in Arabidopsis (Yeh et al., 2012). The results of thermotolerance assays showed that expression of HSFA2 in *QK* dramatically increased the basal thermotolerance (BT) of *A2QK-7* and *A2QK-10*, which had a higher viability than the wild type (Fig. 7; Supplemental Fig. S1). Intriguingly, *A2QK-7* and *A2QK-10* also had a higher viability than the wild type under the assay conditions for short-term and long-term acquired thermotolerance and were comparable to that of the *A2Wt* (Fig. 7; Supplemental Fig. S1), suggesting that overexpression of HSFA2 is sufficient to confer a high level of acquired thermotolerance even in the absence of the master regulators. Expression of HSFA2 in the *QK* background also rescued the defect in thermotolerance to moderately high temperature (TMHT) but provided no obvious advantage as compared with the wild type (Fig. 7; Supplemental Fig. S1). By contrast, the low level of expression of HSFA2 in *A2QK-9* and *A2QK-12* could not rescue the thermotolerance defects of *QK*. Notably, the *A2QK* seedlings tended to form calluses at the apical meristems after the thermotolerance assays (Fig. 8). The number of calluses formed was positively correlated to the expression level of HSFA2. Calluses did not form in the *A2Wt* plants despite having amounts of HSFA2 similar to that of *A2QK-7* and *A2QK-10*, suggesting that the heat-induced callus formation is mediated by overexpression of HSFA2 in the absence of the HSFA1s.

QK and *hsf1a-hsf1b-hsf1d* Triple Mutants Are Hypersensitive to Prolonged Exposure to Temperatures as Low as 27°C

HSFA1a, HSFA1b, and HSFA1d redundantly confer tolerance to prolonged heat stress at 35°C in Arabidopsis (Liu et al., 2011). However, it is not known whether they are also important for tolerating long-term exposure to milder temperatures below 30°C, which do not kill Arabidopsis plants but result in adaptation by reprogramming the developmental processes (Franklin, 2009; Kumar and Wigge, 2010). To test this, phenotypes of the *QK* and the four triple KO mutants of the *HSFA1*s (labeled as *aTK*, *bTK*, *dTK*, and *eTK*, with the prefix letters representing the remaining functional HSFA1) were compared to that of the wild type after prolonged treatments at 25°C to 29°C. *QK* and *eTK* seedlings were not viable when grown continuously at temperatures above 27°C for 7 d, while the viability of the wild type, *aTK*, *bTK*, and *dTK* were not affected (Fig. 9A; Supplemental Fig. S2). Although HSFA2 is not required for viability in this temperature range (Supplemental Fig. S3), ectopic expression of HSFA2 in *A2QK-7* and *A2QK-10* complemented the function of the HSFA1s to confer tolerance to the mild heat stress (Fig. 9A). Similar results were obtained for the adult plants. In the wild type, the growth of rosette leaves, elongation of inflorescences, and development of siliques after switching growth temperature from 22°C to 27°C were similar to those of plants at 22°C (Fig. 9, B and C). The elongation of inflorescences was slightly enhanced at 27°C (Fig. 9C) but not at 29°C. The *QK* mutant showed obvious defects at 27°C, including stunted growth of inflorescences and

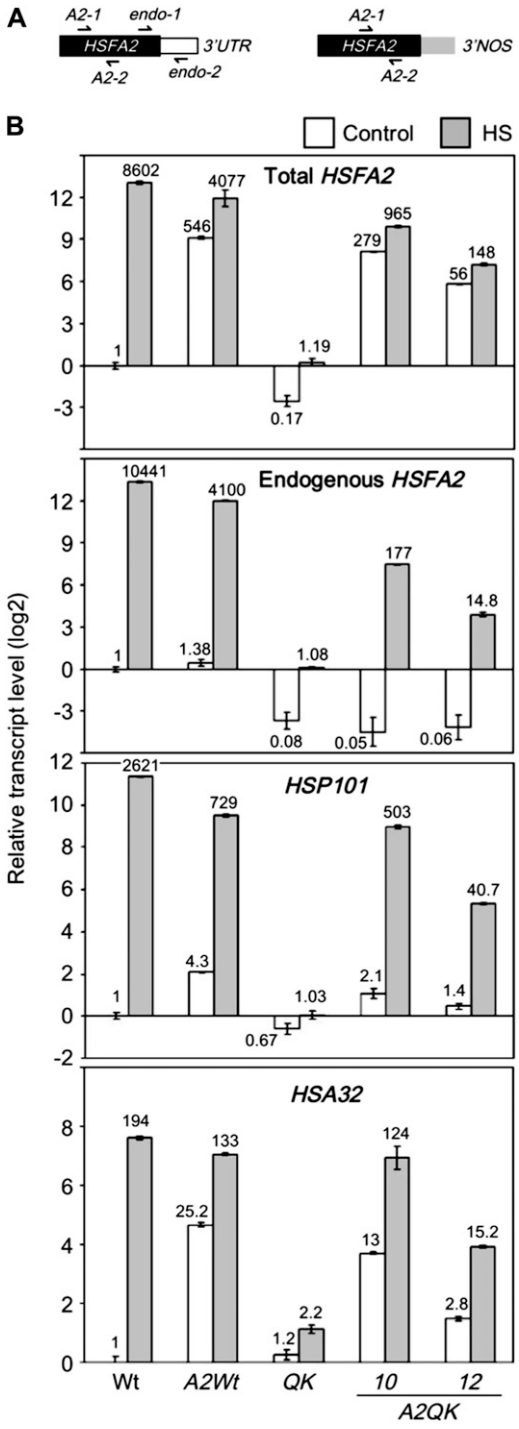

Figure 4. Effect of constitutive expression of HSFA2 on the transcript levels of *HSP101*, *HSA32*, and endogenous *HSFA2*. A, The schemes of the endogenous (left) and recombinant (right) *HSFA2* mRNAs. Black, white, and gray blocks indicate the coding region of *HSFA2*, 3′ untranslated region of endogenous *HSFA2*, and 3′ untranslated region of the nopaline synthase (NOS) terminator of the transgene, respectively. The arrows indicate the locations of the primers used for quantitative RT-PCR. B, The relative transcript levels of *HSFA2*, *HSP101*, and *HSA32* in wild-type (Wt), *QK* mutant, and *35S:HSFA2* transgenic plants (*A2Wt*, *A2QK-10*, and *A2QK-12*) with or without heat treatment. Total *HSFA2* transcript was detected by the primer set *A2-1* and *A2-2*, and endogenous *HSFA2* transcript was detected by the primer set *endo-1* and *endo-2*. White bars indicate the control samples without heat treatment, and gray bars indicate the samples treated at 37°C for 1 h. The numbers at the top or bottom of bars are the transcript level relative to the wild-type sample under control conditions.

aborted siliques, despite of normal growth of leaves (Fig. 9, B and C). These defects were more obvious at 29°C than at 27°C. The rosette leaves of the *QK* mutant were bleached at 29°C, while those of the wild type remained green. Ectopic expression of HFSA2 also complemented the thermotolerance defects of *QK* at these stages (Fig. 9, B and C). These results suggest that HSFA1a/HSFA1b/HSFA1d play pivotal roles in the thermotolerance of reproductive as well as vegetative tissues under chronic heat stress at temperatures as low as 27°C.

The HSFA1s and HSFA2 Have Differential Roles in Tolerance to Salt, Osmotic, and Oxidative Stresses

Previously, we demonstrated that the HSFA1s are involved in response and tolerance to salt, osmotic, and oxidative stresses during seedling establishment (Liu et al., 2011); however, the individual roles of the four HSFA1s were not clear. Overexpression of *HSFA2* implicated the gene in salt and oxidative stress tolerance (Ogawa et al., 2007), but whether HSFA2 can function against these stress factors independently of the HSFA1s is not known. Here, we compared the responses of the wild type, *QK* mutant, *HSFA1* triple KO mutant, and *A2QK-10* seeds sown on the growth media containing 125 mM NaCl, 300 mM mannitol, or 5 mM H_2O_2. Consistent with a previous report (Liu et al., 2011), the *QK* mutant showed a significantly higher percentage of severely stunted growth than the Col-0 wild type after germination under stress conditions (Fig. 10). Unexpectedly, the four *HSFA1* triple KO mutants showed differential responses to these stress factors. The *aTK* mutant showed severely stunted growth in response to high salt concentration, while the other three triple KO mutants behaved like the wild type (Fig. 10). The four triple KO mutants showed different degrees of defect under high concentrations of mannitol, with the *eTK* and *dTK* mutants least affected. In response to H_2O_2, the *bTK* and *dTK* mutants behaved like the wild type, while the growth of *aTK* and *eTK* were more significantly retarded, but to less severe extent than the *QK* mutant. Interestingly, overexpression of HSFA2 in the *QK* background significantly rescued the defect in tolerance to H_2O_2, but provided little or no protection to salt and osmotic stresses (Fig. 10). However, the phenotype of the *hsfa2* mutant under these stress conditions was similar to that of the wild type (Supplemental Fig. S3), suggesting that HSFA2, at least for seedling establishment, does not play an important role in response to these stress conditions as compared with the functions of the HSFA1s.

DISCUSSION

The presence of diverse HSF proteins in plant species is presumably associated with their success in

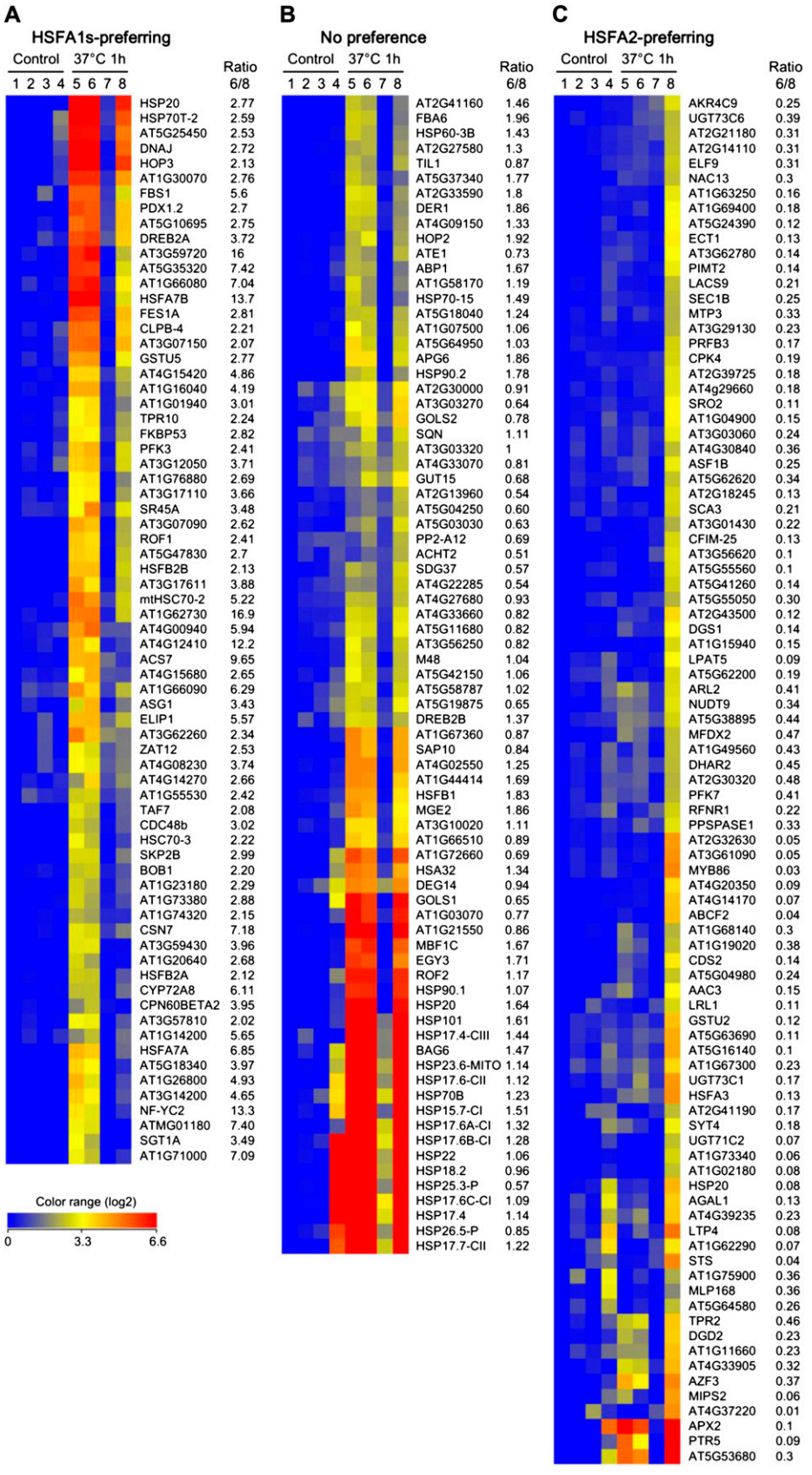

Figure 5. Heat shock-induced genes differentially regulated by HSFA1s and HSFA2. Heat maps of selected microarray expression profiles in 7-d-old seedlings of wild-type, *hsfa2*, *QK*, and *A2QK-10* transgenic plants under control and heat shock (37°C for 1 h) conditions. The 7-d-old seedlings of the wild type (lanes 1 and 5), *hsfa2* (lanes 2 and 6), *QK* (lanes 3 and 7), and *A2QK-10* (lanes 4 and 8) under control condition (lanes 1–4) or after heat shock treatment (lanes 5–8) were collected for the analysis of microarray expression profiles. A total of 239 selected genes (excluding *HSFA2*) depending on HSFA1s or HSFA2 for heat shock induction are clustered into three major groups. The genes preferentially regulated by HSFA1s (A) or HSFA2 (C) were identified by having a signal ratio of lanes 6/8 greater than 2 or less than 0.5, respectively. The genes with signal ratio between 0.5 and 2 were considered as having no preference for HSFA1s or HSFA2 (B). The color range represents the relative level (log2) compared with the wild-type sample under control conditions.

Table I. *Classifications of the heat-induced genes differentially regulated by HSFA1s and HSFA2*

Heat shock-induced genes in three major groups (HSFA1s preferring, no significant preference, and HSFA2 preferring) were categorized based on published information functions predicted in The Arabidopsis Information Resource (Lamesch et al., 2012).

Functional Categories	HSFA1s Preferring		No Significant Preference		HSFA2 Preferring	
	nos.	*%*	*nos.*	*%*	*nos.*	*%*
Signaling	2	2.8	3	3.9	4	4.4
Transcription	11	15.3	3	3.9	5	5.5
Posttranscriptional regulation	1	1.4	1	1.3	3	3.3
Chaperone and cochaperone	17	23.6	27	35.1	4	4.4
Redox homeostasis	4	5.6	2	2.6	8	8.8
Metabolism	3	4.2	4	5.2	19	20.9
Enzyme	5	6.9	8	10.4	13	14.3
Development	2	2.8				
Heat tolerance			3	3.9		
Metal homeostasis					1	1.1
Transporter					4	4.4
Unknown	27	38	26	33.8	30	33

adapting to changing environments that could have negative impact. Unraveling the biological function of each HSF may provide a better understanding of how plants cope with stresses and the evolution of plant HSFs. However, the roles of individual plant HSFs are masked by the redundant functions they share with other members of the protein family. Genetic disruption of one *HSF* gene may not necessarily produce an obvious phenotype, as exemplified in recent studies of Arabidopsis A1-type HSFs (Lohmann et al., 2004; Liu et al., 2011; Nishizawa-Yokoi et al., 2011; Yoshida et al., 2011). Stable or transient overexpression of a *HSF* transgene may also generate misleading results, as the overexpressed protein may derepress the endogenous master HSFs by competing with the attenuators, such as HSP70, HSP90, and HSBP (Voellmy, 2004; Fu et al., 2006; Yamada et al., 2007; Hsu et al., 2010; Hahn et al., 2011). For example, HSFA2 was shown to interact with HSP90 and HSBP (Meiri and Breiman, 2009; Hsu et al., 2010), and therefore overexpression of HSFA2 in the wild-type plant could derepress the HSFA1s, trigger a stress response, and enhance stress tolerance. This possibility makes interpreting overexpression results difficult. Here, to assess the independent function of HSFA2, we ectopically expressed it in an Arabidopsis mutant lacking all four HSFA1s. This strategy did not allow the exclusion of possible interference from the other remaining HSFs, but any such interference is likely to be much weaker in the absence of the HSFA1s, as the expression of several HSFs, such as *HSFA3*, *HSFA7a/HSFA7b*, *HSFB1*, and

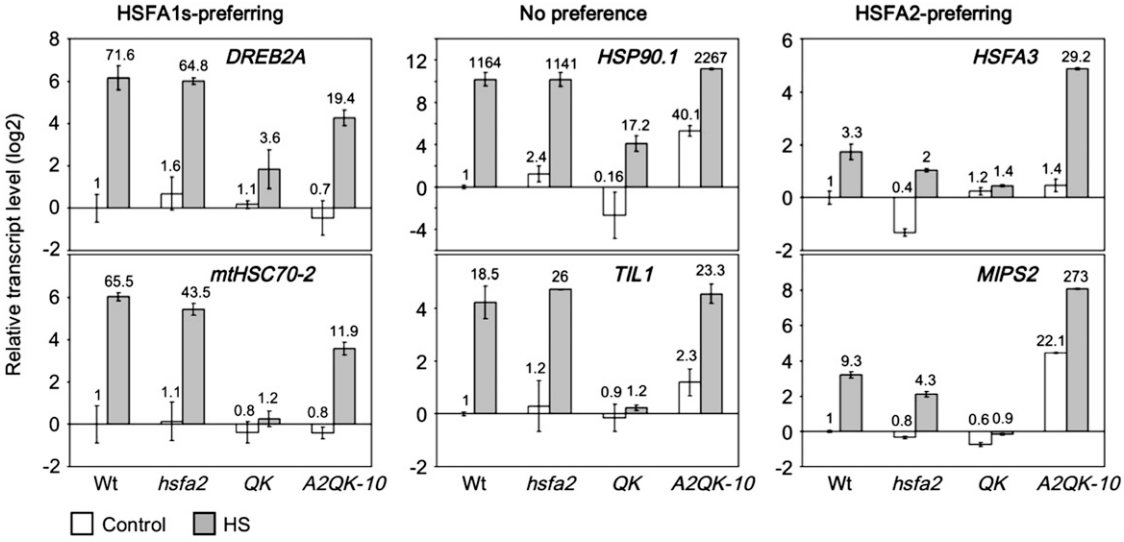

Figure 6. Confirmation of the target preference of HSFA1s and HSFA2 by quantitative RT-PCR. The relative transcript levels of selected genes with a preference for regulation by the HSFA1s or HSFA2 according to the microarray data were analyzed by quantitative RT-PCR and relative to the level of the wild type under control conditions. The samples and treatment conditions were the same as those in Figure 5. The relative transcript levels of each sample are indicated at the top of the bars. White bars indicate the control samples without heat treatment, and gray bars indicate the samples treated at 37°C for 1 h.

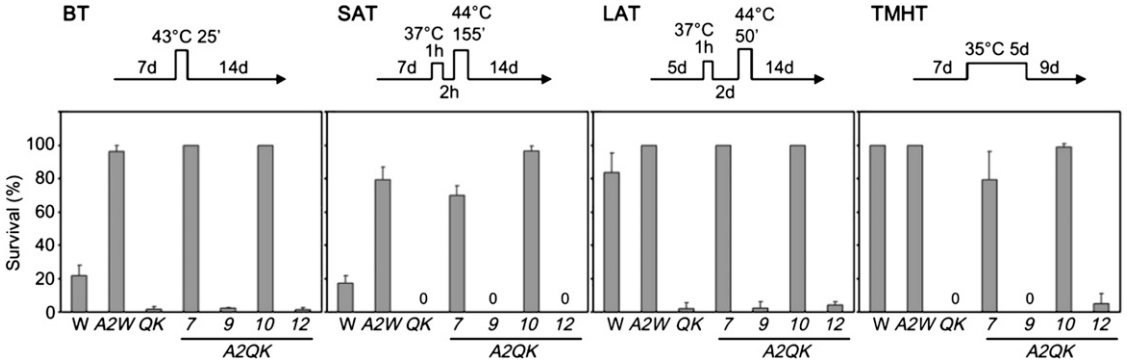

Figure 7. Constitutive expression of HSFA2 conferred high level of thermotolerance in the absence of the HSFA1s under different heat stress regimes. The viabilities of the wild type, *QK* mutant, and *35S:HSFA2* transgenic lines (*A2Wt*, *A2QK-7*, *A2QK-9*, *A2QK-10*, and *A2QK-12*) after treatment with four different heat stress regimes (shown by the schemes) for BT, SAT, LAT, and TMHT. The viable plants were defined as those that generated new rosette leaves after heat stress. Images of SAT, BT, and TMHT are shown in Supplemental Figure S1, and an image of LAT is shown in Figure 8A. Results are presented as mean values of three replicates ± SD ($n \geq 40$ each).

HSFB2a/HSFB2b, also requires the HSFA1s (Liu et al., 2011).

In this study, we demonstrated that HSFA2 could perform certain functions in the absence of the HSFA1s, such as triggering heat stress response and conferring thermotolerance. These results suggest that HSFA2 can act as a transcription activator independent of the HSFA1s, including in nuclear localization and transcription activation of heat stress genes. Our findings are in agreement with a previous study that demonstrated that transiently expressed Arabidopsis HSFA2 can shuttle between the cytoplasm and nucleus without HSFA1 (Kotak et al., 2004), a prerequisite for HSFA2 to act as transcription factor. Because the expression of HSFA2 in seedlings is strictly controlled by the HSFA1s upon stress, the independent role of HSFA2 can only be fulfilled after prolonged or reexposure to stress conditions or by ectopic overexpression, where HSFA2 preferentially targets a subset of genes with different physiological functions from those conferred by the HSFA1-preferring genes (Fig. 5).

In response to heat stress, HSFA2 seems to have higher activity than the HSFA1s in activating the genes involved in redox homeostasis (*ASCORBATE PEROXIDASE2* [*APX2*] and *ALDO-KETO REDUCTASE4 SUBFAMILY C9* [*AKR4C9*]) and the metabolism of carbohydrates (*MIPS2*) and lipids (*LONG-CHAIN ACYL-COA SYNTHETASE9* [*LACS9*], *CYTIDINEDIPHOSPHATE DIACYLGLYCEROL SYNTHASE2* [*CDS2*], *DIGALACTOSYL DIACYLGLYCEROL DEFICIENT2* [*DGD2*], and *DGD1 SUPPRESSOR1* [*DGS1*]; Fig. 5; Table I; Supplemental Table S1). According to the functions reported in previous studies (Kelly et al., 2003; Rossel et al., 2006; Simpson et al., 2009; Haselier et al., 2010; Moellering and Benning, 2010; Zhao et al., 2010; Valluru and Van den Ende, 2011), these genes may contribute to maintenance of cellular homeostasis during prolonged heat stress. In addition, HSFA2, when expressed at high levels, functioned in a similar manner to the HSFA1s regulating a large number of chaperones and cochaperones, such as HSP101, HSP90, HSP70B, HSP70-15, HSP60-3B, small HSPs, MITOCHONDRIAL GRPE2 (MGE2), and ROTAMASE FKBP2 (ROF2; Fig. 5; Table I; Supplemental Table S1), explaining its capacity to confer a wide spectrum of heat tolerance. By contrast, HSFA2, as compared with the HSFA1s, is a relatively weak activator of the heat-induced transcription regulators such as *HSFA7a*, *HSFA7b*, *NUCLEAR FACTOR Y SUBUNIT C2* (*NF-YC2*), *DREB2A*, *HSFB2b*, and *HSFA2* itself (Figs. 4 and 5; Table I; Supplemental Table S1), which may prevent these transcription factors from being amplified and overaccumulated. Overaccumulation of stress-related transcription regulators could be detrimental to growth and is not deemed preferable (Ogawa et al., 2007; Zhu et al., 2009). Recently, a heat-induced splice variant of *HSFA2* was identified and suggested to be involved in the autoregulation of *HSFA2* (Liu et al., 2013). However, the protein level of this HSFA2 variant was not determined, and whether it could exert an effect in heat stress response is unclear. Unlike the above-mentioned transcription factors, *HSFA3* is preferentially regulated by HSFA2, which is in good agreement with its late induction profile under heat stress (Schramm et al., 2008). Interestingly, HSFA3 was shown to be partially regulated by DREB2A (Sakuma et al., 2006; Schramm et al., 2008). Hence, two pathways control the heat induction of HSFA3, one through HSFA1s-DREB2A and another through HSFA1s-HSFA2. It would be of interest to find out why HSFA3 is subjected to regulation via two independent pathways.

Several genes involved in diverse signaling pathways are also under the differential regulation of the HSFA1s and HSFA2. For example, *CSN7* (COP9 signalosome, Dessau et al., 2008) and *ACS7* (ethylene, Dong et al., 2011) are preferentially regulated by the HSFA1s; *BAG6* (calcium, Kang et al., 2006) and *ABP1* (auxin, Braun et al., 2008) are regulated by both HSFA1s and HSFA2; and *UGT73C6* (brassinosteroids, Husar et al., 2011) and *CPK4* (abscisic acid, Zhu et al., 2007) are preferentially

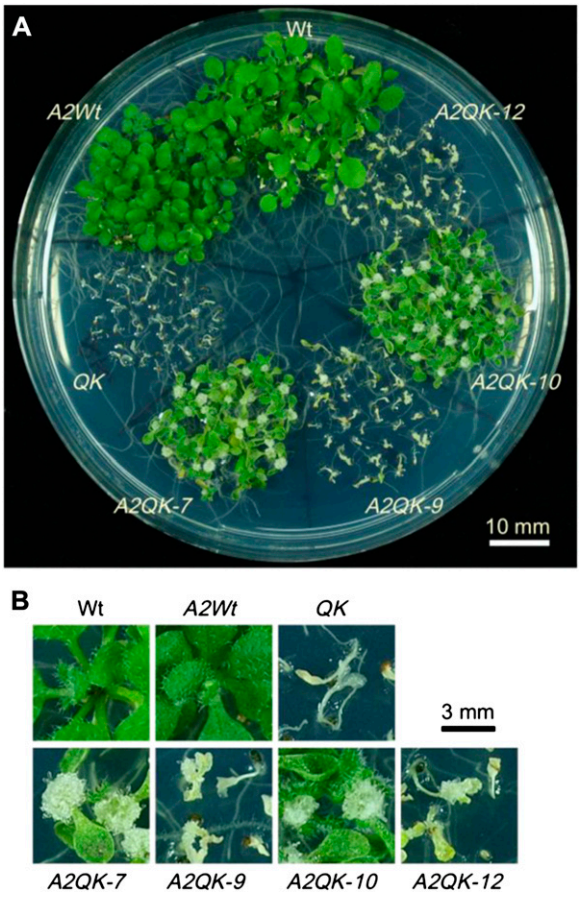

Figure 8. Constitutive expression of HSFA2 promoted the formation of calluses after heat treatment in the absence of the HSFA1s. A, The phenotypes of wild-type, *QK* mutant, and *35S:HSFA2* transgenic plants (*A2Wt*, *A2QK-7*, *A2QK-9*, *A2QK-10*, and *A2QK-12*) after treatment with heat stress regime for LAT as indicated in Figure 7. B, Enlarged images of the representative plants in A.

regulated by HSFA2. These connections suggest that the HSFA1s and HSFA2 can differentially modulate these diverse signaling pathways despite the HSFs being considered the terminal components of signal transduction (Kotak et al., 2007). Due to the short duration of the single heat treatment performed in this study, we were not able to investigate the synergetic effect of heterooligomers formed by HSFA1s and HSFA2 as shown in tomato (Chan-Schaminet et al., 2009); this direction should be explored in the future.

Although we showed that HSFA2 can promote growth and development in the absence of the HSFA1s (Fig. 2), overexpression of HSFA2 in the wild-type background resulted in dwarfism (Ogawa et al., 2007). Consistently, we also observed that the *A2Wt* lines showed dwarf phenotype (Supplemental Fig. S4A) that is associated with reduced cell number, but not cell size, in expanded leaves (Supplemental Figure S4, B and C). These observations suggest that HSFA2 acts as a positive regulator in the absence of the HSFA1s but as a negative regulator if HSFA1s are present. In the absence of HSFA1s, it is possible that HSFA2 replaces the function of HSFA1s in growth and development due to the high degree of sequence homology shared between the two classes of HSF. However, it is not clear why a high level of HSFA2 suppresses the growth of the transgenic plants in the presence of the HSFA1s. Previous reports showed that disruption of *HSFA2* does not affect growth and development of Arabidopsis plants under nonstress conditions

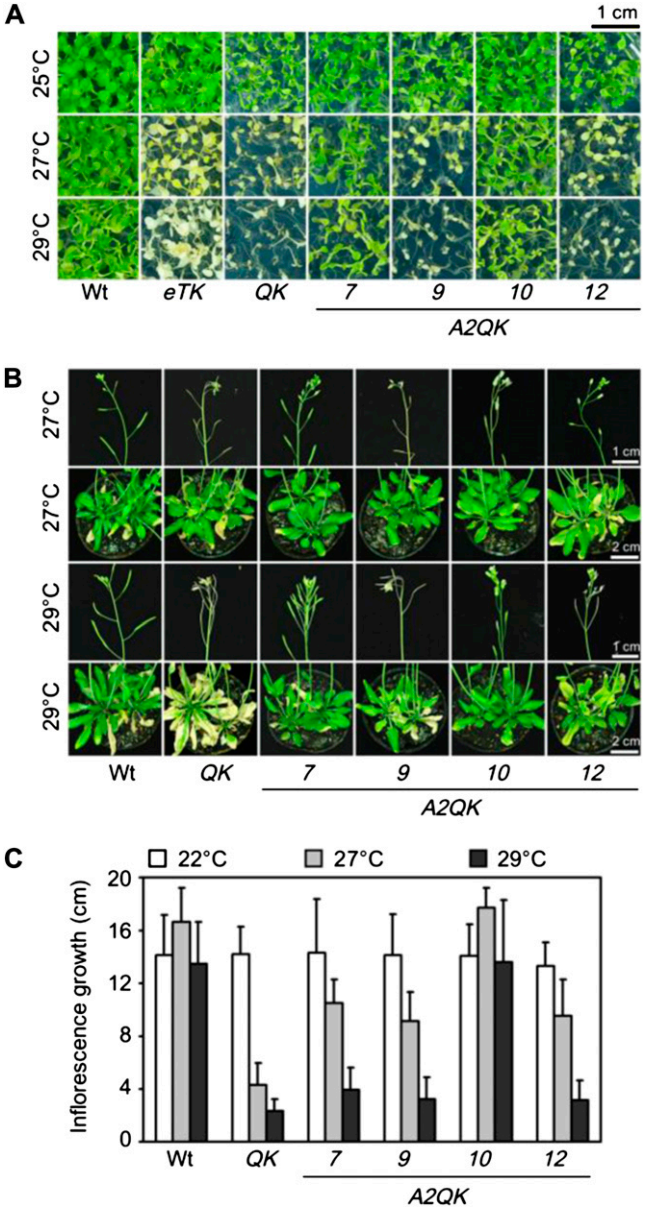

Figure 9. HSFA1a/HSFA1b/HSFA1d are required for tolerance to chronic heat stress at temperatures above 27°C in seedlings and flowering plants. A, Phenotypes of 14-d-old seedlings of the wild type (Wt), *eTK*, *QK*, *A2QK-7*, *A2QK-9*, *A2QK-10*, and *A2QK-12* after treatment at 25°C, 27°C, or 29°C for 7 d. B, Phenotypes of 40-d-old plants of indicated lines after treatment at 27°C or 29°C for 8 d. C, The elongated length of inflorescences of the plants in B after treatment at 22°C, 27°C, or 29°C for 8 d.

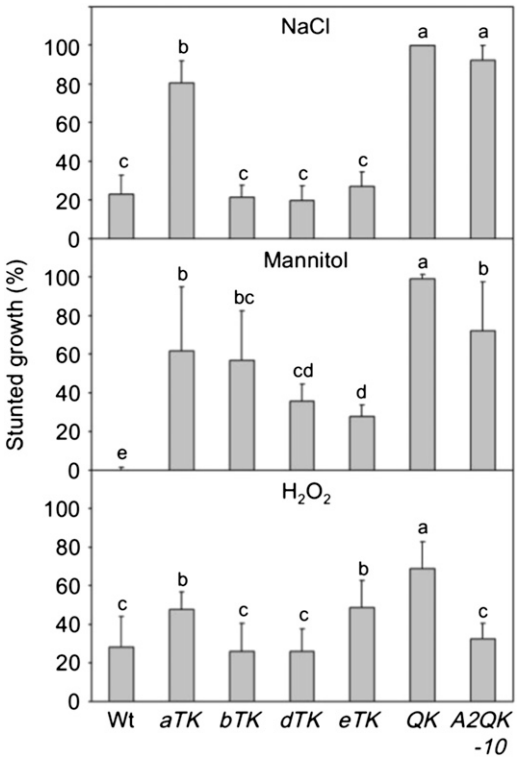

Figure 10. Contributions of individual HSFA1s and constitutive expression of HSFA2 in tolerance to salt, osmotic, and oxidative stresses. The growth of wild-type, *HSFA1* triple KO mutant (*aTK*, *bTK*, *dTK*, and *eTK*), *QK*, and *A2QK-10* transgenic plants sown on 0.5× MS medium with 125 mM NaCl, 300 mM mannitol, or 5.5 mM H_2O_2 was assessed after 12 d. Stunted growth was defined as plants without green cotyledons and true leaf emergence. The growth rates under stress conditions were normalized by the growth rate of each line sown on plates without the stress factors. Results are the mean values of six replicates ± SD ($n = 45$). Groups labeled with different letters of the alphabet indicate those with statistically significant differences (LSD, $\alpha = 0.05$).

(Nishizawa et al., 2006; Schramm et al., 2006; Charng et al., 2007). However, whether HSFA2 plays a role in regulating growth and development after being induced to a high level by heat stress has not been investigated.

The notion that HSFA2 affects development is supported by the association of HSFA2 overexpression and callus formation after heat stress (Fig. 8). A callus is a mass of dedifferentiated cells often induced in wounded tissues exposed to auxin-rich media (Skoog and Miller, 1957; Birnbaum and Sánchez Alvarado, 2008). It has been shown that heat treatment promotes callus formation in explants (Miyoshi, 1996; Parra-Vega et al., 2013) and that the expression of *HSFA2* is up-regulated during the process of callus formation (Che et al., 2002). Interestingly, overexpressing HSFA2 in the wild type did not promote callus formation in heat-stressed seedlings (Fig. 8, compare the *A2Wt* and *A2QK* lines) but was shown to enhance formation of calluses in callus-inducing, auxin-rich medium (Ogawa et al., 2007). The phenotypic difference in the promotion of callus formation in HSFA2 overexpressing plants in the wild-type and *QK* backgrounds suggests that the HSFA1s affect the function of HSFA2. This is consistent with the observation mentioned above, i.e. that the presence or absence of the HSFA1s alters the function of HSFA2 as a regulator of growth and development. Taken together, these findings suggest that HSFA2 plays an important role in growth and development, but its intrinsic role and mechanism of regulation remain to be elucidated in the future.

In *Drosophila* spp., HSF is required for oogenesis and early larval development (Jedlicka et al., 1997). Absence of HSF1 in mice is not lethal but increases female infertility and prenatal lethality and retards growth (Xiao et al., 1999). Here, we showed that disruption of all four *HSFA1*s, in addition to impairing growth of young seedlings, increases the chances of seed abortion (Fig. 2E), which is associated with the female gametophyte of the *QK* mutant (Fig. 2F). These findings indicate a conserved role of HSF in the development of female organs and embryos in plants and animals. It would be of interest to know how HSFA1s control these processes. Of note, a similar seed abortion phenotype was shown in the KO mutant of *HSBP*, a negative regulator of the HSFA1s (Hsu and Jinn, 2010; Hsu et al., 2010). The seed abortion rate in the *HSBP* KO mutant is about 35%, higher than the 20% observed in the *QK* mutant (Fig. 2F). This phenotypic similarity suggests that HSFA1s and HSBP may function in concert in seed development despite their functions in heat stress response being antagonistic (Hsu and Jinn, 2010; Hsu et al., 2010). The small HSPs, HSP17.4 (AT3G46230) and HSP17.6A (AT1G59860), have been shown to be involved in early embryogenesis, and double KO of these genes leads to seed abortion (Dafny-Yelin et al., 2008). Because *HSP17.4* and *HSP17.6A* are the targets of the HSFA1s in heat stress response (Fig. 5; Supplemental Table S1), it is likely that they are the target genes of HSFA1s during seed development as well. Overexpression of HSFA2 in the *QK* mutant was able to rescue the seed abortion phenotype (Fig. 2, E and F), probably due to its ability to interact with HSBP (Hsu and Jinn, 2010; Hsu et al., 2010), and was as proficient as the HSFA1s in inducing *HSP17.4* and *HSP17.6A* (Fig. 5; Supplemental Table S1).

Overexpression of HSFA2 in Arabidopsis wild-type background was shown to enhance tolerance of seedlings germinating on medium with high salt or mannitol concentrations (Ogawa et al., 2007). However, our results showed that in the absence of HSFA1s, overexpression of HSFA2 does not seem to rescue the tolerance to salt stress and has little effect on osmotic stress tolerance (Fig. 10). This observation suggests that HSFA2 plays a very minor, if any, role in tolerance to these adverse conditions, at least at the stage of germinating young seedling. This finding correlates with the results showing that the *hsfa2* null mutant is not more sensitive than the wild type to salt and osmotic stresses (Supplemental Fig. S3). Although *hsfa2* mutant showed similar sensitivity to H_2O_2 as the wild type, overexpression of HSFA2 in *QK* mutant did restore tolerance to this oxidative agent (Supplemental

Fig. S3), suggesting that a high level of HSFA2 could provide protection independent of the HSFA1s, probably by inducing genes involved in redox homeostasis as mentioned above. These results are in good agreement with evidence demonstrated previously that implicates in HSFA2 in oxidative stress response (Li et al., 2005).

In this study, we further investigated the capacity of individual HSFA1s to confer tolerance to salt, osmotic, and oxidative stresses by using the four triple KO mutants. To our surprise, among the four HSFA1s, HSFA1a, the most potent inducer of thermotolerance (Liu et al., 2011), is the least effective inducer of tolerance to salt and osmotic stresses (Fig. 10). By contrast, HSFA1e, which does not confer thermotolerance in the absence of HSFA1a, HSFA1b, and HSFA1d, strongly induces salt and osmotic stress tolerance. HSFA1d seems to be involved in the response to all three stress factors. These results suggest that during evolution, the four members of the HSFA1 group have evolved divergent functions in stress response by subfunctionalization. It is tempting to speculate that the existence of multiple, subfunctionalized HSFA1s are beneficial for plants to cope with combined stresses of varying degrees of severity. This hypothesis awaits testing with vigorous challenges of combined stresses on mutants containing different combinations of HSFA1s. Of note, several monocots, such as rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), and *Brachypodium distachyon*, have a single *HSFA1* in their genomes (Scharf et al., 2012). It will therefore be of interest to know whether or how the monocot HSFA1 can mediate multiple stress tolerances.

The finding that the HSFA1s have a role in tolerance to mild heat stress at ambient temperature around 27°C (Fig. 9) was quite unexpected, as this temperature is not linked to heat stress response, which is usually tested at temperatures 10°C to 15°C above that for optimum growth (Lindquist, 1986). It has been previously shown that Arabidopsis plants respond to high ambient temperature by promoting morphological and developmental alterations, such as petiole elongation, in which the basic helix-loop-helix transcription factor PHYTOCHROME-INTERACTING FACTOR4 (PIF4) plays a pivotal role (Koini et al., 2009; Franklin et al., 2011; Kumar et al., 2012; Sun et al., 2012). Because the *eTK* mutant still showed the phenotype of elongating petioles (Fig. 9) and the *pif4* mutant seemed to be viable under this high ambient temperature (Koini et al., 2009), these results suggest that HSFA1a/HSFA1b/HSFA1d and PIF4 trigger two independent pathways for thermotolerance and morphological adjustment, respectively. Our data reveal a new type of thermotolerance, which constitutes thermotolerance diversity orchestrated by the master regulators of heat stress response (Yeh et al., 2012). It remains to be seen how HSFA1a/HSFA1b/HSFA1d regulate transcription reprogramming in this temperature range.

In conclusion, our work reveals common and divergent roles of Arabidopsis class A1 and A2 HSFs in development and response to different stresses. The approach shown here may be applicable to the elucidation of the functions of other Arabidopsis HSFs as well as the posttranslational modifications of HSFA2 recently identified (Cohen-Peer et al., 2010; Evrard et al., 2013).

MATERIALS AND METHODS

Plant Materials and Growth Condition

The mutant lines of *QK*, *aTK*, *bTK*, *dTK*, *eTK*, and *hsfa2* used in this study were obtained as previously described (Charng et al., 2007; Liu et al., 2011). The normal growth conditions in Murashige and Skoog (MS) medium and soil were also as previously described (Liu et al., 2011).

Generating Transgenic Plants

The complementary DNA of HSFA2 was amplified by RT-PCR using mRNA isolated from heat-stressed samples (37°C, 1 h) of 7-d-old Col-0 wild type as a template. The PCR product was cloned into pCR8/GW/TOPO (Invitrogen) and then subcloned into Gateway overexpression vector pB2GW7 (Karimi et al., 2002) to yield the binary vector pYC125. The construct was then transferred into *Agrobacterium tumefaciens* GV3101 strain and transformed into *QK* mutant and the Col-0 wild type by the floral dip method to generate *A2QK* and *A2Wt*, respectively. The *A2Wt* homozygous lines with high levels of HSFA2 were sterile, and only few viable seeds could be obtained. Therefore, the heterozygous line of *A2Wt* was used for this study.

Evaluating Seed Size, Seed Abortion Rate, and Growth Rate of Young Seedlings

The method of seed size evaluation was as described in (Liu et al., 2011). The development of immature seeds in the ninth and tenth siliques from the top of inflorescences was observed under a stereomicroscope (Olympus), and the seed abortion rates of 15 siliques from five independent plants were scored. The growth rate from the imbibed seed to the two rosette leaves stage was measured as previously described (Liu et al., 2011). The root length of 7-d-old seedlings grown in 0.5× MS medium was calculated using ImageJ software (National Institutes of Health).

Thermotolerance Assays

The thermotolerance assays were performed as previously described with modifications (Charng et al., 2006; Liu et al., 2011). For the BT assay, 7-d-old seedlings were treated for 25 min at 43°C. For short-term acquired thermotolerance (SAT) assay, 7-d-old seedlings were acclimated for 1 h at 37°C, recovered for 2 h at 22°C, and then treated for 155 min at 44°C. For long-term acquired thermotolerance (LAT) assay, 5-d-old seedlings were acclimated for 1 h at 37°C, recovered for 2 d at 22°C, and then treated for 50 min at 44°C. After exposure to these heat stress regimes, the plants were recovered for 14 d at 22°C. For the TMHT assay, 7-d-old seedlings were treated for 5 d at 35°C under a light/dark cycle of 16 h/8 h (120 mmol m^{-2} s^{-1}) and recovered for 9 d at 22°C. At the end of recovery, photos were taken and the survival rates were evaluated. To test the tolerance to chronic heat stress at 27°C or 29°C, 7-d-old plants grown in 0.5× MS medium plates were placed in a growth chamber set at the indicated temperature for 7 d under a light/dark cycle of 16 h/8 h (120 mmol m^{-2} s^{-1}), then the phenotypes were recorded. Heat tolerance of 40-d-old plants grown in potted soil was assessed by incubating in a growth chamber set at the indicated temperature and treated for 8 d under a light/dark cycle of 16 h/8 h (120 mmol m^{-2} s^{-1}). After treatment, the phenotypes were photographed and the elongating length of inflorescences was measured.

Oxidative, Osmotic, and Salt Stress Treatments

The seeds were sterilized and sown on 0.5× MS medium containing 1% (w/v) Suc with 125 mM NaCl and 300 mM mannitol or 0.1% (w/v) Suc with 5 mM H_2O_2 imbibed at 4°C for 3 d and then grown at 22°C for 18 d before photograph taking and evaluation of the growth phenotype.

Immunoblotting and Quantitative RT-PCR

The methods of protein extraction and immunoblotting were as described previously (Chi et al., 2009). Antibodies against HSFA2, HSP101, HSP90,

HSA32, sHSP-CI, and tubulin were also previously described (Charng et al., 2006; Chi et al., 2009; Liu et al., 2011). Total RNA extraction and quantitative RT-PCR were performed as described in Liu et al. (2011). The primers used for RT-PCR are listed in Supplemental Table S2.

Microarray Analysis

Seven-day-old seedlings grown at 22°C on 0.5× MS plates containing 1% (w/v) Suc were first heat-shocked for 1 h at 37°C (Heat-shocked) or left at 22°C (control) and collected for RNA extraction. Total RNA used for microarray analysis was purified by the RNeasy procedure (Qiagen). Examination of the RNA quality and processing of ATH1 GeneChip arrays with 22,810 features (Affymatrix) were performed by Vita Genomics. Two independent biological replicates were processed for the analysis. Assessment of experimental quality and statistical analyses were performed as previously described (Liu et al., 2011). The microarray result, including hierarchical clustering, was analyzed using GeneSpring 11.5.1 (Agilent Technologies). The annotations of the Arabidopsis (*Arabidopsis thaliana*) genes were downloaded from The Arabidopsis Information Resource (http://www.arabidopsis.org). The microarray data can be accessed in the Gene Expression Omnibus at the National Center for Biotechnology Information (accession no. GSE44655).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers*HSFA1a* (At4g17750), *HSFA1b* (At5g16820), *HSFA1d* (At1g32330), *HSFA1e* (At3g02990), and *HSFA2* (At2g26150). The accession numbers of other genes mentioned in this article can be found in Supplemental Table S1.

Supplemental Data

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

Supplemental Figure S1. Constitutive expression of HSFA2 conferred a high level of thermotolerance in the absence of HSFA1s.

Supplemental Figure S2. HSFA1a/b/d are required for tolerance to chronic heat stress at temperature above 27°C.

Supplemental Figure S3. HSFA2 is not essential for growth at 29°C, or tolerance to oxidative, salt, or osmotic stresses.

Supplemental Figure S4. Constitutive expression of HSFA2 in wild-type background caused dwarfism with small rosette leaves.

Supplemental Table S1. List of HSFA1s-preferring, no preference, and HSFA2-preferring genes shown in Figure 5.

Supplemental Table S2. Primers used in this study.

ACKNOWLEDGMENTS

We thank Jen Sheen for critical reading of the manuscript, Yun-Ru Lai for technical support, Miranda Loney for English editing, and two anonymous reviewers for constructive comments.

Received May 8, 2013; accepted July 4, 2013; published July 5, 2013.

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