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Functional analysis of an *Arabidopsis* heat-shock transcription factor *HsfA3* in the transcriptional cascade downstream of the DREB2A stress-regulatory system

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Abstract

A transcription factor DREB2A functions as a key regulator not only in drought stress responses but also in heat stress (HS) responses, and activates expression of many abiotic stress-responsive-genes involved in drought and HS tolerance. *HsfA3* is one of the most up-regulated heat-inducible genes in transgenic plants overexpressing DREB2A. In this study, the analyses of *HsfA3* expression profile and the transactivation analysis of *HsfA3* showed that the expression of *HsfA3* was directly regulated by DREB2A under HS. Microarray analysis using transgenic plants overexpressing *HsfA3* also showed that overexpression of *HsfA3* induces many heat-inducible genes. Furthermore, we showed that thermotolerance of the *HsfA3* overexpressors was increased, and that of the *hsfA3* T-DNA tagged mutants was decreased. These results indicate that *HsfA3* regulates expression of many heat-inducible genes in the transcriptional cascade downstream of the DREB2A stress-regulatory system and functions in acquisition of thermotolerance under the control of the DREB2A cascade.
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High temperature is a key stress factor with a negative impact on the growth of plants and the productivity of crops. Many genes involved in the stress tolerance and response are induced under the high temperature stress condition. These genes include heat-shock transcription factors (HSFs) and heat-shock proteins (HSPs). HSFs regulate the expression of heat-shock-regulated genes including HSPs as transcription factors [1]. In *Arabidopsis*, 21 members of HSFs were identified and divided into three classes, A, B, and C, based on structural features of their oligomerization domains [2]. Among them function of the several HSFs has been reported. Using knockout mutants, HsfA1a and HsfA1b were shown to be important for the immediate stress-induced activation of heat-shock-responsive gene expression [3] and heat-inducible HsfA2 was characterized to be essential for extending the duration of acquired thermotolerance [4,5]. HsfA4a and HsfA8 are hypothesized to function as sensors of reactive oxygen species (ROS) [6] and

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HsfA5 was reported to function as a specific repressor of *HsfA4* [7]. Furthermore, *HsfA9* was shown to be regulated by ABI3 and function as a master regulator for expression of *Hsp* genes during seed development [8]. Thus, HSFs form a complex regulatory network in *Arabidopsis* and their complex regulation of gene expression is considered to be important for causing flexible responses of plant to environmental conditions.

The dehydration-responsive element (DRE) with the core sequence A/GCCGAC was identified as a *cis*-acting promoter element that regulates gene expression in response to drought, high-salinity, and cold stresses in *Arabidopsis* [9]. A similar motif was identified as the C-repeat and low-temperature-responsive element in cold-inducible genes [10]. *Arabidopsis* cDNAs encoding DRE-binding proteins, *DREB1A/CBF3*, and *DREB2A* were isolated by using the yeast one-hybrid screening method [9,10]. These proteins are AP2/ERF type transcription factors binding the DRE sequence and activate the expression of genes driven by the DRE sequence. In *Arabidopsis*, there are three DREB1/CBF proteins, DREB1A/CBF3, DREB1B/CBF1, and DREB1C/CBF2 [9,10] and two DREB2 proteins, DREB2A and DREB2B [11]. While expression of the *DREB1/CBF* genes is induced by cold stress, expression of the *DREB2* genes is induced by drought and high-salinity [9,12]. These *DREB2* genes are also induced by heat stress (HS) [13]. However, overexpression of the intact *DREB2A* gene did not result in any remarkable alterations in plant phenotype and expression of the downstream genes [9]. Recently, a negative regulatory domain was found in the central region of DREB2A and deletion of this region transformed DREB2A to a constitutive active form (DREB2A-CA) [12]. Overexpression of DREB2A-CA resulted in improved tolerance to both drought stress and HS in the plants [12,13]. Microarray analysis indicated that the expression of many drought- and/or heat-shock-inducible genes are up-regulated in the DREB2A-CA overexpressor [12,13]. The highest up-regulated gene was *HsfA3* which is a member of the *HSF* gene family and belongs to class A. *HsfA3* is a unique *HSF* gene up-regulated by DREB2A-CA and phylogenetically distinct from other *HsfAs* (Supplementary Fig. 1). In the transgenic plants many HSPs were also up-regulated, which allows us to expect that *HsfA3* contributes to expression of these HSPs in the transcriptional cascade downstream of the DREB2A regulatory system and improves thermotolerance.

In this study, we carried out expression and transactivation analyses of *HsfA3* and demonstrated that expression of *HsfA3* is directly regulated by *DREB2A* under the HS condition. We also characterized the function of *HsfA3* using *HsfA3* overexpressors and knockout mutants and found that *HsfA3* regulates many heat-shock-related genes in response to HS. Furthermore, we report thermotolerance of the overexpressors and the mutants, and discuss the role of *HsfA3* in the transcriptional cascade in response to HS.

Materials and methods

Plant materials. Plants (*Arabidopsis thaliana* ecotype Columbia) were grown on germination medium agar plates for 10–48 days, as described previously [12]. The dreb2a-1 (379F02), dreb2a-2 (179C04) and hsfa3-2 (208B08) mutants were obtained from the GABI-KAT [14]. The hsfa3-1 (SALK_011107) was obtained from ABRC. For HS treatment, Arabidopsis seedlings were grown on agar plates at 22 °C for 2–3 weeks and then transferred to 37 °C. Arabidopsis T87 suspension-cultured cells were maintained as described previously [12].

Quantitative RT-PCR analysis. Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantitative RT-PCR was carried out as described on a 7300 Real Time PCR system (Applied Biosystems) using POWER SYBR GREEN PCR MASTER MIX (Applied Biosystems) [13].

Plant transformation. The 35S:*HsfA3* and 35S:sGFP-*HsfA3* plasmids were constructed by cloning of cDNAs encoding the *HsfA3* protein into a pGreenII0029 El2-35S-Ω vector and a pGreenII0029 El2-35S-Ω NsGFP vector [13], respectively. The constructed plasmids were introduced into *Agrobacterium tumefaciens* GV3101 cells. Plants were transformed as described previously [12].

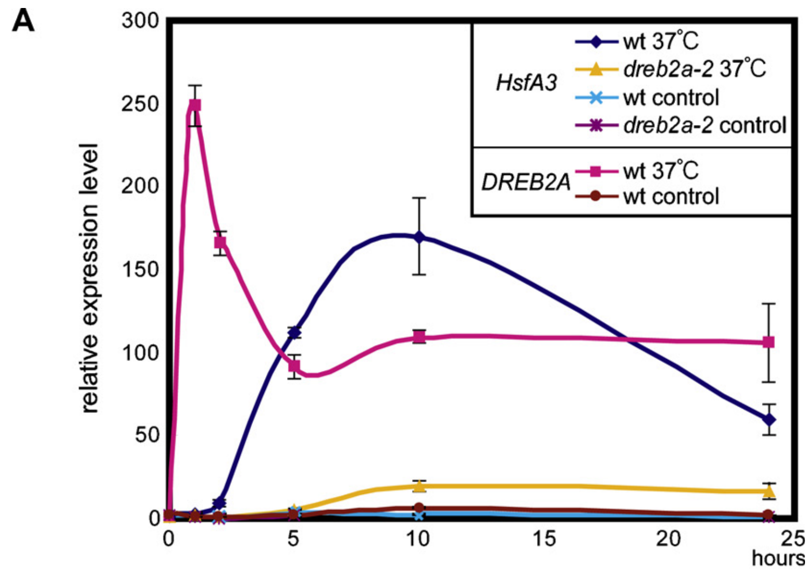
Histochemical analysis. For the *HsfA3* promoter:GUS plasmid, a 1000-bp fragment of the *HsfA3* promoter was inserted into the pGreenII0029GUS vector, which has a GUS sequence in the ApaI site. Histochemical localization signals of GUS activity were detected as described [15].

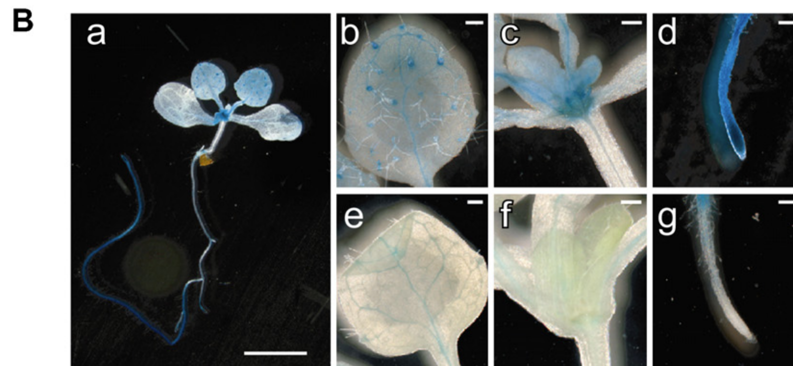
Observation of subcellular localization of green fluorescent signals in transgenic plants. The 1000-bp *HsfA3* promoter and the sGFP fused with the *HsfA3* coding sequence and the Nos terminator of the 35S:sGFP-*HsfA3* plasmid were cloned into the pGreenII0029 vector. GFP fluorescence was analyzed with a confocal laser-scanning microscope LSM5 PASCAL (Zeiss, Oberkochen, Germany).

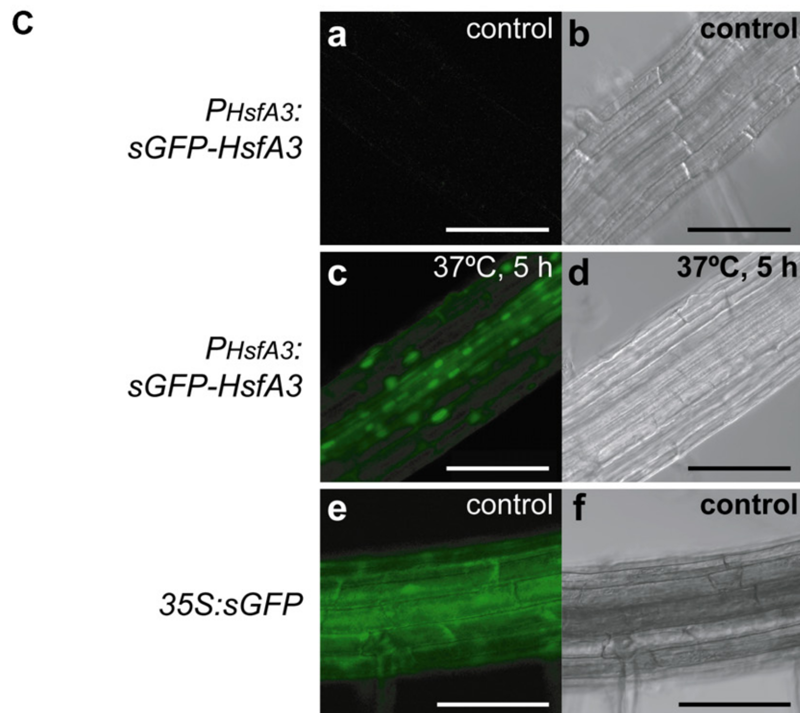
Transient expression experiments. For effector plasmids, we used 35S:DREB2A-CA [12] and 35S:DREB1A [11]. For reporter plasmids two copies of the 50-bp fragments containing DRE or mutated DRE core motifs of the *HsfA3* promoter were inserted into the RD29AmTATA-GUS plasmid [12]. The *HsfA3* promoter:GUS plasmid was also used for a reporter plasmid. Transient expression assay was performed as described previously [12].

Microarray analysis. Total RNA was isolated from two lines of transgenic plants overexpressing *HsfA3*, *35S*:*HsfA3-a*, and *35S*:*HsfA3-b* and used for the preparation of Cy5- and Cy3-labeled cRNA probes. All microarray experiments, including the data analysis, were performed

Fig. 1. Stress-inducible and tissue-specific expression of the *HsfA3* gene and subcellular localization of the *HsfA3* protein. (A) Expression of *HsfA3* and *DREB2A* in response to HS (37 °C) treatment. Total RNA was prepared from about 3-week-old *Arabidopsis* plants that had been heated at 37 °C or incubated at 22 °C for several hours. Accumulation of the *HsfA3* and *DREB2A* mRNAs was analyzed by quantitative RT-PCR. Bars indicate SD ($n = 3$). The expression level under control condition was defined as 1.0. (B) Histochemical localization of GUS activity in the 10-day-old T2 transgenic plants containing the *HsfA3 promoter*:*GUS* fusion gene. Transgenic plants were incubated at 37 °C for 10 h and stained for 2 h. Plants (a–d) and (e–g) were stained after incubation and before incubation, respectively. Scale bar = 5 mm (a) and 0.4 mm (b–g). (C) Confocal microscope images of sGFP fluorescence (a,c, and e) and Nomarski microscope images (b,d, and f) of the *HsfA3 promoter*:*sGFP-HsfA3* plants and the 35 S:*sGFP* plants. The root tissues were observed under microscope before (a,b,e, and f) or immediately after (c and d) incubation at 37 °C for 5 h. Scale bar = 100 μm.







according to Agilent methods. The reproducibility of microarray analysis was assayed by biological and technical (dye swap) replicates in each experiment.

Thermotolerance of plants. Thermotolerance test was performed as described [13].

Results and discussion

Analysis of HsfA3 expression and its localization

We performed quantitative RT-PCR to investigate the expressions of *HsfA3* and *DREB2A* during heat stress (HS) in wild-type plants (Fig. 1A). The expression of *HsfA3* was gradually induced by 37 °C treatment, especially increased after 2 h, and peaked at 10 h. In contrast, the expression of *DREB2A* was rapidly and transiently induced under HS condition and peaked around 1 h. These phased expression patterns of *HsfA3* and *DREB2A* suggest that *HsfA3* and *DREB2A* act for acclimation to HS in sequence. To clarify the effect of *DREB2A* on the expression of *HsfA3*, we analyzed the expression of *HsfA3* in the *DREB2A* knock out mutant, *dreb2a-2*. Repression of *DREB2A* expression in the *dreb2a-2* plants was confirmed in a previous study [13]. The HS-inducible expression of *HsfA3* was decreased markedly in *dreb2a*. The phased expression patterns of *HsfA3* and *DREB2A* and the strong repression of *HsfA3* expression in *dreb2a* indicate that *HsfA3* is mainly regulated by *DREB2A*.

Then we examined the tissue-specific expression with 10-day-old T_2 transgenic *Arabidopsis* plants harboring a *HsfA3* promoter:*GUS* fusion construct (P_{HsfA3}:*GUS*). Weak histochemical staining of GUS activity was observed in roots and veins of leaves under normal growth conditions (Fig. 1B). To examine the staining patterns of these transgenic plants under stress condition, we incubated the plants on agar plates at 37 °C for 10 h. We found significant GUS staining in roots and leaves. Especially the trichomes, nodes of petioles, and root tips presented stronger GUS staining than other regions of plants. We also analyzed *DREB2A* promoter:*GUS* (P_{DREB2A}:*GUS*) transgenic plants [15], after 2 h HS treatment. We observed similar *GUS* staining patterns in roots and leaves of the P_{DREB2A}:*GUS* transgenic plants to those of the P_{HsfA3}:*GUS* transgenics (Supplementary Fig. 2). These results indicate that the *HsfA3* mRNA was accumulated in leaves and roots under HS condition. Moreover, similar expression patterns between *HsfA3* and *DREB2A* suggest that these two genes function in the same tissues under HS condition.

To ascertain that HS induces accumulation of the *HsfA3* protein, we generated transgenic plants harboring a *sGFP-HsfA3* construct driven by the *HsfA3* promoter (P_{HsfA3}:*sGFP-HsfA3*). We used the same promoter region as the P_{HsfA3}:*GUS* construct. Ten-day-old plants grown on agar plates were incubated at 37 °C for 5 h. Then these plants were immediately observed with confocal microscopy. GFP fluorescence was mainly detected in the nuclei of root tissue (Fig. 1C) and also leaves (data not shown), which indicates that the *HsfA3* protein is accumulated in nuclei under HS condition. Interestingly, before HS treatment, we detected almost no GFP fluorescence in roots and leaves, although weak GUS staining was observed in P_{HsfA3}:*GUS* transgenic plants. There might be a posttranscriptional regulatory system to stabilize the *HsfA3* protein under HS condition.

Transactivation of the P_{HsfA3}:GUS fusion gene by DREB2A

To analyze the transcriptional regulation of *HsfA3* by DREB2A in vivo, we performed transactivation experiments using protoplasts prepared from *Arabidopsis* T87 suspension-cultured cells. We used two effector plasmids expressing *DREB2A-CA* and *DREB1A*, respectively, and the P_{HsfA3}:*GUS* construct as a reporter plasmid [13]. These plasmids were cotransfected into *Arabidopsis* protoplasts, and transactivation of the reporter gene was analyzed. As shown in Fig. 2A, expression of DREB2A-CA and DREB1A resulted in 17 and 3 times higher transactivation of the reporter gene than that of the vector as a control, respectively. These results indicate that the *HsfA3* promoter is activated by both DREB2A-CA and DREB1A and DREB2A-CA has five times higher transactivation ability than DREB1A.

To determine whether DRE elements in the *HsfA3* promoter were actually activated by DREB2A-CA, we constructed the *GUS* reporter gene fused to two copies of the DRE or mutated DRE elements (Fig. 2B). We used two kinds of 50-bp sequences that contained independent DRE core motifs of the *HsfA3* promoter (Fig. 2B, DRE1 and DRE2). We also used 50-bp sequences mutated in the DRE or not in DRE as shown in Fig. 2B. Coexpression of DREB2A-CA resulted in more than five times higher transactivation of the reporter genes fused to wild-type DRE1 (DRE1 WT) and DRE2 (DRE2 WT) than the reporter genes fused to a minimal TATA promoter (control). The reporter genes fused to mutated DRE1 M2 and DRE2 M2 were also activated by DREB2A-CA. Both DRE1 M2 and DRE2 M2 have mutations not in the DRE motifs. In contrast, the reporter genes fused to DRE1 M1 and DRE2 M1, which have mutations in the DRE motifs were not activated by DREB2A-CA. These results indicate that two DRE core motifs are key sequences for expression of the *HsfA3* gene under the control of DREB2A. Recently, Schramm et al. reported independently that DREB2A could activate the *HsfA3* promoter using transient GUS reporter assays in mesophyll protoplasts [16].

Overexpression of HsfA3 in Arabidopsis

To determine the function of *HsfA3*, we overexpressed *HsfA3* in *Arabidopsis*. Plants were transformed with vectors carrying fusions of the enhanced CaMV 35S promoter [11] and the *HsfA3* cDNA (*35S*:*HsfA3*). Eight transgenic *Arabidopsis* plants were generated using the vacuum infiltration method [17]. To investigate the expression level of *HsfA3* in these transgenic plants, we performed quantitative RT-PCR analysis and selected three transgenic lines, *35S*:*HsfA3-a*, *35S*:*HsfA3-b*, and *35S*:*HsfA3-c* that showed strong, moderate and weak but sufficiently high level of

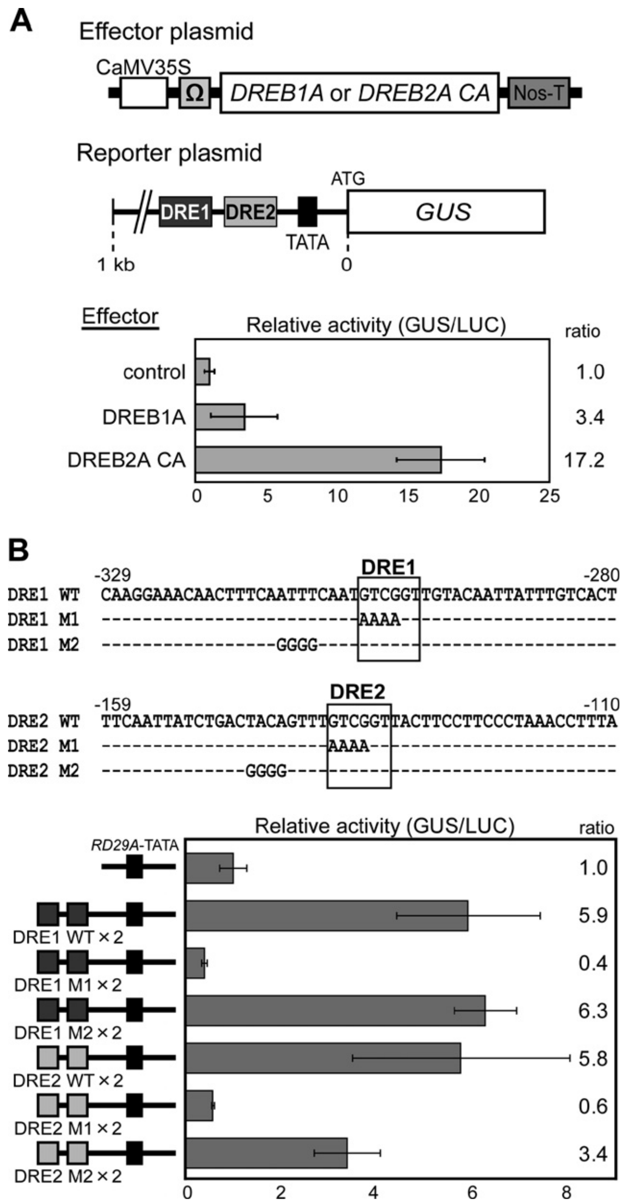


Fig. 2. Transactivation of the *HsfA3 promoter*:*GUS* fusion genes by DREB2A in protoplasts prepared from *Arabidopsis* T87 Cells. The 35 S *promoter*:luciferase (*LUC*) plasmid was cotransfected in each experiment to normalize for transfection efficiency [12]. Bars indicate SD ($n = 3$). (A) Transactivation of the *HsfA3* promoter:*GUS* fusion gene by the DREB1A and DREB2A-CA proteins. Ratios indicate the multiples of expression compared with the value obtained with the vector. The reporter gene fused to the 1000-bp fragments of the *HsfA3* promoter was transfected with each effector plasmid or the vector as a control. (B) Transactivation of the *GUS* reporter gene fused to the DRE elements of the *HsfA3* promoter by DREB2A-CA. Ratios indicate the multiples of expression compared with the value obtained with the *GUS* reporter gene fused to only TATA sequences. The reporter genes driven by two copies of 50-bp fragments which contain the wild-type or mutated DRE core motifs of the *HsfA3* promoter were transfected with the effector plasmid which expressed DREB2A-CA protein.

the transgene expression, respectively, for further analysis (Fig. 3A).

We observed the growth of the *35S*:*HsfA3* plants under normal conditions and compared them with vector control plants. The *35S*:*HsfA3-a* plants that have strong expression of the transgene showed a severely dwarfed phenotype. Among the generated 8 transgenic plants, 2 lines exhibited similar dwarfism including *35S*:*HsfA3-a* and seed productivity of these plants decreased to less than 1% of that of the wild-type plants. The *35S*:*HsfA3-b* plants with moderate growth retardation and the *35S*:*HsfA3-c* plants with little change of phenotype had thicker petioles and showed similar seed productivity compared with the wild-type plants (Fig. 3B).

Microarray and quantitative RT-PCR analyses of transgenic Arabidopsis plants overexpressing HsfA3

To analyze genes downstream of *HsfA3* in *Arabidopsis*, we performed array analysis using an Agilent *Arabidopsis* 3 Oligo Microarray (Agilent Technologies, Palo Alto, CA), covering >44,000 genes. We used two independent transgenic *Arabidopsis* plants overexpressing *HsfA3*, *35S*:*HsfA3-a* and *35S*:*HsfA3-b*. Cy3- and Cy5-labeled cRNA probes were prepared from mRNAs isolated from the *35S*:*HsfA3* and control plants, respectively, grown under unstressed control conditions. In comparison to vector control plants, 121 genes with the average of fold changes four times greater than in the *35S*:*HsfA3* plants are detected (Supplementary Table 2 and Supplementary Fig. 3). Among the 121 up-regulated genes, 29 genes were up-regulated more than 2-fold in the *DREB2A-CA* overexpressor [13] and 45 genes were characterized to be HS-inducible, while 22 of them were overlapped.

For further analysis, we focused on 20 up-regulated genes in the *35S*:*HsfA3* plants that showed remarkable fold changes greater than 50 times. Among the 20 genes, 11 genes were up-regulated in the *DREB2A-CA* overexpressor and also shown to be HS-inducible, whereas 5 genes were characterized to be only HS-inducible. The HSE sequence (nGAAnnTTCn or nTTCnnGAAn) [18], which is a recognition sequence of HSFs, was found in the 1000-bp promoter region of 15 genes among these 20 up-regulated genes. Expression of several up-regulated genes in the *35S*:*HsfA3* plants identified by the microarray was confirmed by using quantitative RT-PCR and shown to increase in the *35S*:*HsfA3* plants (Fig. 3C). Among the 20 up-regulated genes, 12 genes encode HSP-related proteins which are proposed to act as molecular chaperones in protein quality control and expected to function in acquisition of HS tolerance. Intriguingly, three *Hsfs*, *HsfA1e*, *HsfA7b*, and *HsfB2a*, were identified as up-regulated genes in the *35S*:*HsfA3* plants. All three genes have the HSE sequence in the promoter region. The up-regulated expression of *HsfA1e* and *HsfB2a* in the *35S*:*HsfA3* plants was confirmed by quantitative RT-PCR analysis (Fig. 3C). Further transcriptional cascades in response to HS are expected downstream of *HsfA3* because of the up-regulation of these *Hsf* genes. Thus, *HsfA3* plays an important role in the HS responsive gene expression.

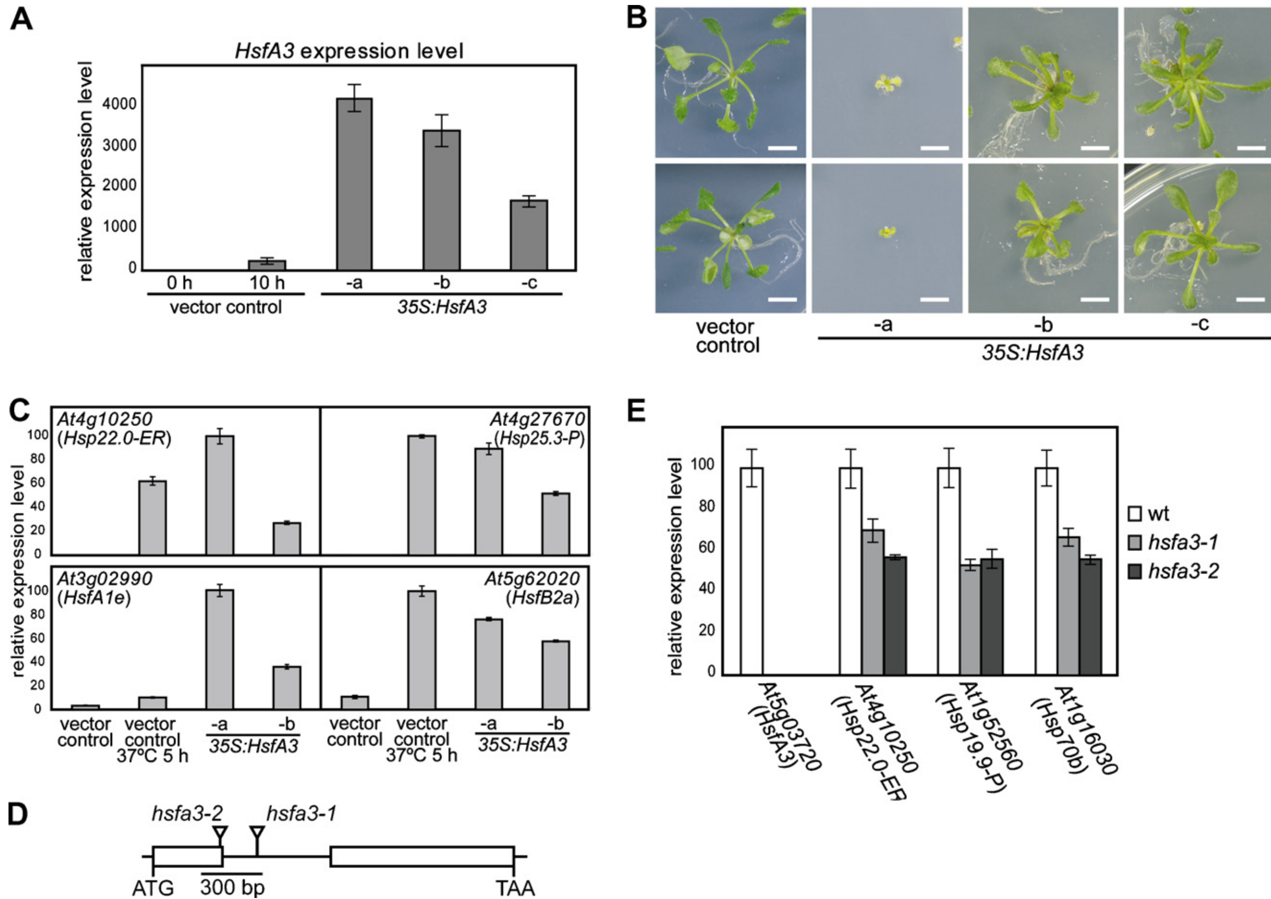


Fig. 3. Effects of overexpressing *HsfA3* in transgenic plants. (A) Quantitative RT-PCR analysis of *HsfA3* in transgenic plants and vector control plants. Total RNA of the plants except for *35S:HsfA3-a* was prepared from 17-day-old plants. Total RNA of *35S:HsfA3-a* was prepared from 43-day-old plants to align the growth stage with other plants. Vector control plants were heated at 37 °C for 10 h. Bars indicate SD ($n = 3$). The expression level of vector control plants under the control condition was defined as 1.0. (B) Photographs of vector control plants and transgenic plants overexpressing *HsfA3*. The plants were grown on germination medium agar plates for 3 weeks. (C) Quantitative RT-PCR analysis of *HsfA3* downstream genes in transgenic plants and vector control plants. Total RNA preparation was performed as shown in (A). Bars indicate SD ($n = 3$). The highest expression level was regarded as 100. (D) A scheme of the *Arabidopsis HsfA3* gene. Exons (open boxes) and an intron (line) are indicated. The positions of the T-DNA insertions are shown. (E) Quantitative RT-PCR analysis of HS-responsive genes in the *hsfa3* mutant plants. Plants grown for about 3 weeks were subjected to HS treatment at 37 °C for 24 h. Bars indicate SD ($n = 3$). The highest expression level was set to 100.

Analysis of HsfA3 knockout mutants

In order to further understand the physiological function of *HsfA3* in HS response, we analyzed two *HsfA3* knockout mutants, *hsfa3-1* and *hsfa3-2*. T-DNAs were inserted into the intron and the first exon of the *HsfA3* gene in *hsfa3-1* and *hsfa3-2*, respectively (Fig. 3D). *HsfA3* expression was abolished in both *hsfa3-1* and *hsfa3-2* homozygous lines, as shown in the quantitative RT-PCR analysis (Fig. 3E). These two mutants did not show any apparent morphological phenotypes under normal growth conditions. Expression levels of three up-regulated genes, *Hsp70b*, *Hsp19.9-P*, and *Hsp22.0-ER*, in *35S:HsfA3* was analyzed both in the *HsfA3* mutants and wild-type plants (Columbia ecotype) after HS treatment for 24 h. In both mutants, transcript levels of these three genes were reduced compared with those in the wild-type plants (Fig. 3E). These results confirm that expression of these genes is under control of *HsfA3*.

Thermotolerance of the 35S:HsfA3 plants and the HsfA3 knockout mutants

We identified that many HS-inducible genes are up-regulated in the *35S:HsfA3* plants and down-regulated in the *HsfA3* knockout mutants. As most of these genes downstream of *HsfA3* are related to acquisition of HS tolerance, we evaluated thermotolerance of the *35S:HsfA3* plants. Vector control and transgenic plants were grown on selective agar plates for 7 days, and then plants were moved onto filter paper premoistened with liquid germination medium. The plants grown on filter paper for another 3 days were exposed at 46 °C for 45 min. As shown in Fig. 4A, only 5% of vector control plants survived, whereas 72% of *35S:HsfA3-b* and 85% of *35S:HsfA3-c* survived during subsequent 7-day recovery at 22 °C. These results indicate improvement in thermotolerance of the *35S:HsfA3* plants.

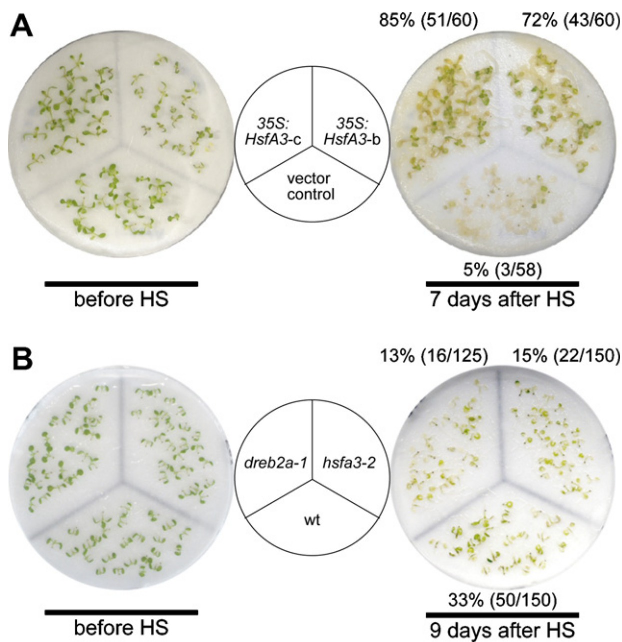


Fig. 4. Thermotolerance of the *35S:HsfA3* and *hsfa3* mutant plants. (A) Ten-day-old seedlings of the vector control or the *35S:HsfA3* plants were treated at 46 °C for 45 min. Photographs were taken after a 7-day recovery period in 22 °C. (B) Seven-day-old seedlings of the wild-type, *dreb2a-1*, and *hsfa3-2* plants were treated at 45 °C for 1 h. Photographs were taken after a 9-day recovery period in 22 °C. These plants had significantly lower or higher tolerance than the vector control or the wild-type plants (χ^2-test, $p < 0.001$). Percentages of surviving plants and numbers of surviving plants per total numbers of tested plants are indicated around the photographs.

Furthermore, we investigated the sensitivity of the *HsfA3* knockout plants to HS. Seven-day-old *dreb2a-1*, *hsfa3-2* mutants, and wild-type plants, were exposed at 45 °C for 1 h. When 33.3% wild-type plants survived in this treatment, only 12.8% of *dreb2a-1* and 14.7% *hsfa3-2* plants displayed viability after a 9-day recovery at 22 °C (Fig. 4B). Reduced thermotolerance was also observed in *hsfa3-1* mutant (Supplementary Fig. 4). Thus, we showed that *HsfA3* plays a positive role in the response of plants to HS and functions in the acquisition of thermotolerance under the control of the DREB2A stress-regulatory system.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.01.134.

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