A Heat-Inducible Transcription Factor, HsfA2, Is Required for Extension of Acquired Thermotolerance in Arabidopsis[1][W][OA]

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The expression of heat shock proteins (Hsps) induced by nonlethal heat treatment confers acquired thermotolerance (AT) to organisms against subsequent challenges of otherwise lethal temperature. After the stress signal is removed, AT gradually decays, with decreased Hsps during recovery. AT of sufficient duration is critical for sessile organisms such as plants to survive repeated heat stress in their environment, but little is known regarding its regulation. To identify potential regulatory components, we took a reverse genetics approach by screening for Arabidopsis (*Arabidopsis thaliana*) T-DNA insertion mutants that show decreased thermotolerance after a long recovery (2 d) under nonstress conditions following an acclimation heat treatment. Among the tested mutants corresponding to 48 heat-induced genes, only the heat shock transcription factor *HsfA2* knockout mutant showed an obvious phenotype. Following pretreatment at 37°C, the mutant line was more sensitive to severe heat stress than the wild type after long but not short recovery periods, and this could be complemented by the introduction of a wild-type copy of the *HsfA2* gene. Quantitative hypocotyl elongation assay also revealed that AT decayed faster in the absence of *HsfA2*. Significant reduction in the transcript levels of several highly heat-inducible genes was observed in *HsfA2* knockout plants after 4 h recovery or 2 h prolonged heat stress. Immunoblot analysis showed that Hsa32 and class I small Hsp were less abundant in the mutant than in the wild type after long recovery. Our results suggest that HsfA2 as a heat-inducible transactivator sustains the expression of Hsp genes and extends the duration of AT in Arabidopsis.

Eukaryotic cells respond to elevated temperature or heat shock (HS) by inducing the transcription of genes encoding proteins such as molecular chaperones. Many of these proteins are involved in preventing or repairing the damage caused by heat stress and thus confer increased thermotolerance (Lindquist and Craig, 1988; Vierling, 1991). This phenomenon, known as HS response (HSR), is initiated by the activation of the HS transcription factor (Hsf), a conserved protein present in all eukaryotic organisms studied to date (Wu, 1995; Nover et al., 2001).

The exact number of Hsf genes differs greatly among various eukaryotic organisms. For example, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* each has a single *Hsf* gene playing an important biological role in addition to the HSR (Sorger and Pelham, 1988; Wiederrecht et al., 1988; Jedlicka et al., 1997; Hsu et al., 2003). The mammalian genome contains three *Hsf* isoforms, *Hsf1*, *Hsf2*, and *Hsf4*, each with a distinct biological function (Xiao et al., 1999; Bu et al., 2002; Fujimoto et al., 2004; Xing et al., 2005). In contrast to the low numbers of *Hsf* genes found in animals and yeasts, the model plant Arabidopsis (*Arabidopsis thaliana*) contains 21 Hsf homologs that can be sorted into three classes (classes A, B, and C; Nover et al., 2001). Most class A Hsfs of Arabidopsis contain the AHA motifs essential for transcription activation, while the class B and C Hsfs do not (Kotak et al., 2004). Genetic evidence shows that *HsfA1a* functions as a master regulator of HSR in tomato (*Lycopersicon esculentum*; Mishra et al., 2002). In Arabidopsis, *HsfA1a* and *HsfA1b* play important roles in the induction of a number of HS protein (Hsp) genes in the early phase of HSR (Lohmann et al., 2004). A rice (*Oryza sativa*) mutant, *spl7*, which is sensitive to environmental stress and exhibits a spotted leaf phenotype under elevated temperature, was shown to have a missense mutation in an *Hsf* gene belonging to the *HsfA4* group (Yamanouchi et al., 2002).

Intriguingly, several plant *Hsf*s are HS-inducible genes themselves, which among eukaryotic systems is a feature unique to plants (Nover et al., 2001). For example, *HsfA2* is strongly induced by HS in tomato (Scharf et al., 1998) and Arabidopsis (Busch et al., 2005).

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The presence of HsfA2 exclusively after HS treatment suggests its role in the transcriptional regulation of Hsp genes during prolonged heat stress or in recovery after HS (Baniwal et al., 2004) and, hence, its importance in the development of acquired thermotolerance (AT).

Recently, two research groups have independently reported the function of *HsfA2* by characterizing the same Arabidopsis T-DNA knockout line. Li et al. (2005) found that disruption of *HsfA2* caused a slight reduction in expression of some Hsp genes upon HS treatment and that the knockout mutant displayed reduced basal and AT as well as oxidative stress tolerance, while overexpression of HsfA2 enhances tolerance under these stress conditions. Schramm et al. (2006) identified a subset of genes whose expression was reduced upon HS treatment in the knockout mutant. They showed that HsfA2 bound to the promoter regions of selected target genes and activated a β-glucuronidase (*GUS*) reporter that was fused to them. However, in contrast to the observation made by Li et al. (2005), no obvious phenotype was observed for the mutant under the relatively comprehensive assay conditions (Schramm et al., 2006). The discrepancy in these studies obscures the biological function of this important component of plant HSR.

In the postgenomic era, functional study of target genes by reverse genetic approaches has become the norm, but knockout mutation often leads to no significant change in phenotype. This approach is especially difficult for the study of stress-induced genes because plants without these genes often show no or very subtle difference from the wild type even under the stress condition that induces them. Apparently, developing a more subtle and effective assay is essential for elucidating the biological function of HSR genes because so far only a few successful cases have been reported (Hong and Vierling, 2000; Queitsch et al., 2000; Panikulangara et al., 2004; Li et al., 2005; Charng et al., 2006; Chen et al., 2006). Recently, we have demonstrated the function of a plant-specific Hsp, Hsa32, in Arabidopsis by employing a novel assay method (Charng et al., 2006). The mutant plants lacking Hsa32 became more sensitive to severe HS than the wild type after long (48 h) but not short recovery following heat acclimation treatment, suggesting that the protein is associated with the duration of AT. AT of significant duration is presumably an important feature for plants to cope with repeated heat stress with intervening periods of milder temperature, but very little information is available on this issue so far.

Here, we report the screening of Arabidopsis T-DNA insertion mutants of 48 heat-induced genes under the same assay conditions used to characterize Hsa32; only the *HsfA2* knockout plants showed a significant heat-sensitive phenotype, which could be rescued by introducing a wild-type copy of *HsfA2*. Physiological studies showed that HsfA2 is essential for AT after long but not short recovery. Further diagnosis by microarray and reverse transcription (RT)-PCR suggested that the heat-induced Hsf is not required for initial regulation of HSR genes but for sustaining the transcript level of Hsp genes during recovery and prolonged heat stress. Semiquantitative RT-PCR and immunoblot assays suggest that the cause of the phenotype was at least partly due to the reduced level of Hsps, such as Hsa32, in the mutant after long recovery following an acclimation treatment. The results not only provide direct genetic evidence of the biological function of HsfA2 but also of the existence of a regulatory component for the duration of AT, at least in Arabidopsis, but probably in other higher plants as well.

RESULTS

Reverse Genetic Screening for Mutants Sensitive to Severe HS after Long Recovery from Acclimation

To identify genes involved in duration of AT, we took a reverse genetic approach by randomly screening Arabidopsis T-DNA insertion mutants corresponding to 48 genes (Table I), which previous microarray analyses had shown to be up-regulated by HS treatment (Busch et al., 2005; Charng et al., 2006). Most of the mutant lines had T-DNA inserted in the exon region of the target gene, which should have resulted in loss-of-function alleles. Homozygous lines of these mutants were isolated by PCR analysis. RT-PCR experiments confirmed that all the T-DNA insertion lines were unable to accumulate corresponding transcripts under normal and HS conditions (data not shown). Three-day-old seedlings of these mutants grown on agar plates were treated with severe HS (44°C for 50 min) after a 2-d recovery from previous acclimation treatment (37°C for 1 h). These conditions killed the *Hsa32* knockout but not the wild-type plants (Charng et al., 2006) that were included in each plate as positive and negative controls, respectively, to ensure the effectiveness of the treatment. After two rounds of screening, a lethal phenotype under the test condition was repeatedly observed only for the *HsfA2* T-DNA insertion mutant (Fig. 1A). We therefore carried out further studies on this putative *HsfA2* mutant.

The defect of *HsfA2* T-DNA insertion was associated with the homozygous line containing both disrupted *HsfA2* alleles with T-DNA inserted within the second exon near the 5′ end (Supplemental Fig. S1A), whereas about 75% and 100% of the offspring of the hemizygous or azygous sister lines, respectively, showed the wild-type phenotype. These results indicated that the T-DNA insertion caused a loss-of-function mutant allele, hereafter designated as *hsfA2-1*. RT-PCR analysis showed that transcripts of *HsfA2* were induced by HS treatment (37°C, 1 h) in the wild type but not in the *hsfA2-1* plants, whereas the expression of *Hsa32* and *Hsp101* control genes was not significantly affected in the mutant (Supplemental Fig. S1B). The RT-PCR results confirmed that *hsfA2-1* was a null mutant of *HsfA2*. Because Southern-blot analysis showed that

Table I. *List of Arabidopsis T-DNA insertion mutants of heat stress-responsive genes*

AGI No.	Gene Description[a]	Change (HS/C)[b]	Mutant Stock No.[c]
At4g25200	Hsp23.6-M	397.5	CS817244(e)
At5g12020	Hsp17.6-CII	347.0	SALK_086201(e)
At2g26150	HsfA2	318.1	SALK_008978(e)
At5g51440	Hsp23.5-M	296.0	SALK_118536(i)
At2g46240	BAG domain-containing protein	283.3	SALK_004760(e)
At3g09640	L-Ascorbate peroxidase (Apx2)	253.0	SALK_057686(e)
At1g16030	Hsp70b	202.6	CS809145(e)
At5g59720	Hsp18.2 (Hsp18.1-CI)	181.4	SALK_042001(e)
At5g37670	Hsp15.7-CI-related	172.5	SALK_107711(e)
At4g12400	Stress-inducible protein (sti)	125.3	SALK_000794(e)
At2g20560	Hsp, putative	107.2	SALK_051100(e)
At4g26780	Cochaperone grpE family protein	86.3	SALK_082197(e)
At4g36990	HsfB1	85.9	SALK_012292(e)
At5g52640	Hsp81-1	67.0	SALK_007614(e)
At5g01180	Proton-dependent oligopeptide transport family protein	61.1	SALK_020787(e)
At1g30070	SGS domain-containing protein	56.8	GABI_106C03(e)
At1g03070	Expressed protein, low similarity to *N*-methyl-D-Asp receptor-associated protein	56.4	SALK_066103(e)
At3g63350	HsfA7b	51.5	SALK_152004(e)
At1g07500	Expressed protein	49.5	SALK_100918(e)
At5g10695	Expressed protein	45.0	SALK_121527(e)
At3g09350	Armadillo/β-catenin repeat family protein	37.4	SALK_072075(e)
At3g07090	Expressed protein	31.7	SALK_011655(e)
At2g47180	Galactinol synthase, putative	25.1	SALK_128044(e)
At4g11660	HsfB2b	24.4	SALK_047291(e)
At5g27660	DegP protease, putative	23.1	SALK_042659(e)
At3g13470	Chaperonin	21.6	SALK_014547(i)
At3g51910	HsfA7a	16.0	SALK_080138(e)
At4g15420	PRLI-interacting factor K	15.2	SALK_063032(e)
At3g03520	Phosphoesterase family protein	14.6	SALK_078975(e)
At2g22240	Inositol-3-P synthase isozyme 2	13.5	SALK_031685(e)
At4g23570	Phosphatase-related	11.1	SALK_026971(e)
At5g56000	Hsp81.4	10.9	SALK_147967(e)
At2g24100	Expressed protein	10.2	SALK_059512(e)
At1g23180	Armadillo/β-catenin repeat family protein	10.0	SALK_032366(e)
At5g51740	Peptidase M48 family protein	9.9	SALK_088054(e)
At4g04020	Plastid-lipid associated protein PAP, putative	9.6	SALK_026661(e)
At3g12050	Aha1 domain-containing protein	9.5	SALK_048367(e)
At4g28390	Mitochondrial ADP/ATP carrier protein, putative	7.8	SALK_053581(e)
At5g04940	SET domain-containing protein (SUVH1)	7.6	SALK_114175(e)
At1g49710	Fucosyltransferase-like protein	7.4	SALK_063355(e)
At2g47730	Glutathione transferase	5.4	SALK_039887(e)
At1g79550	Phosphoglycerate kinase	5.4	SALK_066422(e)
At3g44110	DnaJ hsp, putative	5.4	SALK_132923(e)
At3g10020	Expressed protein	4.9	SALK_078717(e)
At1g78600	Zinc finger (B-box type) family protein	4.2	SALK_105367(e)
At3g17790	Acid phosphatase type 5 (ACP5)	4.1	SALK_046785(e)
At1g52870	Peroxisomal membrane protein-related	3.9	SALK_133672(i)
At5g64400	Expressed protein	2.1	SALK_126353(i)

[a]According to The Arabidopsis Information Resource, except HsfB1. [b]The relative change in gene expression in response to 37°C for 2 h (HS) versus control in 15-d-old seedlings of wild-type Arabidopsis (Col-0). The data were extracted from the microarray data (accession no. GSE4062) published previously (Charng et al., 2006). [c]The stock numbers of ABRC or GABI-Kat program are shown. Mutants with T-DNA insertion located at exon or intron region are labeled as (e) or (i), respectively.

multiple T-DNA insertion events occurred in the *hsfA2-1* mutant line (data not shown), we performed a complementation test by transforming a wild-type copy of *HsfA2* genomic DNA into the knockout mutant to ensure that the observed phenotype was not caused by disruption of other genes by T-DNA or by a secondary mutation in the genome of the mutant line. The introduced wild-type gene, covering a 387-bp potential promoter region (Supplemental Fig. S1), rescued the mutant phenotype of *hsfA2-1* (Fig. 1A) and restored

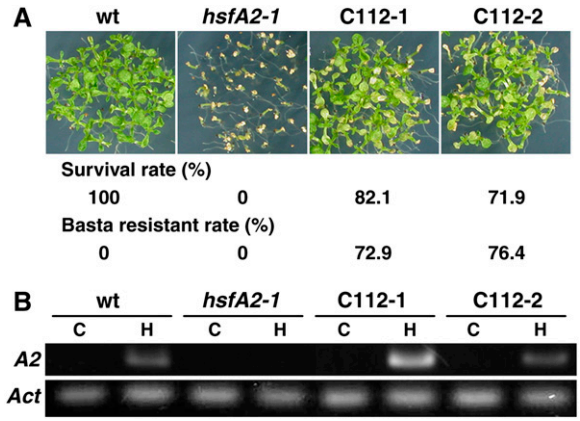

Figure 1. Disruption of *HsfA2* by T-DNA insertion led to a severe defect in AT, which could be complemented by the wild-type copy of the gene. A, The phenotypes of Arabidopsis seedlings of the wild type (wt), *hsfA2-1*, and two independent transgenic lines (C112-1 and -2, at T_2 generation) harboring transgenic *AtHsfA2* and *bar* genes in the background of *hsfA2-1* after severe HS challenge (44°C for 55 min) applied after a 2-d recovery at room temperature following a conditioning treatment at 37°C for 1 h. All the seedlings were germinated, conditioned at the 3-d-old stage, and challenged in the same plate. The photographs were taken 8 d after the challenge and reorganized for presentation. The survival rate after HS challenge and Basta-resistant rate were calculated on the basis of 50 seedlings and indicated at the bottom. B, RT-PCR analysis of the transcripts of HsfA2 in the lines mentioned above. RNA samples were purified from 5-d-old seedlings with (H) or without (C) HS treatment at 37°C for 1 h. The RT-PCR product of *actin* is shown as a loading control.

the heat-inducible expression of *HsfA2* in independent transgenic lines of the T_2 generation (Fig. 1B). The survival rate of the thermotolerance test was about the same as the resistance rate against L-phosphinothricin (Basta; Hoechst AG), conferred by the cotransformed *bar* gene, in lines C112-1 and C112-2, which suggests cosegregation of the transgene and complementation capability. Both tests yielded a survival rate close to 75% (Fig. 1B), indicating that a single T-DNA insertion event may have occurred in these transgenic lines. The results of the complementation experiment confirm that disruption of *HsfA2* caused the mutant phenotype observed in *hsfA2-1* seedlings.

Besides the defect in AT, *hsfA2-1* did not exhibit an obvious phenotypic difference in morphology, germination time and rate, growth rate, time to flowering, and seed yield as compared to the wild type under nonstress conditions, which suggests that HsfA2 is not required for normal growth and development.

HsfA2 Was Essential for the Duration But Not Induction of AT

To investigate how HsfA2 was involved in the development of AT, we compared results of a thermotolerance test between the *hsfA2-1* mutant and the wild type as well as the T-DNA knockout mutants for *Hsa32* (*hsa32-1*) and *Hsp101* (*hsp101*), which show different types of defects in AT. Hsa32 was shown to be essential for protecting Arabidopsis seedlings against severe heat stress after long but not short recovery (Charng et al., 2006), whereas Hsp101 was shown to be required for AT developed during a short (Hong and Vierling, 2000; Queitsch et al., 2000) as well as a long recovery time (Charng et al., 2006).

In a survival rate assay, 3-d-old Arabidopsis seedlings grown on solid agar medium were first conditioned at 37°C for 1 h, allowed to recover for various times at room temperature, challenged by a severe HS (at 44°C for various times), then allowed to recover at room temperature again for more than 7 d. For the wild type, the conditioning treatment prevented the seedlings from being killed by the severe HS, and the strength of AT gradually declined but lasted up to 72 h (Fig. 2, A–E). The *hsfA2-1* mutant significantly differed in survival rate from the wild type if the severe HS challenge was applied following a 2-h (Fig. 2, B and C) or 24-h (data not shown) recovery, which was similar to the case for *hsa32-1* seedlings. In contrast, the *hsp101* plants undergoing the same treatment all died (Fig. 2C). Similarly to the *hsa32-1* and *hsp101* mutant plants, the *hsfA2-1* plants became more sensitive to the severe HS challenge than the wild-type plants after a 2- to 3-d recovery (Fig. 2, D and E). However, the damage caused by the severe HS was less severe in *hsfA2-1* than in *hsa32-1* and *hsp101* mutants. In the *hsfA2-1* mutant population, a small number of the seedlings, despite being severely injured, managed to survive and eventually produce true leaves, whereas the *hsa32-1* and *hsp101* plants were all dead under the same conditions (Fig. 2, D and E). If the treatment time at 44°C was increased to 50 min, all the *hsfA2-1* mutant plants would die, as shown in Figure 1. The decreased thermotolerance after long recovery in all the mutants, however, was reversible. A second conditioning treatment 2 h before the severe HS challenge after 72 h of recovery protected the mutant plants against severe injury (Fig. 2F). These results suggest that HsfA2 is required for longer duration instead of initial induction of AT.

To elucidate further details of this process, we monitored and compared the development of phenotype in the heat-sensitive mutants after the HS regime shown in Figure 2D. The severe HS challenge did not cause any immediately observable damage in the wild-type and mutant plants, but the heat-induced injury syndrome began to develop after 2 d of recovery in the mutants (Fig. 2G). The degree of injury in the *hsfA2-1* plants seemed to be less severe than that in the *hsp101* plants (Fig. 2G). The hypocotyls of the *hsfA2-1* plants looked healthier than those of the *hsp101* mutant plants, which became severely dehydrated and wilted after 6 d of recovery. In the root, severe HS inhibited the growth of the main root in both the wild type and mutants (Fig. 2H). However, vigorous adventitious roots developed in the wild type but only began to emerge in the *hsfA2-1* or did not emerge at all in the *hsa32-1* and *hsp101* mutants (Fig. 2H). Ion leakage analysis (measuring the extent of membrane

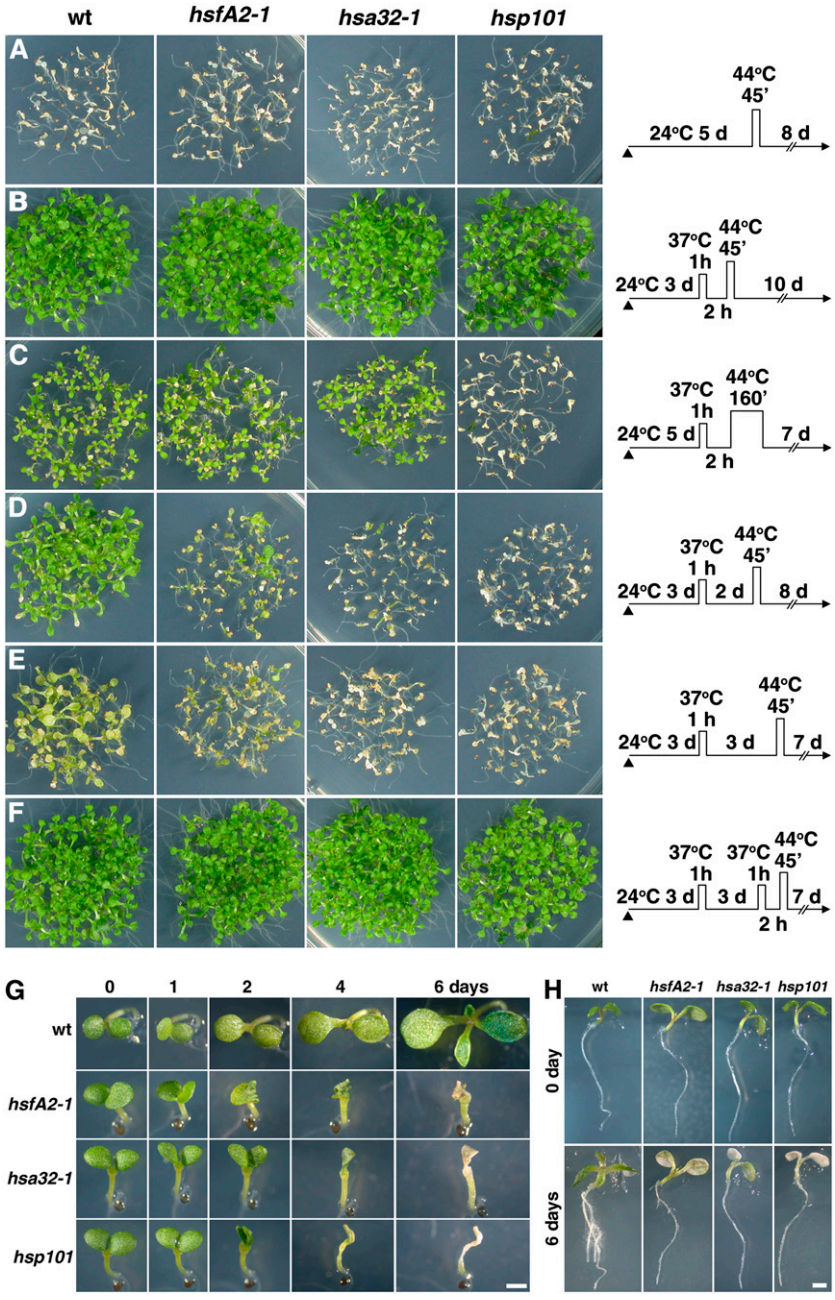

Figure 2. *hsfA2-1* plants were less tolerant than wild type to severe HS challenge after a long but not short recovery following acclimation treatment. A to F, The phenotypes of the wild type (wt), *hsfA2-1*, and T-DNA knockout lines of *Hsa32* (*hsa32-1*) and *Hsp101* (*hsp101*) plants were revealed after treatment by different HS regimes schematically shown on the right of each section. The arrowheads indicate the end of seed imbibitions. The plants were photographed 7 to 10 d after HS treatment. Seedlings of each section were grown on the same plate and reorganized for presentation. G, The progression of phenotypes of representative wild-type and mutant seedlings 0 to 6 d after the second HS treatment as shown in D. H, The morphology of roots of the wild-type and mutant lines at 0 or 6 d after the second HS treatment as shown in D. In this case, plants were grown vertically. Bar = 1 mm in both G and H.

damage) 2 h after the severe HS challenge showed no significant difference between the wild type and the mutants (Fig. 3). However, ion leakage in the wild-type plants gradually decreased after 6 h of recovery, whereas that of the mutants remained high.

We also examined the role of the other 47 heat-induced genes in AT during short recovery. Interestingly, the T-DNA insertion mutant (SALK_088054) of a putative membrane-associated protease gene showed lethal phenotype after severe HS challenge following a short but not long recovery (Supplemental Fig. S2), in contrast to the cases of *hsfA2-1* and *hsa32-1*. These results revealed the temporal roles of these genes during the development of AT and that the function of HsfA2 specifically affects AT at a later phase.

AT Declined Faster in the Absence of HsfA2

Quantitative hypocotyl elongation assay was performed as previously described to determine the decay rate of AT (Charng et al., 2006). In this study, we measured the elongation of hypocotyls of vertically grown etiolated seedlings after severe HS challenge (44°C for 45 min) following conditioning treatment at 37°C for 1 h. The recovery time between the conditioning and HS challenge was varied to reveal the kinetics

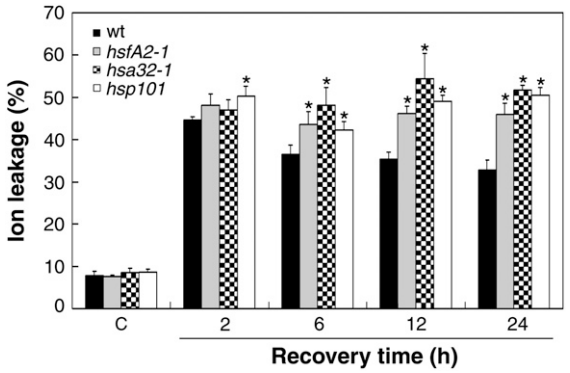

Figure 3. Ion leakage of the wild type and mutants. Seedlings 3 d old were conditioned at 37°C for 1 h, recovered at room temperature for 2 d, and then challenged by 44°C for 45 min. After the second HS treatment, the seedlings were allowed to recover at room temperature again for various times, from 0 to 24 h, before undergoing ion leakage analysis as described in "Materials and Methods." Error bars represent SD based on data in three separate duplicates. The plants with asterisks had significantly higher ion leakage than wild-type plants ($P < 0.05$, independent Student's *t* test).

of thermotolerance decay. Hypocotyls could not elongate if they were directly challenged without conditioning. Figure 4A shows the baseline of hypocotyl elongation after various recovery times following conditioning treatment, and no significant difference was observed among the *hsfA2-1*, *hsp101*, *hsa32-1*, and wild-type plants in the absence of severe HS treatment. Consistent with the results of survival rate assay, *hsfA2-1* plants showed a significant decrease in hypocotyl elongation after the severe HS challenge applied during long (>24 h) but not short (<12 h) recovery times (Fig. 4B). After challenge at 48 h of recovery, the *hsfA2-1* plants still showed some growth, while the *hsa32-1* and *hsp101* plants showed no further growth. This observation agreed with the survival rate data (Fig. 2), which suggests that HsfA2 is essential for extending the duration but not the initial induction of AT.

hsfA2-1 Showed Slightly Increased Sensitivity to HS without Acclimation

We also employed the hypocotyl elongation and survival rate assay to determine whether HsfA2 is required for basal thermotolerance, defined as the capability to tolerate severe heat stress without prior conditioning that induces HSR. We measured the length of hypocotyls elongated for 3 d after the application of a short pulse of severe HS (5 min at 46°C–50°C or 15–30 min at 44°C). During the short treatment at 44°C or above, the expression of the strongly heat-induced genes, such as *Hsp25.3-P* and *Hsp18.1-CI*, was not invoked (data not shown). Figure 5 shows that direct challenge by severe HS increasingly retarded hypocotyl growth of the etiolated seedlings, both with increased treatment time (Fig. 5A) or temperature (Fig. 5B) in all plants. However, only a small difference was observed between the wild-type and *hsfA2-1* plants, whereas the growth of the *hsp101* and *hsa32-1* plants was severely inhibited (Fig. 5, A and B). In a survival rate assay (treatment at 44°C for various times), we did not observe any significant difference between the *hsfA2-1* mutant and wild-type plants. These data suggest that Hsa32 and Hsp101 are required for basal thermotolerance, while HsfA2 is minimally involved.

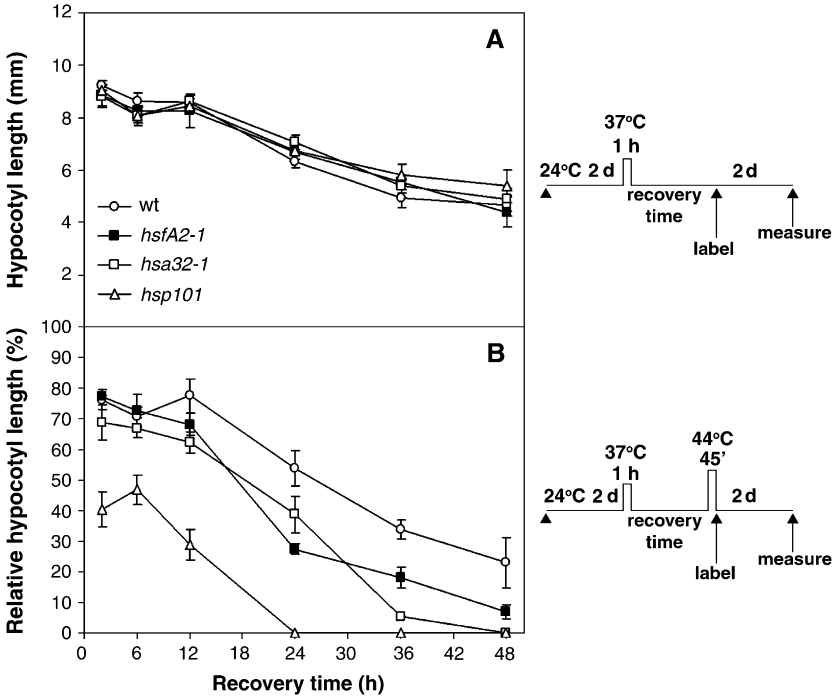

Figure 4. AT declined faster in the absence of HsfA2 as revealed by quantitative hypocotyl elongation assay. The wild-type, *hsfA2-1*, *hsa32-1*, and *hsp101* seedlings were first conditioned at 37°C for 1 h, then without (A) or subjected to (B) severe HS treatment at 44°C for 45 min after recovery for the indicated times as shown schematically at the right. The arrowheads indicate the end of seed imbibition. The positions of the top of hypocotyls were labeled right after HS treatment. The elongation of hypocotyls during 2 d of recovery in the dark was then measured. The relative hypocotyl length in B was expressed as a percentage of the numbers in A. Each data point represents the mean of 25 seedlings. Error bars represent SD based on data in five separate duplicates of the mean of five seedlings.

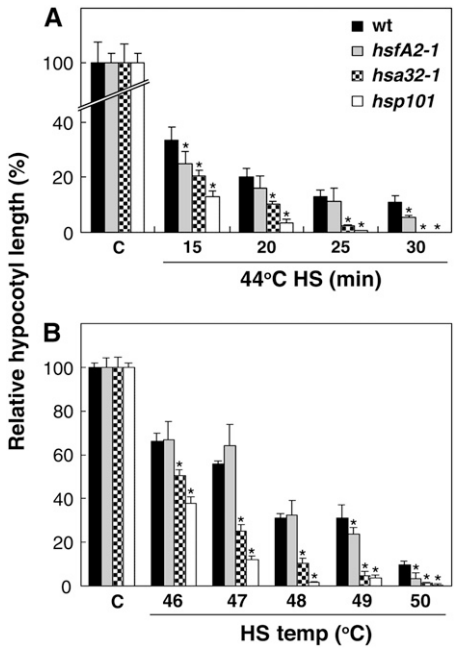

Figure 5. Disruption of *HsfA2* caused a slight reduction in basal thermotolerance. A, Etiolated seedlings 3 d old underwent direct, severe HS challenge at 44°C for 15 to 30 min or, in B, 46°C to 50°C for 5 min. After HS challenge, the seedlings were labeled for position of hypocotyls and then allowed to grow vertically for another 3 d in the dark. The elongation of hypocotyls after HS was expressed as a percentage of that of the nontreated control (C). Each data point represents the mean of 25 seedlings. Error bars represent SD based on data in five separate duplicates of the mean of five seedlings. The plants with asterisks had significantly lower values than wild-type plants ($P < 0.05$, independent Student's *t* test).

In addition, when grown under continual moderate heat stress (see ''Materials and Methods'' for condition), no obvious phenotypic difference was observed between the wild-type and *hsfA2-1* seedlings.

Disruption of *HsfA2* Lowered Expression Levels of Heat-Induced Genes during Recovery and Prolonged Heat Stress

Because AtHsfA2 was previously shown to have transcription factor activity (Port et al., 2004; Schramm et al., 2006), disruption of *HsfA2* may have significantly compromised the expression of certain Hsp genes during recovery, which in turn shortened the duration of AT. However, we could not exclude the possibility that the initial induction of some heat-induced genes required HsfA2, which may be present at a very low level without HS. First, we compared the transcriptome profiles of the wild-type and *hsfA2-1* seedlings after treatment at 37°C (HS) or 24°C (control) for 1 h with use of the Affymetrix ATH1 22 K chip, which contains 22,500 probe sets representing approximately 24,000 genes of the Arabidopsis genome. The correlation of the two biological replicates in each sample was relatively high ($R > 0.96$; Supplemental Fig. S3A). No major difference (3-fold or larger) was observed between the wild-type and mutant plants in the hybridization signals, both HS and control, in expression for all genes, including members of the Hsf family, except *HsfA2* (Supplemental Fig. S3B).

Because the phenotype of *hsfA2-1* was manifested after the severe HS challenge after 48 h or longer recovery, we used the ATH1 chip to query the transcriptome profiles of the wild-type and the mutant seedlings harvested right after the treatment at 44°C for 45 min in the HS regime indicated in Figure 2D. Control samples were seedlings of the same age but without both the conditioning and severe HS treatment. Microarray data revealed only a few genes showing significantly lower HS to control signal ratio in the mutant than in the wild type in two biological repeats. *Hsp18.1-CI* (previously known as *Hsp18.2*; Takahashi and Komeda, 1989) and *Hsp25.3-P* (previously known as *Hsp21*; Osteryoung et al., 1993) were the most affected (Supplemental Table S1). The small number of affected genes was probably due to the time point chosen for the microarray study, when the transcript levels of other Hsp genes were too low to show a difference after the long recovery. Because the expression of *Hsp18.1-CI* and *Hsp25.3-P* was not induced by severe HS at 44°C for 45 min in the wild-type and mutant plants (data not shown), abundance of the transcripts of these genes probably declined faster in *hsfA2-1* than in the wild type during recovery. Thus, we examined the transcript level of *Hsp18.1-CI*, *Hsp25.3-P*, *Hsa32*, and *Hsp101* by semiquantitative RT-PCR during the course of recovery (0–48 h) after conditioning at 37°C for 1 h (Fig. 6A). Apart from *Hsp101*, these genes have been shown to be the potential targets of HsfA1a/b in Arabidopsis because in the *HsfA1a* and *HsfA1b* double knockout mutant their expression was significantly suppressed in response to HS (Busch et al., 2005). Directly after the conditioning treatment or within 2 h of recovery, the mRNA levels of all Hsps tested differed little, which was consistent with the microarray data (Supplemental Fig. S3A). However, the transcript levels of *Hsp18.1*, *Hsp25.3-P*, and *Hsa32* were substantially lower in the *hsfA2-1* than in the wild-type plants after 4 h of recovery, with no significant difference found for *Hsp101* transcript (Fig. 6A). Western-blot analysis showed that after 48 h of recovery, the levels of Hsa32 and sHsp-CI proteins of *hsfA2-1* were lower than in the wild type (Fig. 6B), consistent with the transcript levels.

To determine whether HsfA2 is also required for the duration of HSR under prolonged HS, we also examined the transcript levels of *Hsp18.1-CI*, *Hsp25.3-P*, *Hsa32*, and *Hsp101* in seedlings subjected to continuous heat treatment at 37°C for up to 8 h. RT-PCR analysis again revealed no significant difference in transcript levels of these genes within the first hour of HS treatment but significantly lowered levels in mutant plants after 2 h of prolonged HS (Fig. 7). However, no difference in protein levels of Hsa32 and sHsp-CI was detected in these samples (data not shown),

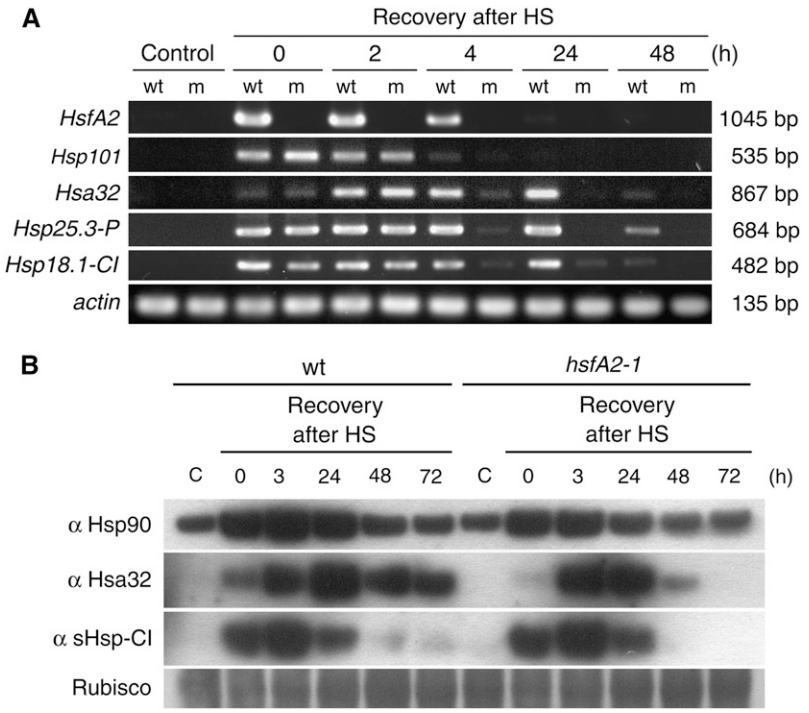

Figure 6. Transcript and protein levels of several Hsp genes declined faster in *hsfA2-1* than in wild type during recovery. A, Semiquantitative RT-PCR analysis of the mRNA levels of *Hsp101*, *Hsa32*, *Hsp25.3-P*, and *Hsp18.1-CI* in the wild-type (wt) and *hsfA2-1* (m) plants during recovery, from 0 to 48 h, after HS treatment of 3-d-old seedlings at 37°C for 1 h. The RT-PCR products of *actin* were shown as a loading control. PCR cycles for *Hsp101*, *Hsa32*, *Hsp25.3-P*, *Hsp18.1-CI*, and *actin* were 30, 30, 30, 25, and 30, respectively. The control (C) samples were seedlings without heat treatment and collected at the same time as the treated samples at 48 h. The expected sizes of the PCR products are indicated at the right. B, Immunoblot analysis of the protein levels of Hsp90, Hsa32, and class I small Hsps detected with corresponding antibodies. The samples were the same as described in A, except the additional samples were collected at 72 h of recovery. In each lane, 50 μg of total protein was loaded. Rubisco large subunit stained by Amido black was shown to ensure equal loading. The control (C) samples were seedlings without heat treatment and collected at the same time as the treated samples at 72 h. Similar results were obtained from two biological repeats, with one shown here.

perhaps due to the stability and/or lower saturation threshold of the amount of mRNA actively engaged in translation of these proteins under continuous heat. These data again suggest that HsfA2 is not required for the initial induction but, rather, for the transcription of certain heat-induced genes during recovery or prolonged HS.

DISCUSSION

In an attempt to identify genetic components involved in the regulation of the duration of AT, we tested T-DNA insertion mutant lines corresponding to 48 heat-induced genes (Table I) by a HS regime that applied severe heat stress after 2 d of recovery from acclimation to mild heat stress. Under this condition, the *HsfA2* knockout line, *hsfA2-1*, exhibited a severely heat-sensitive phenotype (Fig. 1A), while all the other mutants were not substantially different from the wild type. The results suggest that all of these genes except *HsfA2* were not essential for AT duration. Gene redundancy or existence of alternative pathways may explain why some of these mutants did not show a thermotolerance defect under the test conditions. Alternatively, they may be essential for tolerance against heat stress of different magnitude, in combination with other stresses, or at different stages of AT development. Indeed, the finding of a T-DNA knockout mutant of a putative membrane-associated protease gene, which is sensitive to severe HS challenge after a short but not long recovery following acclimation (Supplemental Fig. S2), suggests the existence of distinct stages of AT development during recovery involving different HSR genes. Investigation of this protease mutant is currently under way.

Further characterization of *hsfA2-1* revealed that *HsfA2* was dispensable in the development of AT during the early phase (Figs. 2 and 4). It appears that the gene is essential in extending the duration of AT but not for its induction. The distinct phenotype of *hsfA2-1* was in good agreement with the transcriptomic,

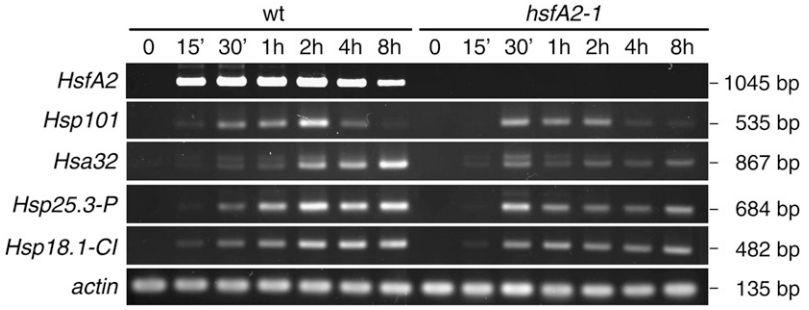

Figure 7. Transcript level of Hsp genes was lower in *hsfA2-1* than in wild type during prolonged HS. Semiquantitative RT-PCR analysis of the mRNA levels of *Hsp101*, *Hsa32*, *Hsp25.3-P*, and *Hsp18.1-CI* in the wild-type (wt) and *hsfA2-1* (m) plants during prolonged HS, from 0 to 8 h, at 37°C. Total RNA was purified from 3-d-old seedlings after treatment. The RT-PCR products of *actin* were shown as a loading control. PCR cycle for *Hsp101*, *Hsa32*, *Hsp25.3-P*, *Hsp18.1-CI*, and *actin* was 30, 30, 25, 25, and 30, respectively. Similar results were obtained from two biological repeats, with one shown here.

RT-PCR, and immunoblot results. According to microarray analysis, disruption of *HsfA2* did not significantly affect the transcript levels of other genes under normal conditions or after 37°C treatment for 1 h. Consistently, RT-PCR semiquantitative analysis indicated that HS induction of several tested Hsp genes was not affected in the absence of HsfA2 in the early phase (Figs. 6 and 7). However, in the *hsfA2-1* mutant, the level of the mRNA of Hsp genes, most prominently *Hsp25.3-P* and *Hsa32*, declined faster than that in the wild type during the recovery period (Fig. 6A). The protein level of Hsa32 in *hsfA2-1* plants was consistent with the transcript result but declined more slowly than the transcripts (Fig. 6B), probably because of its stability. The time course of the changes in protein levels correlates well with the development of the mutant phenotype (Figs. 2 and 4). Hsa32 is a novel Hsp mainly found in land plants (Liu et al., 2006b) whose molecular function is currently unknown (Liu et al., 2006a). It was previously shown to be essential for AT after long recovery (Charng et al., 2006). Therefore, the heat-sensitive phenotype of *hsfA2-1* could be attributed in part to the lower Hsa32 level in the mutant than in the wild type after 48 h of recovery. The lower transcript abundance of other Hsp genes, such as *Hsp25.3-P* and *Hsp18.1-CI*, in the mutant during recovery (Fig. 6A) may result in lower levels of encoded proteins and thus lead to a defect in thermotolerance. However, a T-DNA insertion mutant of *Hsp18.1-CI* (SALK_042001) did not show a heat-sensitive phenotype under the tested condition (Table I; data not shown), which may be due to gene redundancy of class I small Hsps in Arabidopsis (Scharf et al., 2001) or that this gene plays a relatively minor role in AT after long recovery. Our results were consistent with the data of the transcriptome analysis of *hsfA2-1* by Schramm et al. (2006), in which the transcript levels of *Hsa32*, *Hsp25.3-P*, and *Hsp18.1-CI* were substantially affected after a repeated HS (42°C for 1–3 h) treatment.

Several groups (Panchuk et al., 2002; Li et al., 2005; Miller and Mittler, 2006) have reported the connection between plant Hsf and oxidative stress. Studies on transgenic Arabidopsis overexpressing AtHsfA1b suggest that Hsf is involved in the induction of *Apx2*, which encodes the cytosolic hydrogen peroxide (H_2O_2) scavenging enzyme, ascorbate peroxidase (Panchuk et al., 2002). In the studies of Schramm et al. (2006), the heat-induction of *Apx2* is totally abolished by disruption of *HsfA2*. Moreover, a *GUS* reporter gene fused to the *Apx2* promoter can be transactivated by cotransfecting with *AtHsfA2* driven by cauliflower mosaic virus *35S* promoter in tobacco protoplasts. These results suggest that *Apx2* is a potential target of HsfA2. However, it is not clear why the initial induction of *Apx2* is abolished in the mutant after treatment at 44°C for 1 h when HsfA2 was absent in the wild-type plants (Schramm et al., 2006). In our case, the induction of *Apx2* by HS treatment (37°C for 1 h) was not affected by the absence of *HsfA2*, as revealed by microarray and RT-PCR analysis, although it was greatly suppressed in the mutant during recovery and prolonged HS (Supplemental Fig. S4). Heat stress induces production of H_2O_2 (Vacca et al., 2004; Volkov et al., 2006) that causes oxidative stress to the cell. HS-induced Apx2 may be required to remove the reactive oxygen species by compensating the activity of thermolabile Apx1 (Panchuk et al., 2002). The H_2O_2 level was shown to be higher in the *HsfA2* knockout mutant than in the wild-type plants after HS treatment, and the mutant is more sensitive to oxidative stress (Li et al., 2005), suggesting that the decreased Apx2 expression may be responsible. However, we did not observe a substantial decrease of AT in the Arabidopsis knockout mutant of *Apx2* after short or long recovery (Table I; data not shown), which suggests that suppression of the HS-induced reactive oxygen species scavenger alone does not account for the phenotype of *hsfA2-1*.

According to the transcriptome studies of Busch et al. (2005), six *Hsf* genes, *HsfA2*, *HsfA4a*, *HsfA7a*, *HsfB1*, *HsfB2a*, and *HsfB2b*, are significantly up-regulated in Arabidopsis leaves (ecotype Wassilewskija, 5–6 weeks old) by HS treatment (37°C for 1 h). This result is comparable to our microarray data (Supplemental Fig. S3B), except that in our Arabidopsis sample (ecotype Columbia [Col-0] 5-d-old seedlings), *HsfA7b* but not *HsfA4a* was significantly responsive to heat. Our results are consistent with the microarray data generated by von Koskull-Doring's group for heat-stress response of Arabidopsis (ecotype Col-0, 16-d-old seedlings) at 37°C for 1 h, which was examined using the Genevestigator tool (Zimmermann et al., 2004). This discrepancy may result from the use of different Arabidopsis ecotypes or different growth stages or condition of the plants. Nevertheless, the presence of multiple heat-inducible Hsfs in Arabidopsis suggests that there exists a complex feedback control of plant HSR after its initial induction. Our microarray data revealed no significant alteration of the expression of other Hsfs under normal and HS conditions (37°C for 1 h) in the *hsfA2-1* mutant (Supplemental Fig. S3). Although amino acid comparison of DNA-binding domains and HR-A/B regions showed HsfA2 and HsfA7a/b to have a close phylogenetic relationship (Nover et al., 2001), the loss of HsfA2 apparently could not be compensated by the presence of these two heat-induced A-type Hsfs. HsfA2 and other Hsfs may not be functionally redundant because of their differences in structure (Nover et al., 2001). The dominance of HsfA2 following HS treatment may be another possibility (Baniwal et al., 2004). From the HSR microarray data available to date, *HsfA2* is the gene most induced by heat, which suggests a dominant role for the gene in sustaining the HSR, a role that cannot be replaced by *HsfA7a* and *HsfA7b*. This idea is supported by the observations that the T-DNA knockout lines of *HsfA7a* and *HsfA7b* did not show a thermotolerance defect like that of *hsfA2-1* (Table I; data not shown).

The heat-induced B-type Hsfs (*HsfB1*, *HsfB2a*, and *HsfB2b*), which lack the transactivation domain (Nover

et al., 2001), may play a different role from that of HsfA2. Tomato HsfB1, not a transcription activator by itself, synergistically enhances the transactivation activity of LeHsfA1 and LeHsfA2 on the expression of a reporter gene driven by a HS promoter (Bharti et al., 2004). However, despite close homology with tomato HsfB1 (Nover et al., 2001), AtHsfB1 was shown to suppress the expression of heat-inducible reporter gene when transiently coexpressed with AtHsfA4a (Czarnecka-Verner et al., 2000). It is possible that HsfB1 acts differentially as a positive or negative transcription coactivator of some members of the Hsf family. The T-DNA knockout line of *HsfB1* did not show thermotolerance defect like *hsfA2-1* under our test condition (Table I; data not shown), suggesting that HsfB1 is not essential for extending the duration of AT like HsfA2 does. This is consistent with our observation that the expression of *Hsa32*, *Hsp25.3-P*, and *Hsp18.1-CI* was not substantially affected in the *HsfB1* T-DNA knockout mutant during recovery or prolonged heat stress, unlike in the knockout mutant *hsfA2-1* (N.-y. Liu and Y.-y. Charng, unpublished data). The function of AtHsfB2a/b is currently not clear. No significant heat-sensitive phenotype was observed for the *AtHsfB2b* knockout line (Table I; data not shown), probably due to functional redundancy with *AtHsfB2a*, for which we did not obtain a knockout mutant.

In Arabidopsis, HsfA1a/b were shown to be the major transactivators of heat-induced genes in the early phase of AT. The double knockout mutant of *AtHsfA1a/b* failed to accumulate the wild-type level of mRNA of a number of heat-induced genes when treated at 37°C for 1 h (Busch et al., 2005), but when the HS was lengthened to 2 h, the transcripts of several monitored Hsp genes except *Hsp18.2* (At5g59720) reached approximately the same levels as in the wild type (Lohmann et al., 2004). This observation suggests that other Hsfs, such as HsfA2, act as major transactivators during prolonged heat stress. This hypothesis is supported by the finding that in the absence of HsfA2, the expression of tested *Hsp* genes was substantially lower than in the wild type after 2 h of prolonged HS (Fig. 7). HsfA2 acting as a transactivator has been well characterized by previous studies (Boscheinen et al., 1997; Rojas et al., 2002; Kotak et al., 2004; Schramm et al., 2006). It is tempting to speculate that HsfA1s initiates HSR by inducing downstream genes, including HsfA2, which then acts as a positive feedback regulator to sustain the expression of heat-induced genes during recovery or prolonged HS. However, it is to be noted that induction of tomato *HsfA2* by HS requires HsfA1a (Mishra et al., 2002), but in Arabidopsis induction of *HsfA2* seems to be independent of HsfA1a/b (Busch et al., 2005). Whether the other two A1-type Hsfs, HsfA1d/HsfA1e, are responsible for the heat induction of *HsfA2* is currently not known. Our preliminary data showed that neither the *HsfA1d* nor *HsfA1e* knockout led to alteration of the heat-induced expression of *HsfA2*. Further study on a double knockout line of these two genes is under way.

Given the status of *HsfA2* as a heat-inducible gene and its function in sustaining the expression level of tested Hsp genes (Figs. 6 and 7), the role of HsfA2 in extending the duration but not in induction of AT is conceivable. This inference is well supported by the immunoblot result of Schramm et al. (2006) that HsfA2 is a heat-inducible protein greatly accumulated in leaves after 3 h of HS at 42°C. Their data show no or very low abundance of HsfA2 protein without HS or after 1 h of HS, which is in good agreement with our conclusion that HsfA2 is not involved in the initial induction of HSR and AT developed at early phase. This explains why the mutant did not show heat-sensitive phenotype when a short recovery was associated with acclimation treatment both in this study as well as in that of Schramm et al. (2006). However, these observations were not in agreement with the results of Li et al. (2005), who also studied the function of *HsfA2* using the same T-DNA knockout line (SALK_008978). In their study, the mutant plants displayed significantly reduced AT, but their thermotolerance phenotype was demonstrated under rather different conditions (in 7-d-old seedlings, acclimatized at 37°C for 1 h, then challenged at 46°C for 2 h without recovery). In addition, basal thermotolerance of the mutant was also reduced. The authors then showed that the HS (37°C)-induced transcript levels of *Hsp70* (At3g12580) and *Hsp101* (At1g74310) in the *HsfA2* knockout mutant were substantially reduced. These results are inconsistent with the data reported here and by Schramm et al. (2006), in which the expression of most genes, including *Hsp70* and *Hsp101*, were not changed and no significant thermotolerance defect was observed in the mutant after short recovery or without acclimation. One possible cause of this discrepancy may be the different methods or apparatus used for thermotolerance assay. Alternatively, the discrepancy may be due to different T-DNA insertion numbers in the knockout mutants that we used. We did observe multiple copies of T-DNA in homozygous *hsfA2-1* plants by Southern blot (data not shown), and segregation of these T-DNA insertions in its offspring may have resulted in different genetic backgrounds that lead to weaker stress tolerance in some of them. Unfortunately, a complementation experiment was not conducted by Li et al. (2005) to exclude this possibility.

Taken together, our results suggest that HsfA2 is one of the components involved in extending the duration of AT in plants by positively regulating the expression of at least some, if not all, heat-induced genes after their initial induction by HsfA1(s).

MATERIALS AND METHODS

Plant Materials, Transformation, and Growth Condition

The Arabidopsis (*Arabidopsis thaliana*) Col-0 *HsfA2* (At2g26150) T-DNA insertion line SALK_008978 (Alonso et al., 2003) was obtained from the Arabidopsis Biological Resource Center (ABRC; Ohio State University). The T-DNA insertion lines SALK_066374 for *Hsp101* (At1g74310) and GABI-Kat 268A08

for *Hsa32* (At4g21320) were obtained as previously described (Charng et al., 2006). Information on all the other T-DNA insertion lines obtained from ABRC or GABI-Kat program (Rosso et al., 2003) is given in Table I. The T-DNA insertions were confirmed by PCR amplification, and then homozygous lines of the mutant allele were isolated. The design of PCR primers and isolation of homozygous line involved use of the SIGnAL T-DNA Express Arabidopsis gene mapping tool of the Salk Institute (http://signal.salk.edu/tdnaprimers.2.html). The sequence and PCR conditions for all the gene-specific primers used in this study are given in Supplemental Table S2. For complementation of the *hsfA2-1* line, the genomic DNA of Arabidopsis wild-type (Col-0) *HsfA2* was amplified by PCR with a pair of gene-specific primers (Supplemental Table S2). The 2,177-bp PCR product was cloned into pCR8/GW/TOPO (Invitrogen), then sequenced to confirm no missense or nonsense mutation in coding region or any point mutation in the promoter region. The cloned *HsfA2* genomic DNA was then subcloned into pBGW,0 (Karimi et al., 2005) by an in vitro recombinase method to yield the binary vector pYC112, which was then transferred into *Agrobacterium tumefaciens* LBA4404 strain for Arabidopsis (Col-0) transformation by the floral dip method (Clough and Bent, 1998). Transformants were selected on 0.5 × Murashige and Skoog medium plates containing 25 μg/mL Basta. T2 seeds of independent lines underwent herbicide resistance and thermotolerance tests. For propagating seeds, Arabidopsis plants in soil were grown in walk-in growth chambers at 22°C and 16 h of light (120 μmol m^{-2} s^{-1}).

Thermotolerance Test

All the knockout lines were grown under the same condition as the wild-type plants for harvesting seeds for thermotolerance test. For the AT test on Arabidopsis seedlings, survival rate and quantitative hypocotyl elongation assay were performed as previously described (Charng et al., 2006). A modified quantitative hypocotyl elongation assay was applied to evaluate the basal thermotolerance of etiolated seedlings. The seeds were sown and grown vertically in the dark for 3 d at 24°C, and then the plants were heated in a water bath at 44°C for 15 to 30 min or at 46°C to 50°C for 5 min. After this heat treatment, the hypocotyl positions were labeled. The plants were kept grown vertically for 3 d at 24°C, and then the length of hypocotyl elongation was measured. To evaluate the effect of continuously moderate heat stress, 3-d-old seedlings were grown on agar plate up to 3 weeks in growth chambers with temperature set at 35°C during the light period (16 h, 120 mmol m^{-2} s^{-1}) and 33°C during the dark period.

Measurement of Ion Leakage

Three-day-old seedlings were conditioned at 37°C for 1 h, recovered for 2 d, and then heated at 44°C for 45 min. At each time point during the recovery after 44°C HS, 30 seedlings were collected and immersed in 5 mL of deionized water. The samples were kept at room temperature for 5 h, then ion leakage level was measured by a conductivity meter (model no. SC-120, Suntex). The seedlings were autoclaved and the total ion leakage was measured. The relative ion leakage was calculated as (ion leakage level/total) × 100%.

RNA Isolation and RT-PCR Analysis

Total RNA was isolated with TRIZOL reagent (Invitrogen) from plant samples frozen in liquid nitrogen according to the manufacturer's protocol. The presence or absence of transcript of the genes listed in Table I was analyzed by RT-PCR using RNA isolated from the wild-type and mutant plants with or without HS (37°C for 1 h). The sequences of the primers for this purpose were shown in Supplemental Table S2. Semiquantitative RT-PCR analysis of the marker *Hsp* genes was performed basically as previously described (Wang et al., 2001). The sequences of the primers for RT-PCR analysis for each gene are also described in Supplemental Table S2. PCR product was analyzed by agarose gel electrophoresis and staining with ethidium bromide. Florescence images of PCR products were digitized and quantified with Image Gauge Version 3.12 (Fujifilm).

Microarray Analysis

Transcriptome profiling was performed by use of the ATH1 GeneChip array (Affymetrix) as previously described (Charng et al., 2006). For each treatment, two biological replicates and one chip for each replicate were used. The microarray data were processed and analyzed by use of Spotfire DecisionSite 8.0 as previously described (Charng et al., 2006). The microarray data reported here were deposited in the Gene Expression Omnibus at the National Center for Biotechnology Information (GSE4760).

Immunoblotting

The total proteins of plant samples were extracted and quantified and then underwent immunoblot analysis for Hsp90, Hsa32, and sHsp-CI as described previously (Charng et al., 2006).

Supplemental Data

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

Supplemental Figure S1. Schematic structure of *HsfA2* gene carrying the T-DNA insertion and lack of *HsfA2* expression in the *hsfA2-1* mutant.

Supplemental Figure S2. The SALK_088054 line was more sensitive than the wild type to severe HS challenge after a short but not long recovery following acclimation treatment.

Supplemental Figure S3. Transcriptome profile analysis and comparison between the *hsfA2-1* and wild-type plants.

Supplemental Figure S4. The expression of heat-induced *Apx2* was substantially affected in *hsfA2-1*.

Supplemental Table S1. The heat shock response genes most affected by disruption of *HsfA2*.

Supplemental Table S2. Primers used for RT-PCR and genotyping.

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