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# Acquired thermotolerance independent of heat shock factor A1 (HsfA1), the master regulator of the heat stress response

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The heat stress (HS) response in eukaryotes is mainly regulated by heat shock factors (HSFs). Genetic disruption of the master HSF gene leads to dramatically reduced HS response and thermotolerance in several model organisms. However, it is not clear whether organisms devoid of the master regulator can still acclimate to heat. Previously, we showed that Arabidopsis *HsfA1a*, *HsfA1b* and *HsfA1d* act as master regulators in the HS response. In this study, we examined the heat acclimation capacity of the Arabidopsis quadruple and triple T-DNA knockout mutants of *HsfA1a*, *HsfA1b*, *HsfA1d* and *HsfA1e*. Our data showed that in the absence of the master regulators, a minimal but significant level of acquired thermotolerance could be attained in the Arabidopsis mutants after acclimation. The optimum acclimation temperature for the *HsfA1* quadruple mutant was lower than that for the wild type plants, suggesting that plant cells have two HS-sensing mechanisms that can be distinguished genetically. The acquired thermotolerance of the quadruple mutant was likely due to the induction of a small number of HsfA1-independent HS response genes regulated by other transcription factors. Here, we discuss the possible candidates and propose a working model of the transcription network of the HS response by including the HsfA1-dependent and -independent pathways.



In response to elevated temperature, living cells immediately switch on the transcription of a multitude of genes encoding protective proteins or enzymes that enable it to minimize injury or sustain a subsequent harsher heat challenge. This universal phenomenon in all organisms studied to date is known as the heat stress or heat shock response (HSR).[1] In eukaryotic cells, the HSR is mediated by structurally conserved transcription regulators, named heat shock factors (HSFs), which form active trimeric conformations that bind to the conserved *cis*-elements in the promoters of many HSR genes.[2,3] Genetic disruption or knockdown of the master regulator gene(s) has been shown to dramatically diminish the HSR and thermotolerance in chlamydomonas,[4] drosophila,[5] mammalian cells[6,7] and plants.[8-10] However, whether organisms lacking the master regulator can still acclimate to heat is not clear. This question is of great interest in plants as the sessile organisms frequently face the challenge of HS in the natural environment, and the existence of an additional HSR pathway independent of the master HSFs could be advantageous for plants adapting to HS.

In higher plants, homologs of HSFs form a multigene family, whose members can be classified into three major classes (class A, B, and C) and several subclasses.[11] Recently, genetic evidence has revealed diverse functions for some of these HSF homologous genes.[8-10,12-21] The members of the HsfA1 subclass have been shown to be the master regulators of the HSR. Transcriptional knockdown of tomato *HsfA1a* expression dramatically reduced basal and acquired thermotolerance of the transgenic plants, suggesting that HsfA1a plays a major role in the HSR of tomato.[9] In Arabidopsis, there are four members belonging to the *HsfA1* subclass, *HsfA1a*, *HsfA1b*, *HsfA1d* and *HsfA1e*. Disruption of Arabidopsis *HsfA1a* or both *HsfA1a* and *HsfA1b* by T-DNA insertions does not cause significant defect in thermotolerance, as is the case in tomato.[22] Thus, it was thought that the four *HsfA1* genes are likely redundant in function. To evaluate this possibility, we generated a quadruple T-DNA knockout (*QK*) and four triple KO mutants, named *aTK*, *bTK*, *dTK* and *eTK*, where the prefixed letters represent the remaining intact *HsfA1*.[8] These mutants are derived from single mutants of Columbia (Col) or Wassilewskija (Ws) ecotype backgrounds. We showed that the *QK* and *eTK* mutants are extremely sensitive to various HS treatments from seed to adult plant stages compared with either the Col or Ws wild types. Moreover, the HSR was dramatically compromised in these mutants, while in the *aTK*, *bTK* and *dTK* mutants the thermotolerance and HSR were not or were only partially affected. These results suggest that *HsfA1a*, *HsfA1b* and *HsfA1d* share the role of master regulator of the HSR in Arabidopsis.[8] In this study, we were interested to know whether acquired thermotolerance can be attained in the absence of the master transcription regulators.

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First, to determine whether there are differential temperature optimums to induce acquired thermotolerance in the *HsfA1* mutants, 7-d-old seedlings were first acclimated at different temperatures from 31 to 39°C for 1 h, allowed to recover at 22°C for 2 h, then challenged by severe HS at 44°C for 150 min. Viability of the seedlings was then assessed after 7 d of recovery from treatment. The results showed that 37°C was the most effective temperature in inducing acquired thermotolerance in the wild type, *aTK*, *bTK* and *dTK* plants (**Fig. 1A and B**). The acclimation treatments at different temperatures could not confer even slight tolerance to the severe HS treatment in the *QK* and *eTK* mutants (data not shown), which was a more severe result than that seen in the *Hsp101* KO line. *Hsp101* encodes a molecular chaperone that has an important role in thermotolerance.[23,24] These results are consistent with our previous findings: *HsfA1a* is slightly more effective than *HsfA1d*, while *HSFA1b* is the least effective at conferring acquired thermotolerance and the *QK* and *eTK* are unable to attain acquired thermotolerance under the assay conditions.[8] However, we suspected that the challenge treatment at 44°C for 150 min might have been too harsh to reveal the heat acclimation effect in the *QK* and *eTK*, which could both be too weak. Thus, we reduced the severity of our conditions to 43°C for 25 min for the HS challenge. This condition is lethal to non-acclimated *QK* and *eTK* seedlings, but not to the wild type or even the *Hsp101* KO plants. **Figure 1C and D** show that acclimation at 33°C to 37°C could significantly enhance thermotolerance in *eTK* and *QK*, and the optimum acclimation temperature was 35°C. Despite the fact that the survival rate of the *QK* mutant was as high as that of the wild type if acclimated first at 35°C, the mutant plants showed retarded growth after the HS challenge (**Fig. 1C**), indicating a delicate nature of the acquired thermotolerance without the participation of HsfA1s. The viability of the *eTK* was slightly lower than that of the *QK* at different acclimation temperatures (**Fig. 1C and D**), which is consistent with our previous findings that HsfA1e is not required for thermotolerance.[8]

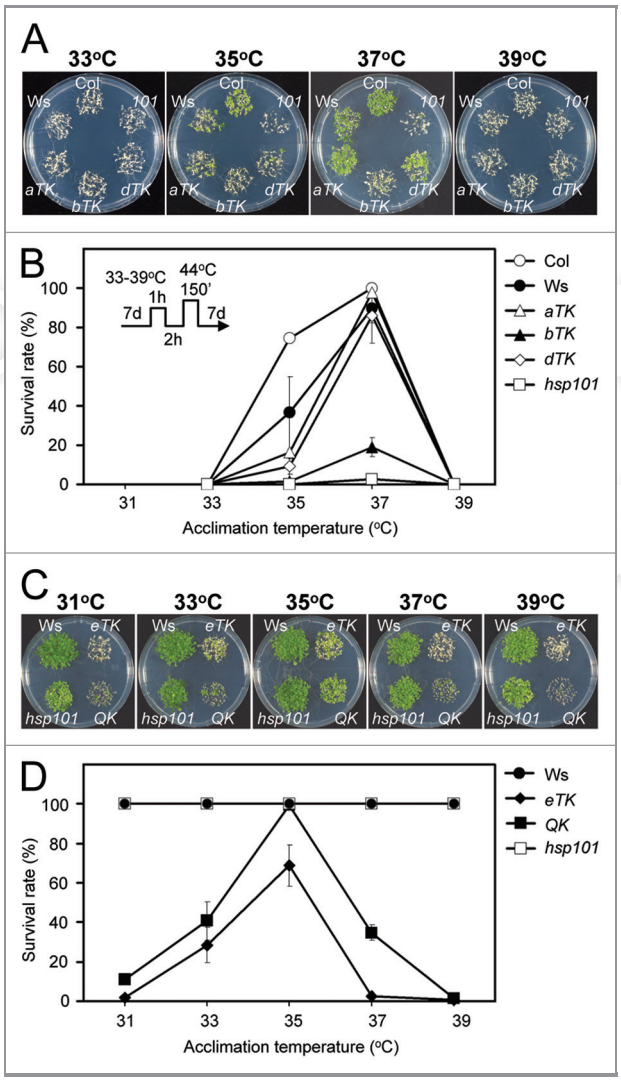


**Figure 1.** Arabidopsis seedlings attained low levels of acquired thermotolerance in the absence of the HsfA1s. Seedlings of the wild type (Col and Ws), *aTK*, *bTK*, *dTK*, *eTK*, *QK* and *Hsp101* KO mutant (*hsp101* or *101*) lines were subjected to HS treatments with HS regimes schematically shown inside (B). The 7-d-old seedlings were first acclimated at 31–39°C as indicated, allowed to recover at 22°C for 2 h, and then challenged at 44°C for 150 min (A and B) or 43°C for 25 min (C and D). The plants were allowed to grow for 7 d after the HS treatments before being photographed. The effect of acclimation temperatures on acquired thermotolerance was measured by growth performance (A and C) and survival rate (B and D). The survival rates are presented as mean values of three replicates ± SD (n ≥ 50 each).

Our data demonstrate that in Arabidopsis the ability to acquire enhanced thermotolerance by acclimation is dramatically reduced in the absence of HsfA1a/b/d, but is not completely abolished. This is consistent with the transcriptional profiles that have previously shown that the heat-induction of a relatively small number of HS-upregulated genes is independent of the master regulators.[8] Moreover, some highly heat-induced genes remain inducible by heat treatment in the *QK* mutant, although to a lesser degree.[8] These genes may contribute to the acquired thermotolerance of the *QK* and *eTK* mutants. So far, it is not clear what transcription factors are responsible for the heat-induction of these genes. Other HSF genes, such as *HsfA4a*, *HsfA4c* or *HsfA7a*, whose transcripts are relatively abundant under normal condition,[11] are possible candidates. These transcription factors might play a minor role in acquired thermotolerance. It was reported that disruption of *HsfA7a* due to T-DNA insertion causes a defect in acquired thermotolerance.[25] However, the mechanism of how this may occur has not been reported. In our work, we did not observe obvious and consistent thermotolerance defects in the same *HsfA7a* KO line (SALK_080138) under various HS conditions (ref. 13 and unpublished data). The role and mechanism of this HSF in acquired thermotolerance remain to be shown. Moreover, we could not exclude the possible participation of HsfA2, Dreb2A and HsfB1/2b, which have been implicated in acquired thermotolerance.[13,14,18,26] Of note, *HsfA2*, *HsfA7a*, *Dreb2A* and *HsfB1/2b* are HsfA1-dependent HSR genes.[8,10]

Alternatively, there might be other types of transcription factors regulating the expression of the HsfA1-independent HSR genes, such as *BiP1* (At5g28540), *BiP2* (At5g42020) and *UTR3* (At1g14360).[8] BiP1 and BiP2 are molecular chaperones localized

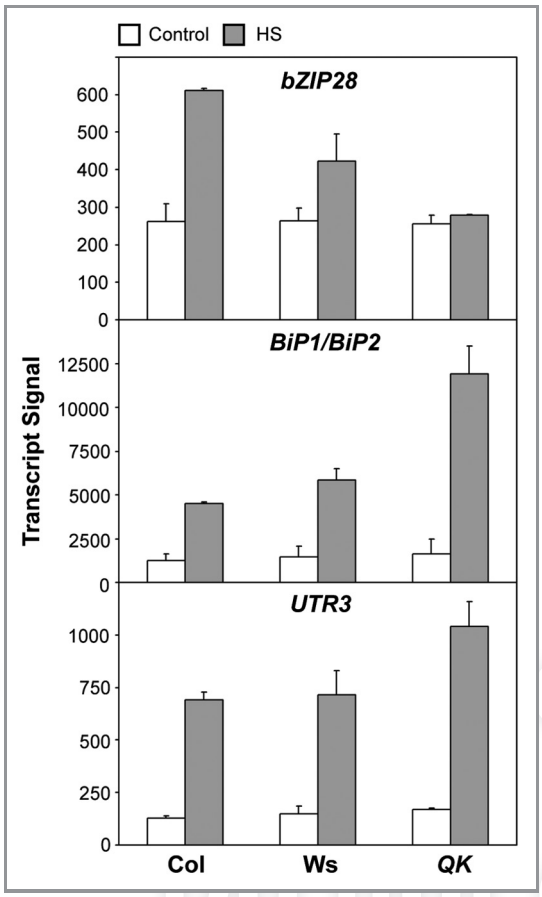


**Figure 2.** The expression levels of *bZIP28*, *BiP1/BiP2* and *UTR3* in the wild type and *QK* mutant. The data were derived from the microarray results published previously.[8] Statistical significance of these data passed the scrutiny of the false discovery rate (FDR) at the stringent level of 0.05.

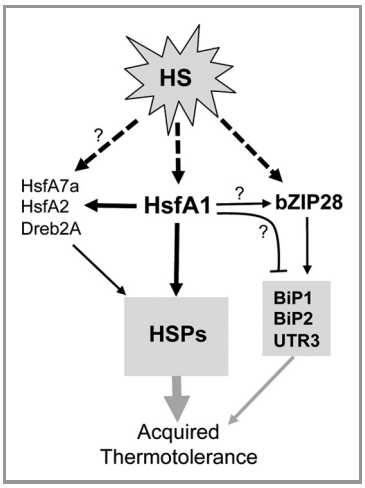


**Figure 3.** Simplified working model of the transcription network of the HSR in Arabidopsis. HsfA1 represents HsfA1a/b/d. The arrows with dashed lines indicate activation of the transcription factors by posttranslational modifications. The black arrows and a bar-head with solid lines indicate positive and negative transcription regulation, respectively. The question marks denote links to be confirmed. The proteins shown in gray boxes are a sample list of target genes of the transcription factors. The gray arrows of different thicknesses indicate speculative degrees of acquired thermotolerance conferred by different HSR pathways.

in the endoplasmic reticulum (ER), while UTR3 is a UDP-galactose transporter localized both at the ER and Golgi.[27] Recently, these genes were shown to be regulated by an ER membrane-tethered basic domain/leucine zipper (bZIP) transcription factor, bZIP28, during ER and heat stresses.[28,29] Upon exposure to stress conditions, the transcription factor domain of bZIP28 is released from the ER membrane by proteolysis and redistributed to the nucleus.[28,29] The Arabidopsis T-DNA KO mutant of *bZIP28* (At3g10800) exhibits increased sensitivity to HS and is unable to trigger the heat-inducible expression of *BiP1/BiP2* and *UTR3*.[28] In contrast, the expression of *Hsp17.4-CIII* (At1g54050), a target gene of HsfA1, is not affected in the *bZIP28* mutant.[28] Although it remains to be seen to what extent bZIP28 is involved in acquired thermotolerance, this membrane-tethered transcription factor may act as a sensor of HS in a pathway parallel to that mediated by HsfA1. Interestingly, *bZIP28* itself is heat-inducible.[28] Our microarray data showed that the heat-induced but not the basal expression of *bZIP28* was substantially reduced in the absence of the HsfA1s (**Fig. 2**), suggesting that at least one of the HsfA1s positively regulates *bZIP28* under HS conditions. The basal expression of *bZIP28* apparently was sufficient to upregulate its target genes. Actually, the expression of *BiP1/BiP2* and *UTR3* were even higher in the *QK* mutant than in the wild type under HS conditions (**Fig. 2**), suggesting that HsfA1s act as negative regulators of these genes under HS conditions. More experiments are needed to confirm these relationships.

Taken together, our results suggest that acquired thermotolerance can be conferred in part by the transcription pathways independent of the HsfA1s in Arabidopsis. A simplified working model is proposed to summarize the current knowledge and the points discussed above (**Fig. 3**). In this model, the HSR is triggered by post-translational modifications of the transcription factors, HsfA1, bZIP28 and possibly others, which then regulate the transcription of HSR genes. It has been well-documented that HSF activity is associated with post-translational modifications in eukaryotes.[30-35] In Arabidopsis, phosphorylation[36] and sumoylation[37,38] of HsfA1a and HsfA2, respectively, have been reported. Activation of bZIP28 by proteolysis has been nicely demonstrated.[28,29] The pathways mediated by HsfA1 and bZIP28 can be independently induced at an early stage of the HSR, but may get cross-wired due to the effect of HsfA1 on the expression of *bZIP28* and its downstream genes. So far, we do not know whether this is a direct or indirect effect and how this effect could physiologically influence the HSR and thermotolerance in the long run. Further studies are anticipated to address these questions.

## Materials and Methods

The plant materials, growth conditions, and thermotolerance assay were described previously.[8] The expression data shown in **Figure 2** were obtained from the microarray data published previously, which can be accessed in the Gene Expression

Omnibus at the National Center for Biotechnology Information (NCBI; accession number GSE26266).[8]

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.


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