
The Transcriptional Cascade in the Heat Stress Response of Arabidopsis Is Strictly Regulated at the Level of Transcription Factor Expression

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Group A1 heat shock transcription factors (HsfA1s) are the master regulators of the heat stress response (HSR) in plants. Upon heat shock, HsfA1s trigger a transcriptional cascade that is composed of many transcription factors. Despite the importance of HsfA1s and their downstream transcriptional cascade in the acquisition of thermotolerance in plants, the molecular basis of their activation remains poorly understood. Here, domain analysis of HsfA1d, one of several HsfA1s in Arabidopsis thaliana, demonstrated that the central region of HsfA1d is a key regulatory domain that represses HsfA1d transactivation activity through interaction with HEAT SHOCK PROTEIN70 (HSP70) and HSP90. We designated this region as the temperature-dependent repression (TDR) domain. We found that HSP70 dissociates from HsfA1d in response to heat shock and that the dissociation is likely regulated by an as yet unknown activation mechanism, such as HsfA1d phosphorylation. Overexpression of constitutively active HsfA1d that lacked the TDR domain induced expression of heat shock proteins in the absence of heat stress, thereby conferring potent thermotolerance on the overexpressors. However, transcriptome analysis of the overexpressors demonstrated that the constitutively active HsfA1d could not trigger the complete transcriptional cascade under normal conditions, thereby indicating that other factors are necessary to fully induce the HSR. These complex regulatory mechanisms related to the transcriptional cascade may enable plants to respond resiliently to various heat stress conditions.

INTRODUCTION

Heat stress is one of the universal stresses faced by all organisms. Heat stress damages cellular components by causing protein denaturation, reactive oxygen species generation, and membrane destabilization (Mittler et al., 2012; Hasanuzzaman et al., 2013). Therefore, it is important for organisms to rapidly activate protective mechanisms to maintain cellular homeostasis upon exposure to heat stress. The current models suggest that a fundamental mechanism of the cellular response to heat stress is highly conserved among eukaryotic cells (Ancker and Sistonen, 2011). The core regulators of the heat stress response (HSR) are transcription factors (TFs) called heat shock transcription factors (HSFs). HSFs are evolutionally conserved among eukaryotes and are the master regulators of the HSR. When cells detect heat stress, HSFs are rapidly activated and enhance the expression of many genes that encode heat shock proteins (HSPs). HSPs protect cellular components as molecular chaperones by preventing protein denaturation and aggregation. HSFs have a modular structure (Åkerfelt et al., 2010; Scharf et al., 2012), and their N-terminal DNA binding and oligomerization domains are conserved among HSFs. Although the C-terminal regions of HSFs are divergent, most HSFs include nuclear localization signals and transactivation domains. Yeast and Chlamydomonas reinhardtii HSFs are constitutively trimeric (Sorger and Nelson, 1989; Schulz-Rafelt et al., 2007), whereas HSFs of animals such as humans and plants such as Arabidopsis thaliana form trimers in response to heat stress (Balter et al., 1993; Hübel et al., 1995). Trimerization is necessary for HSFs to bind to cis-elements called heat shock elements (HSEs; nGAAnnTTCn or nTTcnnGAAn). HSEs commonly exist in the promoters of heat stress (HS)-inducible genes in eukaryotes (Åkerfelt et al., 2010). Although the molecular basis of the HSR is highly conserved, the postulated conditions of heat stress appear to differ among species. For example, animals have the ability to escape from heat stress by moving to a cooler area, which enables animals to actively decrease the heat stress itself. Thus, it is not necessarily important for animals to possess a sophisticated system that protects against heat shock at the cellular level. Instead, animals have developed neural circuits to perceive heat stress and induce an escape behavior (Clark et al., 2007). By contrast, plants are directly and constantly affected by increases in ambient temperature because of their sessile lifestyle. As a result, plants have to adapt not only to daily temperature changes but also to prolonged heat stresses that continue for days or weeks. Therefore, it...
has been hypothesized that plants have had to refine their HSR mechanisms during their evolution.

To cope with various patterns of heat stress, plants have developed large HSF families and a complex transcriptional network composed of many TFs (Kotak et al., 2007; von Koskull-Döring et al., 2007; Scharf et al., 2012). *Drosophila melanogaster* and mammals have up to four HSFs in their genomes and use mainly one HSF for induction of the HSR. By contrast, the plant HSF families include tens of members with various expression patterns and functions in the HSR. Arabidopsis has 21 HSFs assigned to three classes (A, B, and C) that include 14 groups (A1 to A9, B1 to B4, and C1) (Nover et al., 2001; Scharf et al., 2012). Among them, the A1 group includes four members, HsfA1a, HsfA1b, HsfA1d, and HsfA1e. The *hsfa1a hsfa1b hsf1a1d* triple knockout mutant (*hsfa1a/b/d*) is unable to induce expression of most HS-inducible genes and exhibits marked defects in thermotolerance, thus suggesting that HsfA1a, HsfA1b, and HsfA1d redundantly function as master regulators of the HSR (Liu et al., 2011; Yoshida et al., 2011). When plants are exposed to heat stress, HsfA1s induce expression of not only HSPs but also TFs, triggering a transcriptional cascade composed of various TFs, including HsfAs (HsfA1e, HsfA2, HsfA3, HsfA7a, and HsfA7b), HsfBs (HsfB1, HsfB2a, and HsfB2b), DREB2A, and MBF1c (Liu et al., 2011; Yoshida et al., 2011; Liu and Chang, 2013). These HsfA1-downstream TFs include both enhancers and repressors of the HSR. HsfAs, DREB2A, and MBF1c have been reported to be positive regulators of the HSR (Sakuma et al., 2006; Chang et al., 2007; Schramm et al., 2008; Yoshida et al., 2008; Larkin-dale and Vierling, 2008; Suzuki et al., 2011; Nishizawa-Yokoi et al., 2011). These TFs enhance and sustain the expression of HS-inducible genes. In particular, DREB2A forms a multistep transcriptional cascade by activating expression of HsfA3 (Schramm et al., 2008; Yoshida et al., 2008). The transcriptional cascade composed of HsfA1s, DREB2A, and HsfA3 is an amplification system that sustains the HSR for long periods (~24 h). By contrast, HsfBs are repressor-type HSFs and are negative regulators of the HSR that specifically evolved in the plant HSF families (Czamecka-Verner et al., 2004; Ikeda et al., 2011). This involvement of multiple TFs may be valuable for plants to survive under various temperature conditions.

Activation of HSFs is an important step for initiation of the HSR. The regulatory mechanisms of HSF activity have been studied primarily using human HSF1 (Hs-HSF1). Under normal conditions, Hs-HSF1 activity is suppressed by interaction with HSP70 and HSP90 (HSP70/90; Ancar and Sistonen, 2011). Heat stress treatment causes the dissociation of HSP70/90 from HSFs because denatured proteins competitively interact with the HSPs. Moreover, posttranslational modifications, such as phosphorylation, sumoylation, and acetylation, are involved in the regulation of Hs-HSF1 activity during heat stress (Xu et al., 2012b). In plants, regulatory mechanisms of HsfAs have been analyzed using Arabidopsis, tomato (*Solanum lycopersicum*), and Chlamydomonas. HSP70/90 and phosphorylation are also involved in the regulation of HsfA1 activity (Schulz-Raffelt et al., 2007; Liu et al., 2008; Hahn et al., 2011; Schmollinger et al., 2013). Considering the differences between animals and plants in terms of the heat stress conditions that must be overcome, it is presumed that plants have developed at least some unique mechanisms for regulation of the activity of HsfA1s. However, the molecular basis and primary factors in the regulation of HsfA1s are largely unknown. Furthermore, it is unclear whether activation of HsfA1s alone is sufficient to trigger the entire transcriptional cascade downstream of HsfA1s.

In this study, we performed domain analysis of HsfA1d and found that the central region negatively regulates HsfA1d activity in a temperature-dependent manner through interaction with HSP70/90. We generated a constitutively active form of HsfA1d by deleting this negative regulatory region. Overexpression of this constitutively active HsfA1d caused induction of many HSP genes and improved the thermotolerance of the transgenic plants. However, the constitutively active HsfA1d did not induce all of the HsfA1-target genes. These results provide insights into the unique and complex regulatory system of the HSR in plants.

RESULTS

Identification of the Negative Regulatory Domain of HsfA1d

An alignment of the amino acid sequences of HsfA1s from several plant species revealed that not only the characterized functional domains but also the central regions of HsfA1s are highly conserved (Supplemental Figure 1). We hypothesized that the region between the nuclear localization signal and the activation domain, including the conserved central region, might function as a regulatory domain of HsfA1s. We divided this domain into five regions for deletion analysis using Arabidopsis HsfA1d as the model to define the region necessary for regulation of the HsfA1 transcriptional activation activity (Figure 1A; Supplemental Figure 1). Deletion mutants that lacked single or multiple regions were expressed in Arabidopsis mesophyll protoplasts, and transcriptional activation was evaluated using GUS under the control of the *HSP18.2* promoter (*HSP18.2pro:GUS*; Figure 1B). Deletion of a single region, particularly regions 1 and 2, mostly had a positive effect on the HsfA1d activity. Only dΔ5 exhibited lower transcriptional activity than the full-length HsfA1d (dFL). The increase in reporter activity was more significant in the multiple-deletion mutants; dΔ1-2, dΔ1-3, and dΔ1-4 exhibited markedly higher activity than dΔ1. However, deletion of the whole putative regulatory domain (dΔ1-5) resulted in a marked decrease in transcriptional activity.

The DNA binding activity was also analyzed using the 35Spro:*HSE9-GUS* reporter (Treuter et al., 1993), which contains HSEs inserted downstream of the TATA box in the 5′ untranslated region of the *GUS* gene. Note that binding of HSFs to HSE is detected as the repression of reporter activity in this assay. In addition to single-deletion mutants, dΔ1-2 and dΔ1-3 exhibited similar DNA binding activities, which were slightly stronger than that of dFL (Figure 1B). The deletion of additional regions caused a further increase in the DNA binding activity as the region deleted became longer. To examine whether the alteration in transactivation activity and DNA binding activity was due to effects on protein stability, the deletion mutants were expressed in protoplasts as synthetic GFP (sGFP)-fused proteins (Figure 1C). With the exception of dΔ1-5, the amounts of proteins of all of the mutants were similar to that of dFL. The protein level of dΔ1-5 was markedly higher than that of dFL.
Figure 1. Domain Analysis of the Putative Regulatory Domain of HsfA1d in Arabidopsis Protoplasts.

(A) Schematic representation of the HsfA1d structure. The analyzed regions of the putative regulatory domain are indicated by the numbers in open boxes. The numbers on dashed lines indicate the positions of amino acid residues. DBD, DNA binding domain; HR-A/B, oligomerization domain; NLS, nuclear localization signal; AHA, transactivation domain; NES, nuclear export signal.

(B) Effect of deletion of the putative regulatory domain on the transactivation activity and DNA binding activity of HsfA1d. The left panel shows schematic diagrams of the deletion mutants. The middle and right panels show the transactivation activity and DNA binding activity, respectively. Effectors were expressed under the control of the CaMV 35S promoter. The transactivation activity and DNA binding activity were evaluated with the HSP18.2 pro :GUS and 35S pro :HSE9-GUS reporters, respectively. Note that DNA binding of HSFs was detected as repression of GUS expression from 35S pro :HSE9-GUS. To normalize the transfection efficiency, pBI35SV :ELUC was cotransfected in each experiment. The reporter activities obtained with full-length HsfA1d (dFL) and empty vector (Vec) were set to 1 for the assays of the transactivation and DNA binding activities, respectively. The error bars indicate the SD from three replicated samples. Statistically significant differences between effectors are indicated by different lowercase letters (Tukey’s test, P < 0.01).

(C) Protein accumulation levels of the deletion mutants. The proteins of the deletion mutants were expressed as sGFP-fused proteins under the control of the CaMV 35S promoter. A plasmid constitutively expressing a sGFP fused to a 3×FLAG tag was cotransfected in each experiment as an internal control. The levels of the fusion proteins were analyzed via immunoblot analysis with an antibody against GFP.

(D) Repressive effects of regions 1 and 2 on the activity of HsfA1d. The transactivation activity and DNA binding activity of the deletion mutants were analyzed as described in (B).
As demonstrated in previous reports, the sGFP-fused dFL was observed in the nuclei and cytosol of protoplasts (Supplemental Figure 2A; Kotak et al., 2004; Yoshida et al., 2011). Although the single-deletion mutants and dΔ1-2 exhibited subcellular localization that was similar to that of dFL, the deletion mutants that lacked more regions tended to localize primarily in the nucleus. Because this observed alteration in subcellular localization may affect the transactivation activity and DNA binding activity of the deletion mutants, we also analyzed HsfA1d with a mutated nuclear export signal (dmNES) to estimate the effect of nuclear localization on these activities. The NES sequence, LTQQMGLL, was mutated to LTQQMGAA in the dmNES mutant, which caused nuclear localization of HsfA1d (Supplemental Figures 2B and 2C). We compared the transactivation activity and DNA binding activity of dmNES with those of dΔ1-3, which was primarily localized in the nucleus (Supplemental Figure 2D). Although dmNES exhibited DNA binding activity that was similar to that of dΔ1-3, its transactivation activity was markedly lower than that of dΔ1-3. Therefore, the effect of nuclear localization on the transactivation activity of HsfA1d appears to be limited.

The results presented in Figure 1B indicate the possibility that regions 1 and 2 act as negative regulatory domains for HsfA1d activity. Accordingly, we generated deletion mutants that retained these regions (Figure 1D). The transactivation activities of dΔ2-3, dΔ2-4, and dΔ3-4 were similar to those of dΔ2 and dΔ3, which were weaker than those of the mutants that lacked region 1 or regions 1 and 2. The DNA binding activity of these mutants was similar to that of the deletion mutants that lacked a similar length of the regulatory domain, thereby suggesting that the DNA binding activity was affected not by the presence of a specific region but by the length of the putative regulatory domain.

Region 1 Is Responsible for the Inducibility of HsfA1d Activity in Response to Heat Stress

To examine the effects of each region on the heat shock inducibility of HsfA1d activity, the amount of effector constructs was reduced to the level at which the activity of dFL was regulated similarly to endogenous HsfA1s, i.e., such that the HSP18.2_pro/GUS reporter gene was not activated under normal conditions (Figure 2A). We used protoplasts derived from the hsfa1a/b/d triple mutant for this analysis to avoid having endogenous HsfA1s mask the activity of the deletion mutants. We treated protoplasts with a 1-h heat shock followed by a 1-h recovery to allow efficient translation of the GUS mRNA induced during heat stress. Although the activities of dΔ3, dΔ4, and dΔ5 were similar to that of dFL, dΔ1 and dΔ2 were only slightly active under normal conditions (Figure 2A). After heat shock, with the exception of dΔ5, all of the single-deletion mutants were activated, and their activities reached the level of dFL. dΔ5 was also activated, but its activity was lower than those of the other single-deletion mutants. In contrast with the single-deletion mutants, multiple-deletion mutants that lacked region 1 were highly active even in the absence of heat stress (Figure 2B). The increase in the length of the deleted region from dΔ1 to dΔ1-4 resulted in higher activity in the mutants. In particular, the activities of dΔ1-3 and dΔ1-4 were comparable to or higher than that of activated dFL in the absence of heat stress. These two mutants were constitutively active and hardly responded to heat stress. The activity of dΔ1-5 was very low.

Figure 2. Activation of the HsfA1d Deletion Mutants by Heat Stress in the Protoplasts Derived from the hsfa1a/b/d Triple Mutant.

The transactivation activity of the deletion mutants was analyzed with the HSP18.2_pro/GUS reporter under normal (22°C) and heat stress (37°C) conditions. The heat-stressed samples were harvested after 1 h of heat shock treatment followed by 1 h of recovery at 22°C. The reporter activity obtained with dFL under normal conditions was set to 1. The error bars indicate the SD from three replicated samples. Asterisks indicate statistically significant differences between the reporter activities (Student’s t test with Bonferroni correction; *P < 0.05, **P < 0.01, and ***P < 0.001). The effectors used in each panel are as follows: (A) single-deletion mutant; (B) multiple-deletion mutants; and (C) multiple-deletion mutants retaining region 1 or regions 1 and 2.
compared with that of the other multiple-deletion mutants and was not heat inducible.

To investigate whether the putative regulatory domain of other HsfA1s have the same function as that of HsfA1d, we analyzed the analogous deletion series of HsfA1a. Among the single-deletion mutants, only aΔ1 showed higher transactivation activity than aFL (Supplemental Figure 3A). In contrast to HsfA1d, aΔ1 showed higher activity than aFL under both normal and heat stress conditions. The multiple deletion caused a further increase in the transactivation activity of the effectors under both normal and heat stress conditions (Supplemental Figure 3B), which was similar to the result obtained with HsfA1d (Figure 2B). However, the transactivation activity of aΔ1-3 and aΔ1-4 was further activated by the heat stress treatment. Furthermore, the deletion mutant lacking the entire regulatory domain (aΔ1-5) retained activity comparable to that of aFL upon heat stress. Although there were several dissimilarities in the effects of deletions in the regulatory domain between HsfA1a and HsfA1d, the central regions, especially region 1, of both HsfA1s appear to have a similar function as a negative regulatory domain.

We also analyzed the HsfA1d multiple-deletion mutants that retained regions 1 and 2 to examine the repressive function of the regions during treatment with heat stress (Figure 2C). The transactivation activity of these deletion mutants was similar to that of dFL under both normal and heat stress conditions, thereby indicating that the potent activities of dΔ1-3 and dΔ1-4 were eliminated by the presence of regions 1 and 2. The similarity of the activities of dΔ2-3 and dΔ2-4 indicates that the presence of region 1 is sufficient to repress HsfA1d activity. Thus, we primarily focused on region 1 in the following analyses.

Region 1 Is Highly Conserved among HsfA1s, and Its Core Motif Is Important for the Function of Region 1

The results described above indicated that region 1 functions as a repression domain under non-stress conditions and confers heat shock inducibility on HsfA1s. The sequence analysis (Supplemental Figure 1) demonstrated that region 1 is the highly conserved portion of the putative regulatory domain among plant species. Therefore, we designated the most highly conserved part of region 1 as the temperature-dependent repression (TDR) domain (Supplemental Figure 1). We could not find sequences corresponding to the TDR domain in Arabidopsis HSFs of other groups or in Hs-HSF1. Although the N-terminal part of Chlamydomonas Hsf1 (Cr-Hsf1), which includes DBD and HR-A/B regions, shares high similarity with the HsfA1s of land plants, the amino acid sequence of the C-terminal part is so different that we could not align its regulatory domain with that of the HsfA1s. However, we found a motif, QIVKYQP, that was conserved between Cr-Hsf1 and the TDR domain of land plants (Supplemental Figure 3).

**Figure 3.** Mutation of the Tyrosine Residue in the Conserved Motif Disrupts the Repressive Function of Region 1.

(A) Effect of the mutation on Tyr-271 on the transactivation activity (left panel) and DNA binding activity (right panel) of HsfA1d. Tyr-271 was replaced with phenylalanine (dY271F) or aspartic acid (dY271D). The reporter activities obtained with dFL and Vec were set to 1 for the assays of the transactivation and DNA binding activities, respectively. The error bars indicate the so from three replicated samples. Asterisks indicate statistically significant differences between the reporter activities (Student’s t test with Bonferroni correction, **P < 0.01 and ***P < 0.001).

(B) Schematic representation of HsfA1d/VP16 chimera TFs. The VP16AD was fused to the 260 (260/VP) or 296 (296/VP) N-terminal amino acids or the 296 N-terminal amino acids with the Y271D mutation (296YD/VP) of HsfA1d.

(C) Transactivation activity (left panel) and DNA binding activity (right panel) of HsfA1d/VP16 chimeric TFs. The reporter activities obtained with 296/VP and Vec were set to 1 for the assays of the transactivation and DNA binding activities, respectively. The error bars indicate the so from three replicated samples. Asterisks indicate statistically significant differences between the reporter activities (Student’s t test with Bonferroni correction, *P < 0.05 and **P < 0.01).

(D) Activation of the HsfA1d/VP16 chimeric TFs by heat stress in the protoplasts derived from the hsfa1a/b/d triple mutant. The reporter activity obtained with FL under normal conditions was set to 1. The error bars indicate the so from three replicated samples. Asterisks indicate statistically significant differences between the reporter activities (Student’s t test with Bonferroni correction, **P < 0.01 and ***P < 0.001).
Figure 1). We hypothesized that this conserved motif plays an essential role in the repressive activity of region 1. Because the activity of Hs-HSF1 is regulated by phosphorylation (Xu et al., 2012b), we focused on Tyr-271, which is the only amino acid in the conserved motif of the TDR with the potential to be phosphorylated. We replaced Tyr-271 with phenylalanine (dY271F) or aspartic acid (dY271D) to mimic the nonphosphorylated or phosphorylated forms of the protein, respectively (Xu et al., 2012a; Figure 3A). The transactivation and DNA binding activities of dY271D were as high as those of dΔ1, thereby indicating that the phosphomimetic mutant of Tyr-271 had the same effect as deletion of region 1 in terms of disruption of the repressive mechanism of HsfA1d activity. By contrast, dY271F also exhibited higher transactivation activity and DNA binding activity compared with dFL, although the effect of the mutation on the transactivation activity was limited (Figure 3A).

We also substituted Tyr-271 with various other amino acids. Although all Tyr-271 mutants showed increased transactivation activity compared with dFL, dY271D showed the highest activity (Supplemental Figure 4). Mutants of Val-269, which is also highly conserved within the motif, showed increased transactivation activity (Supplemental Figure 4); however, their effects were smaller than that of the dY271D mutant. We generated a d7A mutant, in which all amino acids in the conserved motif were substituted with alanine to completely disrupt the structure of the conserved motif. The d7A mutant was highly active and was comparable to the dY271D mutant (Supplemental Figure 4). These results indicate that the dY271D mutation has the most striking effect among analyzed mutations, and this effect is equivalent to the complete disruption of the structure of the conserved motif.

We similarly examined the function of the analogous conserved tyrosine residue (Tyr-294) of HsfA1a. The aY294D mutant showed potent transactivation activity comparable to that of dΔ1, although the aY294F mutant did not show a remarkable increase in the activity (Supplemental Figure 5). These results indicate that the tyrosine residue in the conserved motif is very important for the function of region 1 in both HsfA1a and HsfA1d.

We investigated whether the tyrosine residue in the conserved motif is phosphorylated in vivo, using transgenic plants that expressed dFL and dY271F as sGFP-fusion proteins in the wild-type background. After the fusion proteins were immunoprecipitated with the anti-GFP antibody from non-stressed or heat stress-treated samples. Because HsfA1d is localized in the cytoplasm and nucleus (Yoshida et al., 2011), we selected the proteins predicted to localize in the cytoplasm or nucleus for further analyses. Among the proteins that satisfied this criterion, five proteins, including HsfA1d, were detected in all of the experiments (Table 1; Supplemental Data Set 1), and three of them were members of the HSP70 family. In a phylogenetic tree, these HSP70s were located in the same branch, which contained six HSP70s (Supplemental Figure 8A and Supplemental Data Set 2).

Among the six HSP70s, HSC70-1, HSC70-2, HSC70-3, and HSP70 interacted with HsfA1d in a yeast two-hybrid (Y2H) assay (Supplemental Figure 8B). HSP70 is a negative regulator of HSF activity in both plant and animal cells (Lee and Schöffl, 1996; Shi et al., 1998; Hahn et al., 2011). We confirmed the repressive effect of HSC70-1, which exhibited the highest score in the LC-MS/MS analysis among the HSP70s, on HsfA1d transactivation activity (Supplemental Figure 8C). To address the possibility that the decrease in reporter activity caused by HSC70-1 was due to a reduced level of unfolded proteins, we tested the effect of co-expression of Escherichia coli GroEL and GroES (GroEL/ES), which are involved in protein refolding (Supplemental Figure 8D; Richter et al., 2010). No significant effect of GroEL/ES on HsfA1d activity was observed.

Although there are several reports regarding the relationship between HsfA1 and HSP70 in plant cells (Kim and Schöffl, 2002; Hahn et al., 2011), the domain of HsfA1d responsible for interacting with HSP70 is unknown. To elucidate whether HSP70s interact with region 1, we examined the binding of truncated HsfA1d derivatives to HSC70-1 via a Y2H assay (Figure 4C). We obtained positive signals from HsfA1d that lacked the region from the C terminus to amino acid 418, 359, or 297 but not from HsfA1d.
that lacked the region from the C terminus to amino acid 260, which did not have region 1. The deletion of amino acids 1 to 144, which includes the DBD, also disrupted the interaction, thereby suggesting that region 1 and the DBD play essential roles in the interaction with HSP70. In human cells, HSP70 interacts with the C-terminal transactivation domain of Hs-HSF1 (Shi et al., 1998). However, we could not find similarity between the amino acid sequences of the transactivation domain of Hs-HSF1 and that of region 1 or that of the DBD. We also tested an HsfA1d derivative that lacked region 1 and the 67 C-terminal amino acids; however,
the yeast strains that harbored the expression vector of 1-260:297-418 grew so slowly that we could not compare their growth with that of other strains (Figure 4C).

To examine whether the interaction of HSP70 with HsfA1d is direct or involves other proteins, we purified recombinant HSC70-1 and HsfA1d proteins and examined them for complex formation. The interaction of these two proteins was quite weak, indicating that additional factors are required for the efficient formation of the HsfA1d-HSP70 complex (Figure 4D).

We confirmed the repressive function of HSC70-1 via region 1 of HsfA1d using HsfA1d/VP16 chimeric TFs as effectors because their activity was regulated though region 1 only. The transactivation activity of 296/VP was repressed by the coexpression of HSC70-1, whereas the activities of 260/VP and 296YD/VP were not affected by HSC70-1, indicating that region 1 is important for the negative regulation of HsfA1d via HSP70 (Figure 4E).

Deletion of Region 1 Causes Constitutive Nuclear Localization of HsfA1d

To investigate the role of the regulatory domain in vivo, we generated transgenic plants that expressed several HsfA1d derivatives (dFL, dmNES, d1, and d1-3) as sGFP-fusion proteins under the control of the CaMV 35S promoter in the wild-type background (dFL_OX, dmNES_OX, d1_OX, and d1-3_OX). Despite similar expression of the transgenes at the mRNA level, the amounts of d1 and d1-3 proteins were less than those of dFL and dmNES (Figures 5A and 5B). The plants overexpressing deletion mutants were smaller than the vector control (VC) plants and those expressing dFL or dmNES (Figures 5C and 5D).

Consistent with previous reports, dFL was localized in the cytosol under normal conditions and moved into the nucleus upon heat shock (Figure 5E; Yoshida et al., 2011). The subcellular localization of dFL under normal conditions was different between protoplasts and transgenic plants (Figure 5E; Supplemental Figure 2A). Because the protoplast isolation process seems to be stressful to plant cells, protoplasts may be in a state of partial stress even under normal conditions, which could lead to the nuclear localization of HsfA1d in protoplasts. By contrast, dmNES exhibited constitutive nuclear localization (Figure 5E). This subcellular localization is similar to that of NES-truncated HsfA1d reported by Yoshida et al. (2011). Regardless of the presence of the intact NES, d1 and d1-3 were localized to the nucleus under both normal and heat shock conditions. The nuclear translocation

Rubisco large subunit (rbcl) stained with Ponceau S is shown as a loading control.

(A) RNA levels of sGFP-HsfA1d derivatives in the transgenic plants (dFL_OX, FL; dmNES_OX, mNES; d1_OX, d1; d1-3_OX, d1-3). HsfA1d derivatives were expressed under the control of the CaMV 35S promoter as sGFP fusions. Lowercase letters indicate two independent transgenic lines that express the same transgene. Ethidium bromide-stained images of rRNA are shown as a loading control.

(B) Protein levels of sGFP-HsfA1d derivatives in transgenic plants. The total protein was analyzed by immunoblot analysis with anti-GFP antibody. The

Figure 5. Generation of Overexpressors of HsfA1d Derivatives.

(A) RNA levels of sGFP-HsfA1d derivatives in the transgenic plants (dFL_OX, FL; dmNES_OX, mNES; d1_OX, d1; d1-3_OX, d1-3). HsfA1d derivatives were expressed under the control of the CaMV 35S promoter as sGFP fusions. Lowercase letters indicate two independent transgenic lines that express the same transgene. Ethidium bromide-stained images of rRNA are shown as a loading control.

(B) Protein levels of sGFP-HsfA1d derivatives in transgenic plants. The total protein was analyzed by immunoblot analysis with anti-GFP antibody. The

Rubisco large subunit (rbcl) stained with Ponceau S is shown as a loading control.

(C) and (D) Growth of transgenic plants that expressed HsfA1d derivatives. Representative images (C) and the maximum rosette radius (D) of 21-d-old plants grown on agar plates under normal conditions are shown. The experiment was performed twice, and a representative result is shown. Asterisks indicate statistically significant differences compared with the VC (Student’s t test with the Bonferroni correction, **P < 0.01; n = 15). Bars = 2 cm.

(E) Subcellular localization of HsfA1d derivatives under normal conditions or after heat stress treatment. Images of the differential interference contrast (DIC) and GFP fluorescence and merged images of the DIC and GFP are shown. Bars = 20 μm.
of HsfA1d is inhibited by interaction with HSP90 (Yoshida et al., 2011). Therefore, these localization data suggested the possibility that region 1 is involved in interactions with not only HSP70 but also HSP90.

### Deletion of Region 1 Decreases the Interactions with HSP70 and HSP90 and Renders HsfA1d Active under Normal Conditions

To examine the contribution of region 1 to the interaction between HsfA1d and HSP70/90 in vivo, we performed coimmunoprecipitation assays with overexpressors of HsfA1d derivatives. Binding of HSP70 to dFL and dmNES was clearly detected (Figures 6A and 6B). However, HSP70 binding to dΔ1 or dΔ1-3 was significantly decreased compared with the binding to dFL and dmNES. Although we did not detect interaction with HSP90 in the LC-MS/MS analysis, it is possible that this lack of detection was because this interaction was not sufficiently stable to survive immunoprecipitation, as observed in human cells (Guo et al., 2001). To overcome this potential problem, we extracted proteins after immobilizing protein-protein interactions by cross-linking with dithiobis(succinimidyl propionate) (DSP). Cross-linking indeed enabled us to detect the interaction between dFL and HSP90. In contrast to HSP70, not only dFL but also dmNES showed reduced interaction with HSP90.

HSP70/90 are thought to dissociate from HSFs in response to heat stress, which causes the activation of HSFs (Richter et al., 2010; Neef et al., 2011). We therefore investigated whether the HsfA1d-HSP70/90 interaction was disrupted by heat shock. Because the ratio of HsfA1d to HSP70/90 might affect the interaction, we generated complementation plants that expressed HsfA1d as an sGFP-fusion protein under the control of an HsfA1d promoter (HsfA1dpro:sGFP-HsfA1d) in the hsf1a/b/d triple mutant (HsfA1dpro:sGFP-HsfA1d/abd; Supplemental Figure 9A). HsfA1dpro:sGFP-HsfA1d/abd plants exhibited restored induction of HS-inducible genes during heat stress depending on the expression levels of HsfA1d (Supplemental Figure 9B). We immunoprecipitated HsfA1d from both HsfA1dpro:sGFP-HsfA1d/abd and 3SSpro:sGFP-HsfA1d/abd (Supplemental Figure 7A) under normal or heat stress conditions. Although HsfA1d constitutively interacted with both HSP70 and HSP90 in the 3SSpro:sGFP-HsfA1d/abd, the interaction between HsfA1d and HSP70 was disrupted in HsfA1dpro:sGFP-HsfA1d/abd specifically under heat stress conditions (Figure 6C). Quantification of the band intensities clearly supported the different patterns of interaction with HSP70 between HsfA1dpro:sGFP-HsfA1d/abd and 3SSpro:sGFP-HsfA1d/abd (Figure 6D). It was difficult to detect HSP90 in HsfA1dpro:sGFP-HsfA1d/abd; the amount of HsfA1d may have been insufficient to communoprecipitate enough HSP90 for detection.

To examine whether deletion of the regulatory domain influences the transactivation activity of HsfA1d in vivo, we analyzed the expression of representative HS-inducible genes in the overexpressers of the HsfA1d derivatives generated in Figure 5A under normal conditions (Figure 6E). Although expression of several genes was activated in dFL_OX and dmNES_OX, the expression levels of the analyzed HS genes were higher in dΔ1_OX and dΔ1-3_OX than in dFL_OX and dmNES_OX despite the lower protein accumulation of these deletion mutants (Figure 5B). These results indicate that dΔ1 and dΔ1-3 were highly active in the transgenic plants. Interestingly, however, expression of HsfA2, which is a direct target gene of HsfA1d (Yoshida et al., 2011), was not activated in any of the transgenic lines (Figure 6E).

To examine whether region 1 of other HsfA1s possesses similar repressive activity in vivo, we generated transgenic plants that expressed aFL or aΔ1 under the control of the CaMV 3SS promoter as sGFP-fusion proteins in the wild-type background (aFL_OX and aΔ1_OX; Supplemental Figure 10A). We did not generate the mNES overexpressor of HsfA1a because HsfA1a was constitutively localized in the nucleus (Yoshida et al., 2011; Supplemental Figure 10B). aΔ1 was also localized in the nucleus under both the normal and heat stress conditions. aΔ1 could activate the expression of HSP genes under normal conditions, but aFL could not (Supplemental Figure 10C). However, similar to the results with the HsfA1d constructs, HsfA2 was not activated in the aΔ1_OX.

We conducted thermotolerance tests using 14-d-old plants grown on agar medium (Figures 6F and 6G). We did not use dΔ1-3_OX in this assay because they produced so few seeds that it was difficult to conduct the thermotolerance tests using the seed pool derived from a single plant. Although all of the overexpressors exhibited higher thermotolerance than the VC, the increase in tolerance conferred by dΔ1 overexpression was dramatic. Even after 80 min of heat stress, dΔ1_OX not only exhibited high viability but also kept most of their leaves green.
Figure 6. Effect of Overexpression of HsfA1d Derivatives on the HSR.

(A) and (B) Coimmunoprecipitation of endogenous HSP70 and HSP90 with HsfA1d derivatives. Extracts from transgenic plants that expressed HsfA1d derivatives were immunoprecipitated using an anti-GFP antibody. The input and immunoprecipitated proteins were analyzed via immunoblot analysis with anti-GFP, anti-HSP70, and anti-HSP90 antibodies. The lower panel of HSP90 shows a longer exposure image of the image in the upper panel. The signal intensities of the bands of coimmunoprecipitated HSP70 and HSP90 normalized by the intensity of the corresponding HsfA1d derivatives are shown in (B). The error bars indicate the SD from triplicate experiments.

(C) and (D) Coimmunoprecipitation of endogenous HSP70 and HSP90 with HsfA1d. A complementation line (HsfA1dpro:sGFP-HsfA1d/abd, Comp) and an overexpressor (35Spro:sGFP-HsfA1d/abd, OX) of HsfA1d in the hsfa1a/b/d background were used. These plants were harvested under normal conditions or after heat stress treatment (37°C for 0.5 h). Extracts from these samples were immunoprecipitated using an anti-GFP antibody. The input and immunoprecipitated proteins were analyzed via immunoblot analysis with anti-GFP, anti-HSP70, and anti-HSP90 antibodies. The lower panels of HSP70 and HSP90 show longer exposure images of the images in the upper panels. The signal intensities of the bands of coimmunoprecipitated HSP70 normalized by the intensity of HsfA1d are shown in (D). The signal intensities for the non-stressed samples were set to 1 for each transgenic plant. The error bars indicate the SD calculated from triplicate experiments.

(E) Expression analysis of HS-inducible genes in transgenic plants. Ethidium bromide-stained images of rRNA are shown as a loading control.

(F) and (G) Thermotolerance test of transgenic plants. Seedlings were treated at 43°C for 50 or 80 min. After recovery for 7 d, photographs of the seedlings (F) were taken, and their survival rates (G) were determined. The viable plants are defined as those that generated new rosette leaves during the recovery period.
Activation of HsfA1d Is Not Sufficient to Induce Expression of Some HsfA1-Downstream Genes, Including TFs

Our preliminary expression analysis (Figure 6E) indicated that dΔ1 and dΔ1-3 were able to induce several HSP genes but not HsfA2 under normal conditions. To explore expression on a global scale, we performed microarray analysis of dΔ1_OX. Under normal conditions, dΔ1_OX had 206 upregulated (fold change ≥ 4) and nine downregulated (fold change ≤ 0.25) genes compared with the VC (Supplemental Data Set 3). We analyzed the expression patterns of the top 100 upregulated genes using the Genevestigator database (Zimmermann et al., 2004) and found that most of them were highly upregulated by heat stress (Figure 7A). Consistent with this result, approximately half of the upregulated genes overlapped with the HsfA1-downstream HS-inducible genes, which were defined as follows: upregulated in Columbia (Col) under the heat stress conditions by at least 4-fold compared with the levels in Col under normal conditions and downregulated in the hsfa1 hsfb1 hsfb2b triple mutant by at least 4-fold under the heat stress conditions compared with the levels in Col or Wassilewskija under the heat stress conditions in the microarray data provided by Yoshida et al. (2011) (Figure 7B). However, the overlapping genes constituted only one-third of the HsfA1-downstream genes.

We designated the HsfA1-downstream genes that were upregulated in dΔ1_OX as “Δ1-responding genes” and the remaining HsfA1-downstream genes as “non-Δ1-responding genes” and analyzed the differences between these groups. The HSEs were similarly enriched in the 1-kb promoters of the genes in each group (Figure 7C), indicating that the number of binding sites of HSFs was not responsible for the differences in the responses between the two groups. The Δ1-responding genes included many coding for chaperones and co-chaperones, such as HSP101, HSP70, and small HSPs (Figure 7D; Supplemental Data Set 4). By contrast, most of the genes encoding TFs, particularly the important amplifiers of the HSR, such as HsfAs and DREB2A, were included in the group of non-Δ1-responding genes (Figure 7D, Table 2; Supplemental Data Set 4). We confirmed the expression of several Δ1-responding chaperone genes (HSP18.2, HSP25.3-P, and HSP22.0-ER) and non-Δ1-responding TF genes (HsfA2, HsfA7a, and DREB2A) in the dFL, dmNES, and dΔ1 overexpressors by qRT-PCR (Figure 7E). Among these genes, HSP18.2, HsfA2, and DREB2A were reported as direct targets of HsfA1d (Yoshida et al., 2011). Although the Δ1-responding chaperone genes were also induced in dFL_OX and dmNES_OX, their expression levels in dΔ1_OX were remarkable and comparable to those in the VC after heat stress. The expression of the non-Δ1-responding TF genes was not induced in any overexpressor.

We also analyzed the responses of Δ1-responding genes and non-Δ1-responding genes to overexpression of HsfA2 (Ogawa et al., 2007) or HsfA3 (Yoshida et al., 2008), which are constitutively active HsfAs, similar to dΔ1. A comparison of the microarray data revealed that 57 (58.7%) of the Δ1-responding genes were upregulated by the overexpression of HsfA2 or HsfA3 (Figure 7F). By contrast, only eight (3.7%) of the non-Δ1-responding genes were upregulated in these overexpressors. These results indicated that the Δ1-responding genes but not the non-Δ1-responding genes were also responsive to other active HsfAs. We also checked the expression of the Δ1-responding genes and non-Δ1-responding genes in the hsfb1 hsfb2b double mutant (Ikeda et al., 2011). These HsfBs suppress the leaky expression of HS-inducible genes under normal conditions (Ikeda et al., 2011). The numbers of upregulated Δ1-responding genes and non-Δ1-responding genes were 20 (20.6%) and 23 (10.5%), respectively (Figure 7G). Although the ratio of the overlapping genes was limited, six of the 25 non-Δ1-responding TF genes were under the control of HsfB1 and HsfB2b (Table 2). Such TF genes included HS-inducible HsfA4 genes and ZAT12, which are important regulators for the induction of stress responses.

Region 1 Is Not Necessary for Attenuation of HsfA1d Activity

A negative feedback regulation system that causes a decrease in the expression of HS-inducible genes by attenuating HsfA1 activity is integrated into the HSR. To examine the function of region 1 in this attenuation, we analyzed the expression patterns of HS-inducible genes during heat stress treatment or recovery in dFL_OX and dΔ1_OX (Figure 8). Their expression was induced to almost the same levels within 1 h of treatment in all of the transgenic plants. After prolonged heat stress or recovery, the expression of HS-inducible genes in dΔ1_OX was attenuated to the same levels as in dFL_OX, thus indicating that region 1 is not necessary for attenuation of HsfA1d activity.

DISCUSSION

In this article, we demonstrated that region 1, which contains the TDR domain, is important for suppressing HsfA1d activity under normal conditions. Furthermore, we found that constitutively active HsfA1d is able to induce the expression of many HSPs but not most of the TFs in the HSR transcriptional cascade. These results indicate that the activation of the transcriptional cascade in response to heat stress is strictly regulated (Figure 9).

In a recent model of the HSR in plants, the activation of HsfA1s was proposed to serve as a master switch to evoke the HSR (Liu et al., 2011; Yoshida et al., 2011). Therefore, we expected the constitutive activation of HsfA1s to cause the induction of most HsfA1-downstream genes even under non-stress conditions. The expression of HS-inducible genes, particularly HSPs, was highly activated in dΔ1 overexpressors under normal conditions (Figures 6E, 7D, and 7E; Supplemental Data Set 4). Importantly, the dΔ1 protein was more active than the dFL and dmNES versions of HsfA1d. A similar pattern of activity was observed for HsfA1a...
Figure 7. Transcriptome Analysis of the dΔ1 Overexpressor.

(A) Stress inducibility of the upregulated genes in dΔ1_OX. 206 genes was significantly upregulated by more than 4-fold in dΔ1_OX compared with VC under normal conditions. The responses of the top 100 upregulated genes in response to abiotic stress or hormone treatments are shown as a heat map.

(B) Venn diagram comparing the upregulated genes in dΔ1_OX with the HsfA1-downstream HS-inducible genes. The total numbers of the genes in each group are shown in parentheses. The HsfA1-downstream HS-inducible genes that were upregulated or not upregulated in dΔ1 were classified as “Δ1-responding genes” or “non-Δ1-responding genes,” respectively.

(C) Number of HSEs in the promoters of all genes registered in TAIR9 (27,684 genes), Δ1-responding genes, or non-Δ1-responding genes. The HSE sequences (nGAnntTnCn or nTTnmmGAn) in 1-kb promoter regions were counted.

(D) Functional categorization of Δ1-responding or non-Δ1-responding genes.

(E) qRT-PCR analysis of several Δ1-responding chaperones and non-Δ1-responding TFs in the overexpressors of HsfA1d derivatives. The expression levels of each gene in VC were measured under both normal and heat stress conditions. For the overexpressors of HsfA1d derivatives, the expression levels were
constructs, in the analysis of aFL and a1 (Supplemental Figure 10C). These results clearly indicate that deletion of region 1 converts HsfA1 proteins into constitutively active forms. Consistent with the constitutive expression of HSPs, dΔ1 {X showed more potent thermotolerance compared with dFL {X and dmNES {X (Figures 6F and 6G). However, contrary to our expectations, the overexpression of dΔ1 failed to upregulate the expression of approximately two-thirds of the HS-inducible HsfA1-downstream genes (non-Δ1-responder genes; Figure 7B). The non-Δ1-responder genes were not upregulated in lines overexpressing HsfA2 or HsfA3 either, whereas over half of the Δ1-responder genes were upregulated in these lines (Figure 7F). These results indicate that the expression of Δ1-responder genes is primarily regulated by a simple mechanism that depends on the presence or absence of active HsfAs. However, non-Δ1-responder genes seem to be subject to other regulation. The distribution of HSEs in the promoters indicates that both Δ1-responder and non-Δ1-responder genes are under the control of Hsfs (Figure 7C). In fact, non-Δ1-responder genes include direct targets of HsfA1d, such as HsfA2 and DREB2A (Table 2; Yoshida et al., 2011). These results raise the possibility that the induction of non-Δ1-responder genes requires not only the activation of HsfA1s but also other events, such as the activation of coactivators or suppression of negative regulators (Figure 9).

HS-inducible TFs are representative genes whose expression is preferentially activated by HsfA1s under heat stress conditions (Liu and Charng, 2013). However, most HS-inducible TFs were classified into non-Δ1-responder genes (Table 2, Figure 7D). Non-Δ1-responder TF genes included those encoding important amplifiers of the HSR, such as HsfA2, HsfA7a, and DREB2A (Chang et al., 2007; Sakuma et al., 2006; Larkindale and Vierling, 2008). Non-Δ1-responder TF genes were only minimally upregulated in lines overexpressing HsfA1d derivatives (Figures 6E and 7E). Overexpressors of aΔ1 were also unable to activate the expression of HsfA2 despite their ability to induce HSPs (Supplemental Figure 10C). Thus, the transcriptional cascade in the HSR cannot be evoked by the activation of HsfA1s alone.

One possible mechanism for the strict regulation of non-Δ1-responsive genes is the cooperative transcriptional regulation mediated by interaction among Hsfs. The plant Hsf family includes several members reported to function as negative regulators. For example, HsfA5 acts as a repressor of HsfA4 activity through specific interaction with HsfA4 (Baniwal et al., 2007). Although specific partners of HsfA1s have not been reported, HsfBs are promising factors for involvement in the regulation of non-Δ1-responsive gene expression. HsfB1 and HsfB2b negatively regulate the expression of HS-inducible genes under normal and mild heat stress conditions (Ikeda et al., 2011). A comparison of the microarray data showed that six of 25 non-Δ1-responder TF genes, including HsfA2 and HsfA7a, are under the control of these HsfBs (Table 2; Ikeda et al., 2011). Under normal conditions, HsfBs may suppress the activity of HsfA1 bound to the promoter of non-Δ1-responding genes through the formation of an HsfA1-HsfB complex, or they may mask the HSEs in the promoter of non-Δ1-responding genes. However, the involvement of HsfBs explains the regulation of only some of the HS-inducible TF genes (Table 2). Further expression analyses of non-Δ1-responding TF genes may help identify more regulators of the HSR.

The negative effect of the constitutively active HsfA1d on plant growth (Figures 5C and 5D) emphasizes the potential value to the plant of using not only a master switch but also multiple coregulators in the HSR transcriptional cascade. dΔ1 {X and dΔ1-3 {X showed growth retardation, and a similar phenotype was observed in the overexpressors of HsfA2 and HsfA3 (Figures 5C and 5D; Ogawa et al., 2007; Yoshida et al., 2008). Although the molecular mechanism of this negative effect on plant growth is unclear, plant growth repression may function during the HSR as reported for the drought stress response (Todaka et al., 2012). Because the expression of TFs potently amplifies the HSR, even leaky expression of TFs has the potential to inhibit plant growth. Plants are directly affected by daily temperature changes and likely repeatedly activate and repress the HSR. To minimize the negative effect of the HSR, plants may have needed to develop a precise mechanism for regulating the HSR at the level of TF expression.

Our domain analysis demonstrated that the region between the trimerization domain and the activation domain negatively regulates HsfA1 activity (Figure 1B; Supplemental Figures 3A and 3B). dΔ1 exhibited the highest transactivation activity among the single-deletion mutants of the regulatory domain despite the similar DNA binding activity, protein stability, and subcellular localization of these deletion mutants (Figures 1B and 1C; Supplemental Figure 2A). Furthermore, the presence of region 1 was sufficient to suppress the activity of the transactivation domains under normal conditions, thus indicating that region 1 confers HS inducibility on the constitutively active deletion mutants and chimeric TFs (Figures 2B, 2C, and 3D). These results indicate that the repressive activity of region 1 is regulated in an HS-dependent manner. The amino acid sequence of region 1 is highly conserved among HsfA1 proteins from various land plants as the TDR domain, although it is not found in HSFs that belong to other groups (Supplemental Figure 1). The QIVKYQP motif, the core sequence of the TDR domain, is also conserved in Chlamydomonas. Hence, temperature-dependent regulation via region 1 appears to be an evolutionarily conserved HsfA1-specific mechanism among plant species. This hypothesis is consistent with the findings of previous studies that reported the important role of HsfA1s in the HSR in tomato (Mishra et al., 2002) and Chlamydomonas (Schulz-Raffelt et al., 2007).
The activity of HSFs is thought to be regulated at the levels of DNA binding and transcriptional activation (Scharf et al., 2012). The DNA binding activities of the deletion mutants were not affected by the presence or absence of region 1, whereas their transactivation activities were significantly affected by region 1 (Figures 1B and 1D). These results suggest that the region 1 represses HsfA1d activity at the level of transactivation activity. Region 1 also has the ability to regulate the transactivation activity of chimeric TFs that harbor VP16AD (Figure 3C). This result indicates that region 1 functions through the recruitment of corepressors and can work independently of the nature of the activation domain.

Interaction assays revealed that one of the region 1-interacting corepressors is HSP70 (Table 1). HSP70 acts as a negative regulator of HSFs not only in plants but also in mammalian cells (Lee and Schöfl, 1996; Shi et al., 1998; Hahn et al., 2011). We confirmed the repressive effect of HSC70-1 on the HsfA1d activity by a reporter assay (Supplemental Figure 8C). Because GroEL/ES did not have any effect on HsfA1d activity in this assay system (Supplemental Figure 8D), the repressive effect of HSC70-1 seems to be direct. The Y2H and coimmunoprecipitation assays showed that region 1 is responsible domain for the interaction with HSP70 (Figures 4C, 6A, and 6B). The inverse correlation between the interaction with HSP70 and the transactivation activity shown by dD1_OX supports the idea that HSP70 is the region 1-interacting corepressor of HsfA1d (Figures 6A, 6B, and 6E). By contrast, overexpression of dmNES did not significantly affect the HSP expression (Figure 6E), although both dmNES and dD1 showed constitutive nuclear localization (Figure 5E). Therefore, the nuclear translocation regulated by HSP90 seems to be less effective in regulating the transactivation activity of HsfA1d.

dD1 retains approximately half of the capacity for interaction with HSP70 compared with dFL (Figure 6B). The remaining HSP70 binding ability of dD1 seems to explain the low activity of dD1 under normal conditions, when the accumulation of dD1 was quite low because of the small amount of effector plasmid used in the assay system (Figures 2A and 2B). By contrast, potent activity of dmNES was observed under normal conditions in the overexpressors (Figure 6D). These results suggest that sufficient HSP70 to repress HsfA1d activity can bind to dD1 if the HSP70:dD1 ratio is substantially high.

If HSP70 is the true repressor of HsfA1d, the interaction should be disrupted in response to heat stress, according to the well-known model for the regulation of HSF activity (Richter et al., 2010; Neef et al., 2011). Although this model is generally accepted, experimental evidence for the dissociation of HSP70 is lacking. In human cells, the amount of HSP70 interacting with Hs-HSF1 is constant across conditions (Shi et al., 1998).

### Table 2. Classification of HsfA1-Downstream TF Genes

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<tr>
<th>Class</th>
<th>Gene Name or Functional Annotation (AGI Code)</th>
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<tr>
<td>D1-Responding</td>
<td>HsfB1 (At4g36990), HsfB2a (At5g62020), DREB2B (At3g11020), MBF1C (At3g24500), A2F3 (At5g43170)</td>
</tr>
<tr>
<td>Non-D1-responding</td>
<td>HsfA1e (At3g52990), HsfA2 (At2g39150), HsfA7a (At3g59110), HsfA7b (At3g32850), HsfB2b (At1g11660), DREB2A (At5g05410), DREB2E (At2g38340), DDF4.1 (At4g09940), NFYC2 (At1g56170), ZAT6 (At5g04340), ZAT7 (At3g46090), ZAT8 (At3g46080), ZAT10 (At1g27730), ZAT12 (At5g59820), BBX8 (At4g48250), BBX22 (At1g78600), RVE7 (At1g18330), GATA3 (At4g34680), PRE5 (At3g28857), TRFL3 (At1g17460), ERF091 (At4g18450), NL4P (At1g20640), Myb7 (At2g16720), MYB family transcription factor (At1g49560), bZIP protein (At5g04840)</td>
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HsfA1-downstream TF genes upregulated or not upregulated in D1 overexpressors are classified as D1-responding or non-D1-responding, respectively. The TF genes upregulated in the hsfb1 hsfb2b double mutant are underlined.

The activity of HSFs is thought to be regulated at the levels of DNA binding and transcriptional activation (Scharf et al., 2012). The DNA binding activities of the deletion mutants were not affected by the presence or absence of region 1, whereas their transactivation activities were significantly affected by region 1 (Figures 1B and 1D). These results suggest that the region 1 represses HsfA1d activity at the level of transactivation activity. Region 1 also has the ability to regulate the transactivation activity of chimeric TFs that harbor VP16AD (Figure 3C). This result indicates that region 1 functions through the recruitment of corepressors and can work independently of the nature of the activation domain.

Figure 8. Region 1 Is Not Required for Attenuation of HsfA1d Activity after Prolonged Heat Stress or during Recovery.

Expression patterns of HS-inducible genes in dFL_OX and dD1_OX during the heat stress treatment and recovery. The plants were treated with heat stress at 37°C for 0 to 6 h (H0 to H6) or allowed to recover for 2 or 5 h after 1 h of heat stress (H1R2 or H1R5). Ethidium bromide-stained images of rRNA are shown as a loading control.
Chlamydomonas, Cr-Hsf1 interacts with HSP70 both before and after heat stress (Schulz-Raffelt et al., 2007; Schmollinger et al., 2013). Thus, the role of HSP70 during the activation of HSFs is unclear. Importantly, our coimmunoprecipitation assay with HsfA1dpro:sGFP-HsfA1d/abd clearly indicated that HSP70 dissociated from HsfA1d in response to heat shock (Figures 6C and 6D). This result strongly supports the function of HSP70 as a repressor of HsfA1d. The difference between results obtained in human and Chlamydomonas implies that the regulatory mechanisms of HSF activity via HSP70 are not necessarily the same among species. Interestingly, the dissociation of HSP70 was not observed in the experiments with 3SSpro:sGFP-HsfA1d/abd (Figures 6C and 6D). The expression of HS-inducible genes was induced by heat stress in 3SSpro:sGFP-HsfA1d/abd similarly to in the wild-type plants (Supplemental Figure 7B), indicating that HsfA1d was sufficiently activated in 3SSpro:sGFP-HsfA1d/abd.

Therefore, we speculate that the ratio of HSP70-free HsfA1d to HSP70-bound HsfA1d in 3SSpro:sGFP-HsfA1d/abd was too low to allow detection of a decrease in coimmunoprecipitated HSP70. In general, the dissociation of HSP70 from HSFs is explained by the “chaperone titration model,” which explains the dissociation of HSP70 by the competitive interaction of unfolded proteins with HSP70 (Richter et al., 2010; Neef et al., 2011). However, this model cannot explain the large difference in the ratio of HSP70-free HsfA1d between HsfA1dpro:sGFP-HsfA1d/abd and 3SSpro:sGFP-HsfA1d/abd. Therefore, unknown mechanisms might enhance the dissociation of HSP70 from HsfA1d upon heat shock, the repressor function of HSP90 seems to be inactivated by heat stress. HSP70-free HsfA1d becomes active and upregulates the expression of HS-inducible genes. However, the induction of non-Δ1-responding genes requires coregulators. TF genes are primarily included among non-Δ1-responding genes; thus, the activity of the transcriptional cascade is under strict control. The solid and dashed arrows between HSP70/90 and HsfA1d indicate strong or weak interactions, respectively. The other arrows and bar-heads indicate positive and negative regulation, respectively. The question marks denote links or factors to be confirmed.

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**Figure 9.** Working Model of the HSR in Arabidopsis.

(A) Conventional activation mechanism of the HSR. HSP70/90 interact with HsfA1d and negatively regulate HsfA1d activity under normal conditions. The HSP70/90 interaction sites of HsfA1d were unclear. Under heat stress conditions, increased amounts of unfolded proteins competively interact with HSP70/90 such that HsfA1d is released from HSP70/90 and becomes active. The activation of HsfA1d enables HsfA1d to induce HS-inducible genes, including HSPs and TFs. TFs trigger the transcriptional cascade and amplify the HSR.

(B) Our proposed activation mechanism of the HSR. HSP70/90 interact with HsfA1d and negatively regulate HsfA1d activity under normal conditions. HSP70 interacts with DBD and region 1 of HsfA1d. By contrast, region 1 and the NES are involved in the interaction between HsfA1d and HSP90. Region 1 is conserved among HsfA1s as the TDR domain (indicated by a light-blue area in region 1). Under heat stress conditions, the interaction between HsfA1d and HSP70 is disrupted by unknown mechanisms. Protein kinases (PKs) may be involved in the activation mechanisms. Although it is unclear whether HSP90 dissociates from HsfA1d upon heat shock, the repressor function of HSP90 seems to be inactivated by heat stress. HSP70-free HsfA1d becomes active and upregulates the expression of HS-inducible genes. However, the induction of non-Δ1-responding genes requires coregulators. TF genes are primarily included among non-Δ1-responding genes; thus, the activity of the transcriptional cascade is under strict control. The solid and dashed arrows between HSP70/90 and HsfA1d indicate strong or weak interactions, respectively. The other arrows and bar-heads indicate positive and negative regulation, respectively. The question marks denote links or factors to be confirmed.
We found that region 1 and NES are involved in the interaction with HSP90 (Figures 6A and 6B). Decrease of the HSP90 interaction caused by deletion of region 1 seems to be a good explanation for the constitutive nuclear localization of d11 and d11-3 because HSP90 is a suppressor of the nuclear translocation of HsfA1s (Figures 5E, 6A, and 6B; Yoshida et al., 2011). Although NES has a similar function in the regulation of HsfA1d subcellular localization to that of HSP90, the relationship between the NES function and HSP90 interaction is unclear. It remains to be elucidated whether HSP90 dissociates from HsfA1d at the same time as HSP70 since we could not detect HSP90 in the coinmunoprecipitated samples obtained from HsfA1dpro:sGFP-HsfA1d/abd (Figure 6C). At least the HS-dependent nuclear localization of HsfA1d does not seem to correlate with the dissociation of HSP90 in 3SSpro:sGFP-HsfA1d/abd because the bulk of HsfA1d was localized in the nucleus after heat shock despite the constitutive interaction with HSP90 (Figures 5E and 6C). Therefore, we speculate that HSP90 functions as a scaffold protein to recruit regulators of the subcellular localization of HsfA1d.

Phosphorylation is an important regulator of HSF activity (Xu et al., 2012b). Thus, we tested the hypothesis that the function of region 1 is regulated by the phosphorylation of a tyrosine residue highly conserved among HsfA1s (Supplemental Figure 1). Although the role of tyrosine phosphorylation in signaling has been unclear in plants, several calcium-dependent protein kinase-related protein kinases have tyrosine transphosphorylation activity (Nemoto et al., 2015), suggesting that tyrosine phosphorylation contributes to signal transduction in plant cells. The dy271D and aY294D mutations had significant effects on the HsfA1s activity as these mutations completely disrupted the function of region 1 (Figures 3A, 3C, 3D, and 4E; Supplemental Figures 4 and 5). Therefore, tyrosine phosphorylation is a promising candidate modification to disrupt the activity of region 1. Although these results support the activation of HsfA1s via tyrosine phosphorylation, we did not obtain direct evidence of Tyr-271 phosphorylation with the anti-pTyr antibody (Supplemental Figure 6B). However, because we used the overexpressors expressing dFL or dy271F under the control of the CaMV 35S promoter in the immunoblot assay, there is a possibility that Tyr-271-phosphorylated, i.e., HSP70-free and active, HsfA1d is sufficiently rare that Tyr-271 phosphorylation is diluted below the detection limit by non-Tyr-271-phosphorylated HsfA1d. Therefore, the enrichment of active HsfA1d may be needed to detect Tyr-271 phosphorylation. Future studies should focus on the precise phosphorylation status of HsfA1d using a large quantity of HsfA1d protein purified from HsfA1dpro:sGFP-HsfA1d/abd.

The activation of HSFs is transient because the HSR includes negative feedback mechanisms (Scharf et al., 2012). Accumulation of HSP70/90 is thought to constitute an important part of this negative feedback (Hahn et al., 2011). However, despite the lower binding of HSP70/90, the activity of d11 was found to be repressed similarly to that of dFL, as indicated by the attenuated expression of HS-inducible genes after prolonged heat stress or during recovery (Figure 8). This result supports the idea that the potent thermotolerance of d11OX was caused by the accumulation of HSPs before the heat stress treatment. The precise regulation of HS-inducible gene expression in d11OX indicates that, in addition to the interaction with HSP70/90, unknown negative regulatory mechanisms, such as posttranslational modifications, may also be involved in the attenuation of HsfA1 activity. Reporter assays indicated that regions 2 to 4 also have the ability to repress HsfA1d activity (Figures 1B and 2B). These regions may function as the dominant-negative regulatory domain during the attenuation phase.

Overall, our study demonstrates that HSP70 is an important regulatory factor for repression of HsfA1 activity in Arabidopsis. The analysis of the effect of heat shock on the interaction between HsfA1d and HSP70 indicates that the regulatory mechanism of the repressor activity is likely different among species. Our results simultaneously revealed dissimilarities in the interaction site of HSP70 between HsfA1d and Hs-HSF1. HsfA1d interacted with HSP70 via the N-terminal half of HsfA1d including the DBD and region 1 (Figure 4C). By contrast, Hs-HSF1 uses the C-terminal activation domain for the interaction with HSP70. We could not find the similarity between the sequences of the HSP70-interacting regions of HsfA1d and Hs-HSF1. Furthermore, it was difficult to reconstitute the HsfA1d-HSP70 complex using recombinant proteins (Figure 4D), whereas Hs-HSF1 has been reported to interact with HSP70 in vitro (Shi et al., 1998). Taking into account the potent interaction between HsfA1d and HSP70 observed in the coimmunoprecipitation assays (Figures 4B and 6A), the formation of the HsfA1d-HSP70 complex appears to need support from other proteins, such as cochaperones. These differences in the repression mechanism may be the cause of the constitutive activation of Arabidopsis HsfA1 expressed in human or Drosophila cells (Hübel et al., 1995). Humans and Drosophilas may be unable to properly regulate HsfA1 activity because of the differences in their molecular mechanisms for suppressing HSF activity via HSP70. Our speculation implies that the molecular mechanisms that underlie the repression of HSF activity via HSP70 could have been flexibly modified during the course of evolution.

In conclusion, we identified region 1 as the negative regulatory domain responsible for the HS-dependent activation of HsfA1d (Figure 9). The function of region 1 was found to be associated with the interaction with HSP70/90. Regulation of HsfA1 activity via region 1 appears to be an evolutionarily conserved mechanism among plant species because the amino acid sequence of region 1 is highly conserved among HsfA1s as the TDR domain. Deletion of region 1 rendered HsfA1d active in the transgenic plants under normal conditions. However, activation of HsfA1d was not sufficient for complete activation of the HsfA1-downstream genes in the transcriptional cascade. Not only the activation of HsfA1d but also other events, such as activation of coactivators or suppression of negative regulators, may be essential to fully activate the transcriptional cascade in response to heat stress (Figure 9). Thus, the HSR in plants is precisely regulated at the transcriptional level in the transcriptional cascade.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Col-0 plants were used in this study. Seeds were sterilized, sown on germination medium (GM) in a Petri dish (90 × 20 mm), and grown in a growth chamber at 22°C with 16 h of light (40 μmol m⁻² s⁻¹) as described previously (Yamaguchi–Shinozaki and Shinozaki, 1994). The
hsfa1a hsfa1b hsfa1d triple knockout mutant was generated by Yoshida et al. (2011). Plant transformation was achieved via the floral dip method (Clough and Bent, 1998) using Agrobacterium tumefaciens strain GV3101 (pMP90) with pSoup (Hellens et al., 2000). Both Columbia and the hsfa1a/b/d triple mutant were used as backgrounds of transgenic plants. The transformants were selected on Murashige and Skoog medium plates that contained 10 mg L\(^{-1}\) hygromycin B and 100 mg L\(^{-1}\) cefotaxime.

**Heat Stress Treatment**

For the heat stress treatment, GM agar plates that contained seedlings were transferred into a growth chamber set to 37°C. For the recovery treatment, the plates were returned to the growth chamber at 22°C. At each sampling point, whole plants were harvested and frozen in liquid nitrogen.

**Thermotolerance Test**

Ten-day-old seedlings grown on GM agar plates were transferred to half-strength Murashige and Skoog medium agar plates. After incubation under the growth conditions described above for 4 d, the plates were sealed with Parafilm M (Bemis Flexible Packaging) and submerged in a water bath (Taitech) at 43°C. After treatment, the plates were returned to the previous growth conditions. After recovery for 7 d, the survival rates were determined. The viable plants were defined as those that generated new rosette leaves during recovery.

**Transient Expression Assays with Arabidopsis**

**Mesophyll Protoplasts**

Protoplast isolation and transfection were performed according to Yoo et al. (2007). For the reporter assays, a plasmid mixture that contained 3 µg of the internal control plasmid (pBG35S-β-GLUC), 5 µg of the reporter plasmid (HSP18.2_gus-GUS or 3SS_puro-HSE9-GUS), and differing amounts of the effector plasmids (pGKH containing effector genes) was used for transfection. Transfections were performed in triplicate. pGKH is a vector expressing the inserted gene under the control of the CaMV 35S promoter. In the assays of transactivation activity, 3 and 0.1 µg of the effector plasmids were used for the protoplasts derived from Col-0 and the hsfa1a hsfa1b hsfa1d triple mutant, respectively. In the assays of DNA binding activity, 0.75 and 0.2 µg of the effector plasmids were used for the assays of the HsfA1d-deletion series and chimeric TFs, respectively. When indicated, the protoplasts were exposed to heat stress at 37°C for 1 h and then allowed to recover at 22°C for 1 h. After incubation, the protoplasts were centrifuged at 100g and lysed in 150 µL GUS extraction buffer (19.5 mM NaH\(_2\)PO\(_4\), 30.5 mM Na\(_2\)HPO\(_4\), 1 mM EDTA, 0.1% [v/v] Triton X-100, and 25 mM NaF, 150 mM NaCl, 1% [v/v] Triton X-100, and 2.5 to 4 g for the complementation line) grown on GM agar plates were transferred to half-strength Murashige and Skoog medium agar plates. After incubation under the growth conditions described above for 4 d, the plates were sealed with Parafilm M (Bemis Flexible Packaging) and submerged in a water bath (Taitech) at 43°C. After treatment, the plates were returned to the previous growth conditions. After recovery for 7 d, the survival rates were determined. The viable plants were defined as those that generated new rosette leaves during recovery.

**Fluorescence Observation**

Protoplasts and 10-d-old seedlings that expressed GFP-fused proteins were observed with a confocal laser scanning microscope (LSM 5 FASC; Carl Zeiss). The heat stress treatment was performed as described above. The experiments were performed two or three times, and representative results are shown.

**Total Protein Extraction**

For extraction of the total protein from seedlings, 2-week-old seedlings (0.1 g fresh weight) grown on GM agar plates were ground in liquid nitrogen with ShakeMaster Auto (BioMedical Science) and homogenized in 1.25× sample buffer (82.5 mM Tris-HCl, pH 6.8, 1.25% [v/v] SDS, 10 M urea, and 894 µM 2-mercaptoethanol). After centrifugation (10,000g at room temperature for 10 min), the supernatants were collected and denatured at 95°C for 3 min. For the extraction of the total protein from protoplasts, the protoplasts were dissolved in 1.25× sample buffer. The extracts were denatured at 95°C for 3 min. After separation via SDS-PAGE, the protein samples were transferred onto a polyvinylidene difluoride membrane (Immobilon-P Membrane; Merck Millipore) and then analyzed via immunoblot analysis. To detect the gGFP-fused proteins, an anti-GFP antibody (Tanaka et al., 2012) and a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Pierce) were used. Signals were detected with the SuperSignal West Dura Extended Duration Substrate (Pierce) and an ImageQuant LAS-4000 system (GE Healthcare). Following the chemiluminescence detection, the membrane was stained with Ponceau S solution (0.1% [v/v] Ponceau S [Sigma-Aldrich] and 5% [v/v] acetic acid) to ensure equal protein loading. The experiments were performed two or three times, and representative results are shown.

**Immunoprecipitation**

Three-week-old seedlings (fresh weight of 1 to 1.5 g) for overexpressors and 2.5 to 4 g for the complementation line) grown on GM agar plates were transferred to half-strength Murashige and Skoog medium agar plates. After incubation under the growth conditions described above for 4 d, the plates were sealed with Parafilm M (Bemis Flexible Packaging) and submerged in a water bath (Taitech) at 43°C. After treatment, the plates were returned to the previous growth conditions. After recovery for 7 d, the survival rates were determined. The viable plants were defined as those that generated new rosette leaves during recovery.

**Peptide Preparation and LC-MS/MS Analysis**

The immunoprecipitates were separated via SDS-PAGE with a precast polyacrylamide gel (10% Perfect NT Gel M; Drem Realization and Communication). The protein samples were visualized by Coomassie Brilliant Blue staining. The gel lanes sliced into 10 pieces were dehydrated by the addition of 100% acetone and then vacuum centrifuged. The cysteine residues were reduced with 10-mM DTT/25-mM NH\(_4\)CO\(_3\) at 56°C for 1 h. The reduced cysteine residues were alkylated with 55-mM iodoacetamide/25-mM NH\(_4\)CO\(_3\) at room temperature for 45 min in the dark. The gel slices were dehydrated by the addition of 50% acetonitrile/12.5 mM...
was electrophoresed and then transferred onto a nylon membrane plates was extracted with RNAiso Plus (Takara Bio) according to the instructions. The experiment was performed twice, and a representative result is shown.

Yeast Two-Hybrid Assays
The Saccharomyces cerevisiae yeast strain AH109 yeast was cotransformed with bait and prey plasmids according to the Yeast Protocols Handbook (Clontech). Interaction assays were performed according to the manufacturer’s instructions for the Matchmaker GAL4 Two-hybrid System 3 (Clontech). The experiments were performed twice, and representative results are shown.

Pull-Down Assays
The GST-HsfA1d and 6×His-HSC70-1 proteins were expressed in Escherichia coli (Rosetta [DE3] pLys) and purified with Glutathione Sepharose 4B (GE Healthcare Life Science) and HisPur Cobalt Resin (Life Technologies), respectively. The purified GST-HsfA1d protein (10 μg) was bound to Glutathione Sepharose 4B. The resin was mixed with 10 μg 6×His-HSC70-1 protein in TEN buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 100 mM NaCl) and rotated for 5 h at 4°C. The resin was washed three times with TEN buffer. The proteins were eluted by incubating the resin in GST elution buffer (50 mM Tris-HCl, pH 8.5, and 10 mM reduced glutathione) overnight at 4°C. After separation via SDS-PAGE, the protein resin in GST elution buffer (50 mM Tris-HCl, pH 8.5, and 10 mM reduced glutathione) overnight at 4°C. After separation via SDS-PAGE, the protein was extracted with 50% acetonitrile/5% formic acid. LC-MS/MS analysis was performed twice, and representative results are shown.

RNA Extraction, RNA Gel Blot Analysis, and qRT-PCR
The total RNA from 2-week-old seedlings (eight plants) grown on GM agar plates was extracted with RNAiso Plus (Takara Bio) according to the manufacturer’s instructions. RNA extraction, RNA gel blot analysis, and qRT-PCR were performed for each cDNA sample, and the obtained values were normalized to the amount of 18S rRNA. The qRT-PCR analyses were performed twice, and representative results are shown.

Microarray Analysis and Data Processing
Transcriptome analysis was performed using an Arabidopsis 3 Oligo Microarray (Agilent Technologies) as described previously (Mizoi et al., 2013). In brief, RNA was extracted from 14-d-old nonstressed seedlings of VC and Δ1 overexpressors as described above. Biological replication was performed by analyzing the RNA obtained from two independent Δ1 overexpressors (lines f and j). RNA labeling, probe hybridization, and slide scanning were performed as described previously (Mizoi et al., 2013). Data analyses were performed with Subio. The signal intensities of the spots were normalized by the Lowess method, and the significance of the expression changes was evaluated by Student’s t-test. The P values were corrected by the Benjamini-Hochberg false discovery rate method, and probes that showed a corrected P value of <0.05 were used for further analyses.

Vector Construction
The coding sequences (CDSs) of HsfA1 genes were amplified by PCR and inserted into the NotI-Xhol sites of the pGKH plasmid, a hygromycin resistance-conferring version of pGKX (Qin et al., 2008; Fujita et al., 2012). The CDS of HSC70-1 was amplified by PCR and inserted into the XbaI-Smal sites of pGKH. The CDSs of GroEL and GroES were amplified by PCR and inserted into the XbaI-Xhol sites of the pGKH. The deletion series of HsfA1s was generated from pGKH-HsfA1a and pGKH-HsfA1d by inverse PCR. Nucleotide substitutions in the CDS of HsfA1s were introduced via the megaprimer method (Sarkar and Sommer, 1990). For generation of GFP-fused genes, HsfA1s and its derivatives were amplified by PCR and inserted into the NotI-Xhol sites of the pGKH-NsGFP plasmid, a hygromycin resistance version of pGKX-NsGFP (Qin et al., 2008; Fujita et al., 2012). To generate the HsfA1dpro:+:GFP-HsfA1d construct for the complementation assay, the 1142-bp promoter region upstream of the HsfA1d start codon was amplified and inserted into SacI-Spel sites of the pGreen0129 (Hellens et al., 2000). The sGFP-HsfA1d-NosT cassette was amplified from pGKH-NsGFP-HsfA1d and inserted into SpeI-KpnI sites of pGreen0129-HsfA1dpro. To generate chimeric Tfs, VP16AD was amplified from pBS5351-VP16AD (Kidokoro et al., 2009) and inserted into the inverse PCR products after Xhol digestion.

Yeast Two-Hybrid Assays
For the Y2H assays, HSC70-2, HSC70-3, HSP70-3, and HSP70T-1 were generated by Koizumi et al. (2014). HsfA1d was inserted into the EcoRI-BamHI sites of the pGBK7 (Clontech). Bait vectors that contained HSC70-1 and HSP70 were generated by Koizumi et al. (2014). HsfA1d was inserted into the EcoRI-Xhol sites of pGAD7 (Clontech). To generate expression vectors for E. coli, HSC70-1 was inserted into the HindIII-XbaI sites of the pCold plasmid (Takara). HsfA1d was inserted into the Xhol-XbaI sites of the pCold GST plasmid, in which the sequence encoding the 6×His tag of pCold was replaced with that encoding GST by inverse PCR. To generate the HSP18.2::GUS reporter construct, the 713-bp promoter region upstream of the HSP18.2 start codon was amplified and inserted into EcoRI-Xhol sites of the pGK-GUS (Qin et al., 2008). Primers and cloning information from this study are listed in Supplemental Tables 1 and 2, respectively.

Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: HsfA1a (AT4G17750), HsfA1d (AT1G32330), HsfA2 (AT2G26150), HsfA7 (AT3G51910), DREB2A (AT5G05410), HSC70-1 (AT5G02500), HSC70-2 (AT5G02490), HSC70-3 (AT3G09440), HSF70 (AT3G12580), HSP70B (AT1G6030), HSP70T-1 (AT1G56410), HSP101 (AT1G74310), HSP18.2
(AT5G59720), HSP17.6I1 (AT5G212020), HSP23.5-P (AT4G27670), and HSP22.0-ER (AT4G10250). All of the microarray data are available at ArrayExpress under accession number E-MTAB-3574.

Supplemental Data

Supplemental Figure 1. Alignment of HsfA1s from Arabidopsis thaliana, Solanum lycopersicum, Gycine max, Oryza sativa, Zea mays, Populus trichocarpa, Physcomitrella patens, and Chlamydomonas reinhardtii.

Supplemental Figure 2. Effect of deletion of the regulatory domain on the subcellular localization of HsfA1d.

Supplemental Figure 3. Activation of the HsfA1a deletion mutants by heat stress in protoplasts derived from the hasfa1a/b/d triple mutant.

Supplemental Figure 4. dY271D mutation has a comparable effect on HsfA1d activity as complete disruption of the conserved motif.

Supplemental Figure 5. The tyrosine residue in the conserved motif is important for the repressive function of region 1 of HsfA1a.

Supplemental Figure 6. Analysis of the tyrosine phosphorylation status of HsfA1d.

Supplemental Figure 7. Generation of sGFP-HsfA1d overexpressors in the hasfa1a/b/d triple mutant background.

Supplemental Figure 8. HSP70s as candidate negative regulators interacting with region 1 of HsfA1d.

Supplemental Figure 9. Generation of complementation lines of HsfA1d in the hasfa1a/b/d triple mutant background.

Supplemental Figure 10. Overexpression of αΔ1 causes activation of HSP expression.

Supplemental Table 1. Primers used in this study.

Supplemental Table 2. Constructs generated in this study.

Supplemental Data Set 1. Peptide sequences of HsfA1d-interacting proteins detected by LC-MS/MS analyses.

Supplemental Data Set 2. Text file of the Hsp70 alignment used for the phylogenetic analysis shown in Supplemental Figure 8A.

Supplemental Data Set 3. Differentially expressed genes in the Δ1 overexpressors.

Supplemental Data Set 4. Responses of the HsfA1-downstream HS-inducible genes to overexpression of HsfAs or knockout of HsfBs.

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AUTHOR CONTRIBUTIONS

N.O., J.M., K.S., and K.Y.-S. conceived and designed the research. N.O. performed most of the experiments. H.Z. performed part of the yeast two-hybrid assay. S. Kidokoro performed the microarray experiment. K.K., J.M., F.T., T.I., and S.Y. performed the LC-MS/MS experiment and analyzed the data. S. Koizumi contributed several constructs. N.O. and K.Y.-S. wrote the article.

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