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HEAT-INDUCED TAS1 TARGET1 Mediates Thermotolerance via HEAT STRESS TRANSCRIPTION FACTOR A1a–Directed Pathways in *Arabidopsis*[C][W]

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Many heat stress transcription factors (Hsfs) and heat shock proteins (Hsps) have been identified to play important roles in the heat tolerance of plants. However, many of the key factors mediating the heat response pathways remain unknown. Here, we report that two genes, which are targets of *TAS1* (*trans*-acting siRNA precursor 1)–derived small interfering RNAs that we named *HEAT-INDUCED TAS1 TARGET1* (*HTT1*) and *HTT2*, are involved in thermotolerance. Microarray analysis revealed that the *HTT1* and *HTT2* genes were highly upregulated in *Arabidopsis thaliana* seedlings in response to heat shock. Overexpression of *TAS1a*, whose *trans*-acting small interfering RNAs target the *HTT* genes, elevated accumulation of TAS1-siRNAs and reduced expression levels of the *HTT* genes, causing weaker thermotolerance. By contrast, overexpression of *HTT1* and *HTT2* upregulated several *Hsf* genes, leading to stronger thermotolerance. In heat-tolerant plants overexpressing *HsfA1a*, the *HTT* genes were upregulated, especially at high temperatures. Meanwhile, HsfA1a directly activated *HTT1* and *HTT2* through binding to their promoters. HTT1 interacted with the heat shock proteins Hsp70-14 and Hsp40 and NUCLEAR FACTOR Y, SUBUNIT C2. Taken together, these results suggest that *HTT1* mediates thermotolerance pathways because it is targeted by *TAS1a*, mainly activated by HsfA1a, and acts as cofactor of Hsp70-14 complexes.

INTRODUCTION

Abiotic stress conditions such as drought, heat, or salinity cause extensive losses in agricultural production worldwide. Global warming is generating rapid changes in temperature that affect plant growth and yield (Mittler, 2006; Mittler and Blumwald, 2010). Thus, crops with enhanced tolerance to field conditions provide a promising avenue to reduce yield losses, improve growth, and provide a secure food supply for a growing world population (Mittler and Blumwald, 2010).

As sessile organisms, plants are subjected to heat stress during their growth and development and have evolved a variety of mechanisms to respond to high temperatures that minimize damage and ensure protection of cellular homeostasis. Central to the heat stress response in plants are the heat stress transcription factors (Hsfs), which belong to a family of proteins conserved throughout the eukaryotic kingdoms (Nover and Scharf, 1997; Schöffl et al., 1998; Kotak et al., 2007). In *Arabidopsis thaliana*, 21 different *Hsf* genes have been identified and assigned to three major classes, A, B, and C, based on a phylogeny of their DNA binding domains and the organization of their hydrophobic repeats (Nover et al., 2001). Class A *Hsfs* contain short peptide motifs characterized by aromatic, large hydrophobic, and acidic amino acid residues (AHA) in the activation domain that are essential for transcriptional activator function. Class B and C *Hsfs* lack the AHA motif and have no activator function on their own (Döring et al., 2000; Nover et al., 2001; Kotak et al., 2004). It has been demonstrated that HsfA1a and other plant Hsf members bind the heat shock element (HSE) consisting of tandem inverted repeats of the short consensus sequence nGAAn (nTTCnnGAAnnTTCn, called the perfect HSE) and the AGGGG motif (von Koskull-Döring et al., 2007; Guo et al., 2008). The roles of several *Hsfs* of class A (15 members) in *Arabidopsis* that show high functional diversification and genetic redundancy are still unknown, and the key elements mediating *HsfA1* genes in the thermotolerance remain obscure.

HsfA1 members (group 1 in class A), including *HsfA1a*, *HsfA1b*, *HsfA1d*, and *HsfA1e*, are major transcriptional activators of the heat shock response (Lohmann et al., 2004; Liu et al., 2011a; Nishizawa-Yokoi et al., 2011; Yoshida et al., 2011). HsfA1a directly senses heat stress and becomes activated, and this process is dependent on the redox state (Liu et al., 2013). Microarray data show that transient mRNA accumulation of a number of heat response genes in the *hsfA1a hsfA1b* double mutant or *hsfA1a hsfA1b hsfA1d* triple mutant is significantly impaired during heat shock (Lohmann et al., 2004; Liu et al., 2011a; Yoshida et al., 2011). The downstream genes of HsfA proteins include several *Hsp* (heat shock protein) and *Hsf* genes and enzyme genes catalyzing the synthesis of metabolites (Busch et al., 2005). Interestingly, there are also a number of *HsfA1*-dependent genes that encode proteins of unknown function.

Hsps function as the central components of responses to various forms of cellular stress in all living organisms through the action of *Hsfs*. Hsps are grouped into five classes based on their

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approximate molecular masses in kilodaltons: Hsp100, Hsp90, Hsp70, Hsp60, and small heat shock proteins (Iba, 2002). Hsp70s are characterized by a typical N-terminal ATPase domain of ~45 kD. They play important roles in folding and refolding, preventing aggregation, and transport and controlled degradation of proteins by interacting with various cochaperones, cofactors, or nucleotide exchange factors (Mayer and Bukau, 2005; Jungkunz et al., 2011). It has been reported that Hsp70 represses the function of HsfA1, HsfB1, and HsfA2 by direct interaction and inhibits the DNA binding activities of these proteins, while Hsp90 enhances this type of function (Hahn et al., 2011).

In addition to common *Hsfs* and *Hsps*, transcriptome analysis of *Arabidopsis* has revealed the involvement of factors in a broad range of physiological processes other than the classical heat stress responsive genes in thermotolerance. An increasing number of mutants with altered thermotolerance have extended our understanding of the complexity of the heat stress response in plants. Recently, much progress has been made in understanding heat shock–responsive small interfering RNAs (siRNAs), which mediate gene silencing by directing protein partners to complementary nucleic acids of target mRNA. Two types of endogenous small RNA have been reported, microRNAs (miRNAs) and siRNAs (Bartel, 2004; Ramachandran and Chen, 2008). miRNAs are single-stranded, ~20- to 22-nucleotide RNA molecules derived from partially complementary RNA precursors, which are mainly transcribed by RNA polymerase II. DICER-LIKE1 (DCL1) is the main enzyme responsible for mature miRNA production. HYPONASTIC LEAVES1 and SERRATE are also important for the biogenesis of miRNA (Bartel, 2004; Chen, 2005). siRNAs are ~20 to 26 nucleotides and are generated from a fully complementary double-stranded RNA by the action of an RNA-dependent RNA polymerase. These small RNAs are known to silence genes transcriptionally by guiding target DNA methylation and post-transcriptionally by triggering target mRNA degradation and by repressing translation (Axtell, 2013).

Plant genomes encode a unique class of sRNAs called *trans*-acting small interfering RNAs (ta-siRNAs), which are also involved in posttranscriptional gene silencing, like miRNAs. ta-siRNA synthesis first requires the direct binding of functional miRNAs to trigger cleavage of noncoding *TAS* (ta-siRNA) precursor RNA, which is subsequently converted into double-stranded RNA from the 3′ fragment by the action of RNA DEPENDENT RNA POLYMERASE6 (RDR6). The resultant dsRNAs are then processed into phased 21-nucleotide ta-siRNAs by the DCL4 enzyme. In the current model, SUPPRESSOR OF GENE SILENCING3 interacts and colocalizes with RDR6 in cytoplasmic granules to stabilize the cleaved transcript followed by recruitment of RDR6. In this way, novel ta-siRNAs are generated from the action of nonhomologous miRNAs, amplifying the diversity of small RNA regulated targets (Peragine et al., 2004; Vaucheret, 2005; Xie et al., 2005; Yoshikawa et al., 2005; Manavella et al., 2012).

Currently, there are four families of noncoding precursor genes generating ta-siRNA in *Arabidopsis*. The *TAS1*, *TAS2*, and *TAS4* families require one miRNA binding site, while *TAS3* needs two. The *TAS1* family has three loci, *TAS1a*, *TAS1b*, and *TAS1c*, which require miR173 to guide cleavage of the transcript (Allen et al., 2005). The *TAS1* loci code for multiple ta-siRNAs with very similar sequences such as siR480(+)/siR255, siR396(+), and siR438(+), which have been demonstrated to target *At1g51670*, *At4g29760*, *At4g29770*, *At5g18040*, and *At5g18065* (Sunkar and Zhu, 2004; Vazquez et al., 2004; Yoshikawa et al., 2005). All *TAS1* transcripts contain at least one conserved sequence when processing the mature *TAS1* siR480(+)/siR255 (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005). siR480(+)/siR255 was first designated as an miRNA (miR389), simply because its precursor transcript has the potential to fold into a hairpin like an miRNA (Sunkar and Zhu, 2004). Unlike miRNA, the biogenesis of ta-siRNA requires RDR6 (Peragine et al., 2004; Vazquez et al., 2004).

In *Arabidopsis*, all five *TAS1*-derived siRNA-mediated targets encode proteins of unknown function (Vazquez et al., 2004; Allen et al., 2005). Some *TAS1*-derived siRNAs are downregulated under salt, dehydration, or cold stress (Sunkar and Zhu, 2004; Kume et al., 2010). Such downregulation is concomitant with upregulation of *At1g51670*, *At5g18040*, and *At4g29760* at 4°C (Kume et al., 2010). Furthermore, overexpression of *At5g18040* can enhance oxidative stress tolerance in transgenic plants (Luhua et al., 2008).

TAS1 genes may play an important role in abiotic stress responses. However, whether and how *TAS1* and *TAS1*-derived siRNAs function in thermotolerance is unclear. Here, we undertook a genetic and molecular analysis of *TAS1* and their targets to define their roles in the heat stress response in *Arabidopsis*. Our results indicated that accumulation of TAS1-derived siRNAs was decreased during the first hour of heat shock. On the other hand, two *TAS1*-targeted genes were directly activated by *HsfA1a* upon heat shock. Proteins encoded by these genes directly interact with the heat shock proteins Hsp70-14 and Hsp40 (AT2G33735) and NF-YC2 and therefore function as cofactors of Hsp70-14 complexes. Our findings reveal a molecular mechanism underlying the thermotolerance of plants.

RESULTS

HTT Genes Are Heat Induced

We used an Affymetrix ATH1 microarray to detect the global expression of the heat-responsive genes using 21-d-old *Arabidopsis* seedlings exposed to 37°C for 1 h. Among the 22,810 genes detected, 3223 were upregulated and 2438 were downregulated more than 1.5-fold by heat stress. These included many genes targeted by miRNAs and ta-siRNAs (Table 1). Among them, *At5g18040* and *At4g29770* were upregulated 18- and 17-fold, respectively, at high temperature. These genes are two targets of *TAS1*-derived siRNAs (Vazquez et al., 2004; Allen et al., 2005). *TAS1*-derived siRNAs include the six reported siRNAs. siR480(+)/siR255, siR396(+), and siR438(+), which have highly homologous RNA sequences, are referred to as TAS1-siRNAs in this study (Figure 1A). *At5g18040* has only one mismatch with the three siRNAs, and *At4g29770* has two mismatches with siR480(+)/siR255 and three mismatches with siR396(+). We designated *At4g29770* and *At5g18040* as *HEAT-INDUCED TAS1 TARGET1* (*HTT1*) and *HTT2*, respectively.

To verify the effects of heat stress on the expression of *TAS1* targets, quantitative real-time RT-PCR (qRT-PCR) were performed using RNA samples derived from seedlings exposed to heat shock at 37°C for 0.5 to 4 h. The expression levels of *HTT1*

Table 1. Expression Levels of miRNA- and ta-siRNA–Targeted Genes at High Temperature

miRNA	Gene Name	Locus	HS versus NT (Fold Change)
miR159	MYB65	AT3G11440	1.57
miR161.2	EMB2654	AT2G41720	3.25
miR165/6	REV	AT5G60690	3.74
miR167	ARF6	AT1G30330	1.52
miR169	HAP2A	AT5G12840	1.53
miR172	TOE2	AT5G60120	1.57
miR400	PPR	AT1G22960	4.40
miR400	PPR	AT4G19440	6.52
miR400	PPR	AT5G46680	2.57
miR472	Disease resistance protein	AT1G62630	2.95
miR472	Disease resistance	AT1G12290	3.97
miR771	eIF-2	AT1G76810	3.53
miR827	BAH1	AT1g02860	4.47
miR838	Unknown protein	AT2g45720	2.00
miR859	F-Box	AT2g18780	4.83
TAS1a	Histone H1/H5 protein	AT1G54260	2.22
TAS1-siRNAs	Hypothetical protein	AT5G18040	18.23
TAS1-siRNAs	Hypothetical protein	AT4G29770	16.97
miR156	SPL3	AT2G33810	−2.44
miR160	ARF16	AT4G30080	−2.63
miR164	NAC1	AT1G56010	−33.33
miR169	HAP2B	AT3G05690	−2.04
miR319	TCP24	AT1G30210	−5.26
miR319	TCP3	AT1G53230	−2.22
miR319	TCP2	AT4G18390	−7.69
miR393	GRH1	AT4G03190	−4.00
miR393	AFB3	AT1G12820	−2.00
miR395	AST68	AT5G10180	−3.13
miR395	APS4	AT5G43780	−3.03
miR396	ATK2	AT4g27180	−3.15
miR400	EMB2745	AT5G39710	−6.25
miR408	ARPN	AT2G02850	−4.00
miR778	SUVH6	AT2G22740	−2.56
TAS3a	ARF3	AT2G33860	−2.86

The RNA samples were harvested from 21-d-old seedlings exposed to heat shock (37°C for 1 h).

and *HTT2* and *At5g18065* were significantly higher in seedlings exposed to heat shock (HS) for 0.5 h than in seedlings exposed to a normal temperature (NT) of 22°C, and the expression increased progressively with time (Figure 1B). We detected that *HTT1* had two alternative-splicing variants. The expression level of *HTT1.1* was much higher compared with that of *HTT1.2* during heat exposure. Like *HTT1*, *HTT2* and *At5g18065*, *At2g29760*, and *At1g51670* were upregulated in seedlings with HS for 0.5 and 2 h, respectively, albeit to a lesser extent. This indicated that all five target genes of *TAS1* siR480(+)/siR255 were heat induced. Therefore, we designated *At5g18065*, *At2g29760*, and *At1g51670* as *HTT3*, *HTT4*, and *HTT5*, respectively.

According to the recent annotations by The Arabidopsis Information Resource (http://www.arabidopsis.org), the *HTT* genes encode 127– to 329–amino acid proteins of unknown function. *HTT1* and *HTT4* share 50% identity in amino acid sequences. HTT1 shows 41% identity with HTT2 and 21% identity with HTT5. We chose *HTT1* and *HTT2* for further experiments as they were remarkably heat inductive in both microarray analysis and real-time PCR experiments.

To examine whether the TAS1-siRNAs are affected by heat stress, we performed small RNA gel blotting using 7-d-old seedlings exposed to heat shock at 37°C for 0.5 to 4 h. The *TAS1* siR480(+)/siR255 probe generated weaker signals in seedlings exposed to HS for 0.5 h than in the NT seedlings (Figure 1C). These signals should represent the overall accumulation of siR480(+)/siR255, siR396(+), and siR438(+), which increased from 0.5 to 1 h and decreased from 2 to 4 h. For accuracy, qRT-PCR was employed to test the expression of mature siR480(+)/siR255. With heat shock for 0.5 h, siR480 (+)/siR255 expression was weaker in seedlings with HS than with NT, consistent with the RNA gel blotting results (Figure 1D). These experiments revealed that high temperature inhibited the production of TAS1-siRNAs.

We linked *HTT* gene expression to TAS1-siRNA accumulation at high temperature. With HS for 0.5 h, the *HTT* genes were upregulated, while the accumulation of TAS1-siRNAs decreased. To examine whether heat-induced expression of *HTT* genes is dependent on TAS1-siRNAs, we analyzed the expression of TAS1-siRNAs and their targets in *rdr6-11* mutants, in which

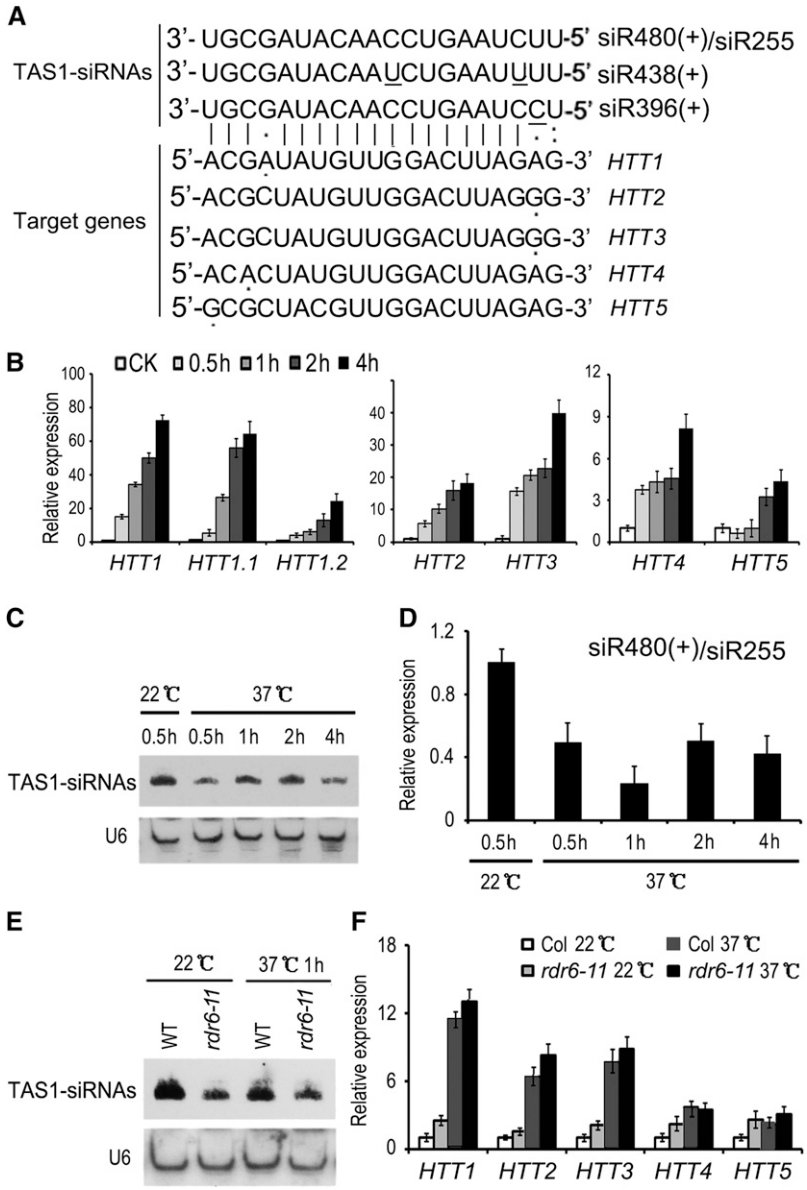

Figure 1. Expression Levels of TAS1-siRNAs and TAS1-Targeted Genes under Heat Shock.

(A) Alignment of three TAS1-siRNAs and the complementary regions in the *HTT* genes. The altered nucleotides in TAS1-siRNAs and the *HTT* genes are underlined and dotted, respectively.

(B) Real-time PCR showing relative expression of the *HTT* genes in the seedlings with heat shock (37°C for 0.5 to 4 h). *HTT1.1* and *HTT1.2* are the two alternative splicing variants of *HTT1*.

(C) RNA gel blotting showing accumulation of TAS1-siRNAs in the plants after heat shock at 37°C for 1 h, using the probe of siR480(+)/siR255.

(D) Real-time PCR showing the expression of siR480(+)/siR255 in the plants after heat shock (37°C for 0.5 to 4 h).

(E) RNA gel blotting showing accumulation of TAS1-siRNAs in *rdr6-11* seedlings with and without heat shock (37°C for 1 h).

(F) Real-time PCR showing the relative expression of the *HTT* genes in *rdr6-11* seedlings with and without heat shock (37°C for 1 h). The RNA samples for RNA gel blotting and real-time PCR were isolated from 7-d-old Col plants. Three biological replicates were taken for real-time PCR. Error bars indicate SD. Noncoding U6 RNA was used as the loading control.

biosynthesis of ta-siRNA was blocked (Olmedo-Monfil et al., 2010). TAS1-siRNA accumulation in *rdr6-11* seedlings with NT was much lower than in the wild-type seedlings (Figure 1E), confirming a previous report that RDR6 is required for the biogenesis of TAS1-siRNAs (Vazquez et al., 2004). Importantly, the *rdr6-11* seedlings did not show an apparent decrease in TAS1-siRNA accumulation under HS conditions compared with those under NT conditions, in contrast with the wild-type seedlings, which displayed a sharp decrease under HS conditions. This implied that the heat stress response of TAS1-siRNAs is dependent on *RDR6*.

In the *rdr6-11* seedlings, the expression levels of all *HTT* genes (except *HTT5*) were much higher than in the wild type under both HS and NT (Figure 1F), suggesting an inverse relationship between *HTT* gene expression and TAS1-siRNA accumulation. Under HS conditions, upregulation of the *HTT* genes in the *rdr6-11* seedlings was nearly as strong as in the wild-type seedlings, meaning that some elements beyond TAS1-siRNAs affect the activities of the *HTT* genes.

All *HTT* genes had a conserved domain complementary to TAS1-siRNAs (Figure 1A). To examine the temporal and spatial expression patterns of TAS1-siRNAs and their target genes, we performed RNA gel blotting of TAS1-siRNAs and real-time RT-PCR of *HTT1* and *HTT2* using RNA samples from various tissues at different stages. TAS1-siRNA accumulation in stems, leaves, inflorescences, and siliques was uniform and much higher than in intact seedlings (Figure 2A). By contrast, the expression of *HTT1* and *HTT2* in the individual tissues examined was slightly weaker than in intact seedlings (Figure 2B). *HTT3*, *HTT4*, and *HTT5* were constitutively expressed in all tissues (Supplemental Figure 1).

To investigate the expression patterns of *HTT* genes, we fused the promoters of *HTT1* and *HTT2* with the β-glucuronidase (*GUS*) gene. In the seedlings of the resultant transgenic plants, the GUS signals of *HTT1:GUS* and *HTT2:GUS* were seen in all tissues (Supplemental Figure 2).

To determine the subcellular localization of the HTT1 and HTT2 proteins, we fused the enhanced green fluorescent protein (EGFP) reporter to both genes at their N termini under the control of the 35S promoter and transiently expressed them in onion (*Allium cepa*) epidermal cells and *Nicotiana benthamiana* leaves. In onion epidermal cells, EGFP-HTT1 and EGFP-HTT2 signals were detected in both the cytoplasm and nucleus (Figure 2C). In *N. benthamiana* leaves, similar EGFP-HTT1 and EGFP-HTT2 signals were also observed (Supplemental Figures 3A and 3B). This indicated that the HTT1 and HTT2 proteins are localized in both cytoplasm and nucleus. Observations of leaf and root cells of *Arabidopsis* plants overexpressing *EGFP-HTT1* and *EGFP-HTT2* supported this conclusion (Supplemental Figures 3C to 3E). These plants showed the enhanced thermotolerance (Supplemental Figure 3F), thus indicating that EGFP-HTT1 and EGFP-HTT2 were functionally normal in planta.

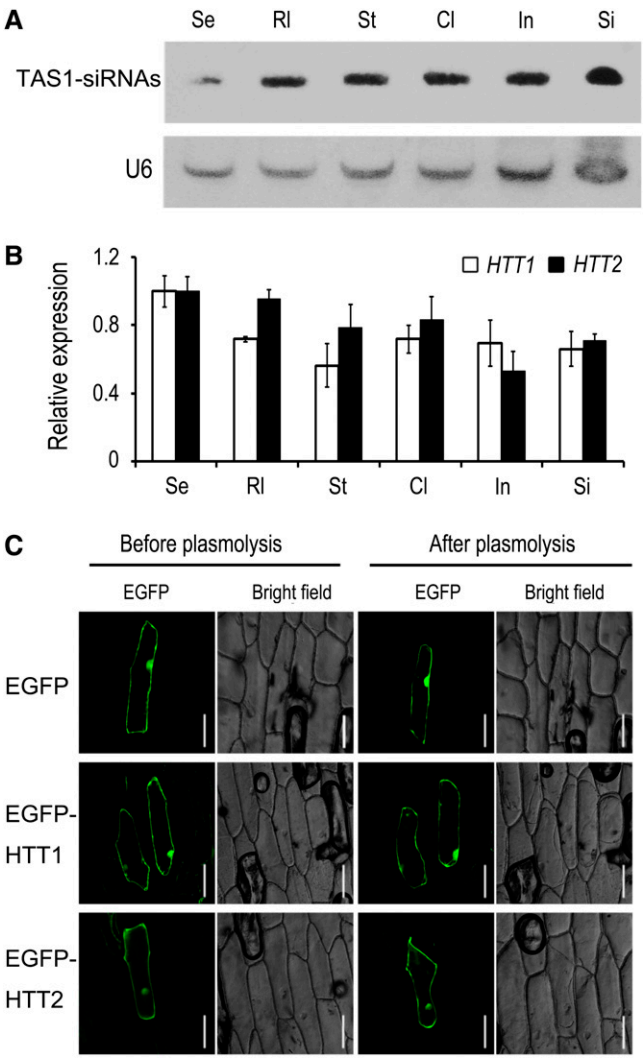

Figure 2. Spatial and Temporal Expression Patterns and Subcellular Localization of *HTT1* and *HTT2.*

(A) RNA gel blotting showing accumulation of TAS1-siRNAs in seedlings and different tissues. Se, seedlings; Rl, rosette leaves; St, stems; Cl, cauline leaves; In, inflorescences; Si, siliques.

(B) Real-time PCR showing expression levels of *HTT1* and *HTT2* in seedlings and different tissues.

(C) Subcellular localization of EGFP, EGFP-HTT1, and EGFP-HTT2 proteins in onion epidermal cells. EGFP and EGFP-HTT fusions were driven by the cauliflower mosaic virus 35S promoter. Three biological replicates were taken for real-time PCR. Bars = 200 μm.

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Overexpression of *TAS1a* Causes Higher Sensitivity to Heat Stress

TAS1 transcripts are noncoding RNAs from which TAS1-siRNAs, which target the *HTT* genes, are derived (Vazquez et al., 2004). To investigate the expression patterns of *TAS1* genes, we fused the promoters of *TAS1a*, *TAS1b*, and *TAS1* with the *GUS* gene. In the seedlings of the resultant transgenic plants, the GUS signals of *TASa:GUS*, *TASb:GUS*, and *TAS3:GUS* were visible in all tissues except hypocotyls (Supplemental Figure 2). In the leaves, GUS signals were preferentially expresses in the primary veins. In the inflorescences, GUS signals were strong in sepals, petals, stamens, and carpels, among which the GUS signal was strongest in stamens. This result showed that the three *TAS1* genes had the same spatial expression pattern.

There are two T-DNA insertion lines of *HTT1* (SALK-102179) and *HTT2* (SALK-056482), but neither is a loss-of-function mutant. Considering that TAS1-siRNAs are processed from a *TAS1* precursor through the cleavage of miR173, we constructed *p35S:MIR173* and *p35S:TAS1a* constructs and cotransferred them into *Arabidopsis* plants. The resulting transgenic plants with co-overexpression of *MIR173* and *TAS1a* were designated as *oeTAS1a*. In total, we selected 11 lines of *oeTAS1a*. In *oeTAS1a* plants, TAS1-siRNAs were overaccumulated (Figure 3A), whereas *HTT1* and *HTT2* were downregulated more than 5-fold (Figure 3B), revealing that the *TAS1a* gene silenced *HTT1* and

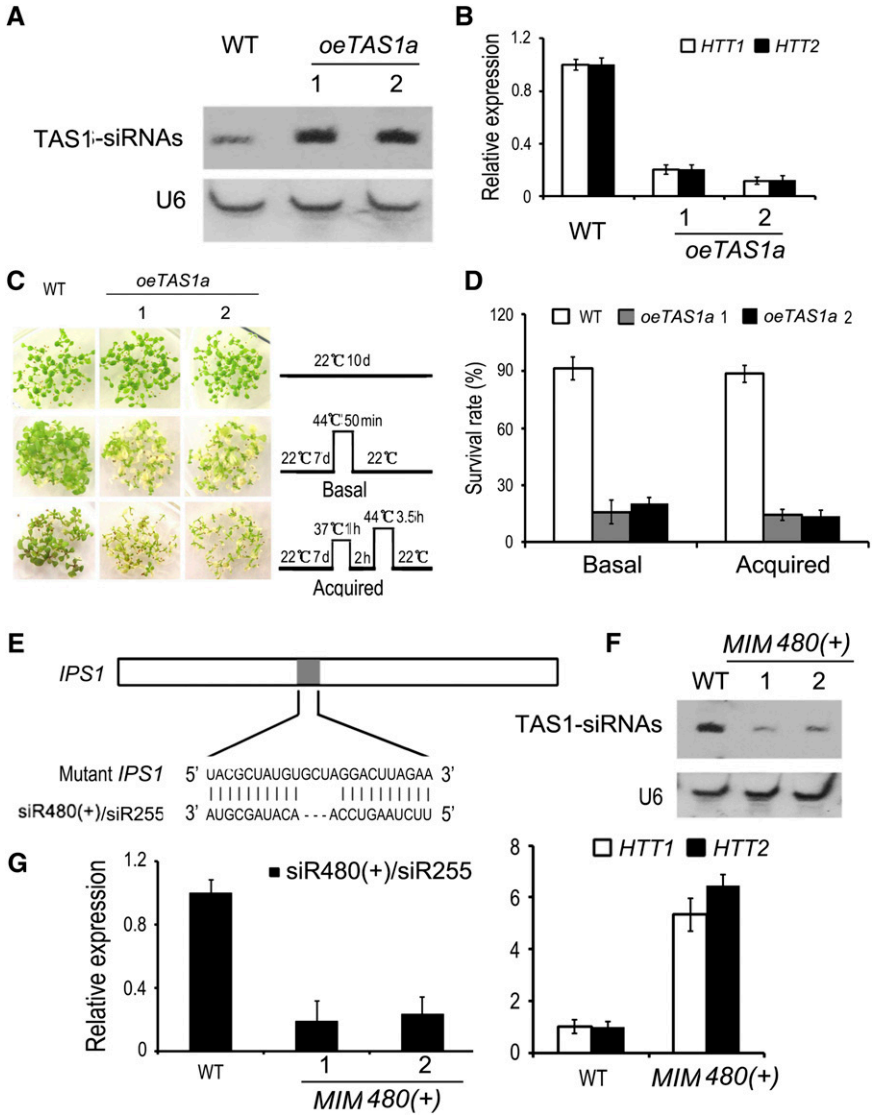

Figure 3. TAS1-siRNA Accumulation, *HTT* Gene Expression, and Thermotolerance in *oeTAS1a* and *MIM480(+)* Plants.

(A) RNA gel blotting showing TAS1-siRNA accumulation in *oeTAS1a* plants (transgenic plants co-overexpressing *MIR173* and *TAS1a*).
(B) Real-time PCR showing the relative expression of *HTT1* and *HTT2* in *oeTAS1a* plants.
(C) The *oeTAS1a* seedlings after heat treatments for basal and acquired thermotolerance detection. For the same heat treatment, the wild-type and transgenic seedlings shown were treated in different plates under the same conditions. Treatment conditions are shown on the right.
(D) Survival rates of *oeTAS1a* seedlings after heat treatments for basal and acquired thermotolerance detection (n = 60 to 100).
(E) Construction of a mimicry sequence for siR480(+)/siR255 [*MIM480(+)*] in the frame of miR399 of *IPS1* (Franco-Zorrilla et al., 2007).
(F) RNA gel blotting showing TAS1-siRNA accumulation in *MIM480(+)* plants (transgenic plants expressing *35S:MIM480(+)*.
(G) Real-time PCR showing relative expression of siR480(+)/siR255 and the *HTT* genes in *MIM480(+)* plants. The seedlings used for heat shock, real-time PCR, and RNA gel blotting were 7 d old. Error bars indicate SD of >30 seedlings.
[See online article for color version of this figure.]

HTT2. For identification of basal thermotolerance, 7-d-old plants were exposed to 44°C for 50 min and then grown at 22°C for 5 d. Under these conditions, 91% of the wild-type plants survived, showing normal phenotype, while only 13% of the *oeTAS1a* plants survived (Figures 3C and 3D), showing remarkable leaf bleaching. For identification of acquired thermotolerance, the plants were exposed to 37°C for 60 min, cooled at 22°C for 2 h, and subsequently transferred to 44°C conditions for 3.5 h and cooled again at 22°C for 5 d. Under these conditions, 89% of the wild-type plants survived, while 13 to 20% of the *oeTAS1a* plants survived. These experiments indicated that the *TAS1a* gene negatively regulates *HTT1* and *HTT2* and reduces both basal and acquired thermotolerance in the transgenic plants.

Overexpression of *HTT1* and *HTT2* Enhances Heat Tolerance

To verify the function of the *HTT1* and *HTT2* genes in heat stress response, we used an artificial miRNA target mimicry technique (Franco-Zorrilla et al., 2007). We modified the miR399 complementary motif of *IPS1* (*INDUCED BY PHOSPHATE STARVATION1*) to mimic target sites for siR480(+)/siR255 (Figure 3E) and transferred it into *Arabidopsis*. In *35S:MIM480(+)* [*MIM480(+)*] plants, TAS1-siRNA accumulation was much lower than in the wild type (Figure 3F), and the expression levels of *HTT1* and *HTT2* mRNAs increased, in agreement with previous reports on the negative effect of target mimicry on miRNA accumulation (Todesco et al., 2010; Yan et al., 2012). This suggested that *MIM480(+)* was antagonistic to TAS1-siRNAs. Real-time PCR using siR480 (+)/siR255-specific primers showed that siR480(+)/siR255 expression in *MIM480(+)* plants was reduced about 6-fold compared with in the wild type (Figure 3G). Importantly, the basal and acquired thermotolerance in *MIM480(+)* was remarkably enhanced because hypocotyls became longer and leaf bleaching was less in *MIM480(+)* plants than in the wild type (Figure 4D). The survival rates of *MIM480(+)* plants were much higher than those of the wild type (Figure 4E). These results indicated that in *MIM480 (+)* plants, TAS1-siRNA accumulation decreases, *HTT* gene expression increases, and thermotolerance is enhanced.

To verify the roles of the *HTT* genes in thermotolerance, we generated transgenic plants expressing the *rHTT1* and *rHTT2* [siR480(+)/siR255-resistant versions of *HTT1* and *HTT2*] under the control of the 35S promoter (Figure 4A). In *35S:rHTT1* (*rHTT1*) and *rHTT2* lines, *HTT1* and *HTT2* were apparently overexpressed (Figure 4B), hypocotyls became longer, and both basal and acquired thermotolerance was much stronger than in the wild type (Figures 4C and 4D). Survival rates of *rHTT1* and *rHTT2* plants at HS were higher than 88% (Figure 4E), in contrast with that of the wild type.

Compared with *rHTT1* and *rHTT2* plants, the *MIM480(+)* plants showed stronger resistance to heat stress (Figures 4C to 4E). In the *MIM480(+)* plants, the hypocotyls were longer and the survival rates were slightly higher than in *rHTT1* and *rHTT2* plants. Altogether, these results indicated that *HTT1* and *HTT2* are required for both basal and acquired thermotolerance.

HTT1 and *HTT2* Act Downstream of HsfA1a

HTT1 and *HTT2* may be activated by some heat stress response factors at the transcriptional level. The Hsfs are central to the heat stress response in plants (Nover and Scharf, 1997; Schöffl et al., 1998; Kotak et al., 2007). To address this question, we analyzed the expression levels of *HTT1* and *HTT2* in the *hsfA1a hsfA1b* double mutant and *35S:HsfA1a* (*HsfA1a*) plants with and without heat stress. The expression levels of *HTT1* and *HTT2* increased in *HsfA1a* plants at NT (22°C) compared with the wild type but decreased in *hsfA1a hsfA1b* plants (Figure 5A). However, at high temperature (37°C; HT), *HTT1* and *HTT2* were strongly upregulated in *HsfA1a* plants, whereas they were moderately arrested in *hsfA1a hsfA1b* plants. These results implied that *HTT1* and *HTT2* act downstream of *HsfA1a* and *HsfA1b*. To examine whether *HsfA1a* regulates *HTT* gene expression via TAS1-siRNAs, we performed RNA gel blotting of TAS1-siRNAs in the *hsfA1a hsfA1b* and *35S:HsfA1a* plants exposed to HT. An effect of *HsfA1a* on the abundance of siR480(+)/siR255 was not found (Figure 5B). In addition, we generated transgenic plants overexpressing *MIM480(+)* in the *hsfA1a hsfA1b* mutants and tested them for basal thermotolerance at 44°C. The survival rate (87%) of *hsfA1a hsfA1b MIM480(+)* plants increased greatly compared with that of *hsfA1a hsfA1b* double mutants (Figures 5C and 5D). This result revealed that *MIM480(+)* is epistatic to the *hsfA1a* and *hsfA1b* alleles and the siR480(+)/siR255-targeted *HTT* genes act downstream of *HsfA1a* and *HsfA1b*.

HsfA1s Activate *HTT1* and *HTT2* through Binding to Their Promoters

HsfA1a, *HsfA1b*, and *HsfA1d* work redundantly as the master regulators of the heat shock response (Lohmann et al., 2004; Liu et al., 2011a). To determine whether the *HTT* genes are in thermotolerance pathways of HsfA1a, we investigated possible protein binding sites in the promoters of *HTT1* and *HTT2*. The sequences of 3 kb upstream of the transcriptional start sites and 5′ untranslated regions (UTRs) were analyzed, and some heat shock elements (HSE:nnGAAnnTTCnnGAAnn) and AGGGG consensus sequences that are considered to be binding motifs of *Hsf* genes were identified (von Koskull-Döring et al., 2007; Guo et al., 2008). There are four homologs of *HsfA1* genes, including *HsfA1a*, *HsfA1b*, *HsfA1d*, and *HsfA1e* in *Arabidopsis*, which likely encode the master regulators of heat shock response genes in *Arabidopsis* (Liu et al., 2011a; Yoshida et al., 2011). We used an inducible expression system based on posttranscriptional activation of the rat glucocorticoid receptor (GR) and transferred a fusion of GR-HsfA1a to *Arabidopsis* plants. The expression levels of *HTT1* and *HTT2* were remarkably increased in *GR-HsfA1a* seedlings treated with the hormone ligand dexamethasone (DEX) (Figure 6A). After treatment with DEX and the translation inhibitor cycloheximide (CHX) for 6 h, the levels of *HTT1* and *HTT2* transcripts increased compared with mock-treated controls. CHX is used to block the translation of mRNAs regulated by HsfA1a (Schneider-Poetsch et al., 2010) and thus prevents secondary effects. This result indicated that *HTT1* and *HTT2* are likely direct targets of HsfA1a.

Hsfs positively regulate downstream genes by directly binding to the conserved HSE and AGGGG motifs in the gene promoters (von Koskull-Döring et al., 2007; Guo et al., 2008). Within the 1.5-kb promoters and 5′ UTRs of *HTT1* and *HTT2*, there are two and three HSE or AGGGG motifs, respectively. To identify whether they are bound by HsfA1a and HsfA1b in vitro, we examined 100-bp segments containing HSE or AGGGG motifs in the *HTT* promoters (Figure 6B). By electrophoretic mobility shift assay (EMSA), we demonstrated the direct binding of HsfA1a and HsfA1b to the *HTT1* and *HTT2* promoter sequences, respectively. Specifically, HsfA1a and HsfA1b bound to the S2 region of *HTT1* and S3 region of *HTT2*, respectively, which contained HSE motifs (Figures 6C to 6F). To confirm that HsfA1a directly interacts with *cis*-regulatory sequences at the *HTT1* and *HTT2* loci in vivo, we performed a chromatin immunoprecipitation (ChIP) assay using a transgenic line expressing a fusion of 6×Myc to HsfA1a under the control of the 35S promoter in the *hsfA1a hsfA1b* mutant. Chromatin extracted from 7-d-old seedlings with and without heat treatment at 37°C was

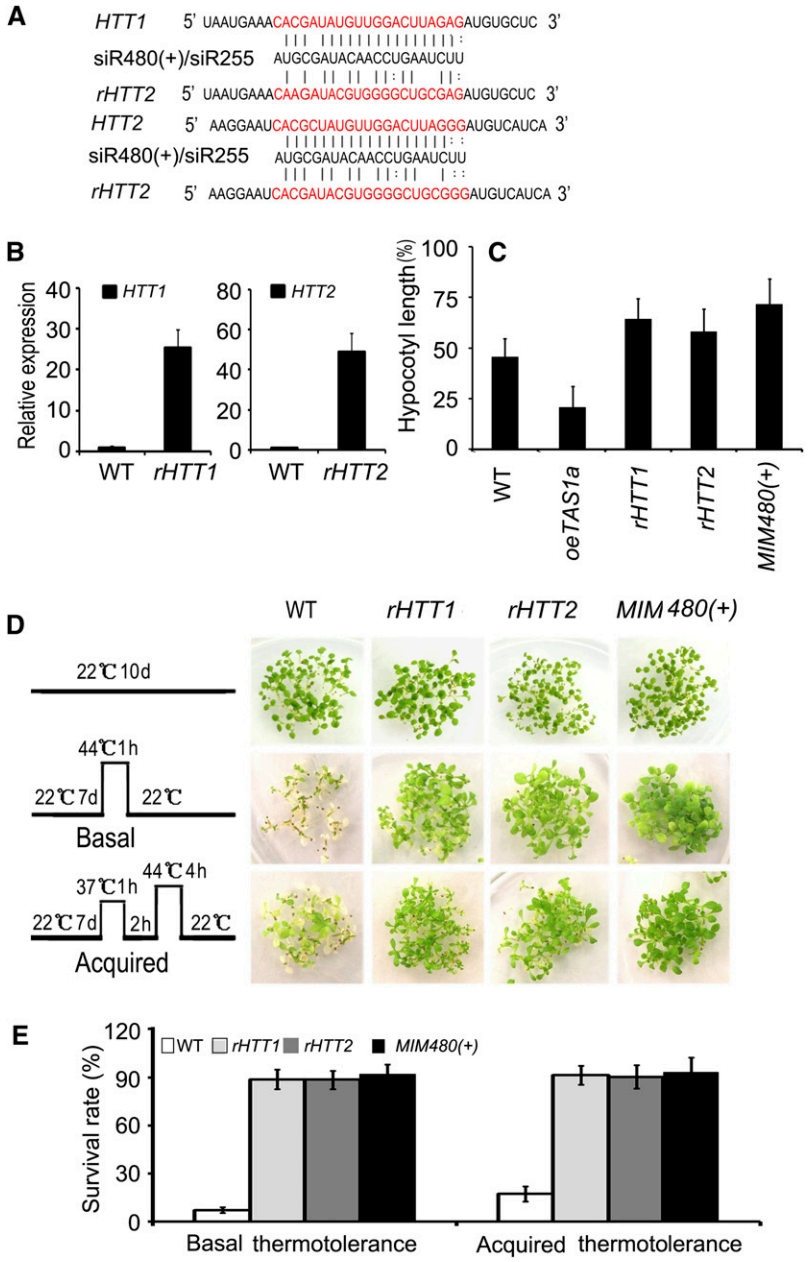

Figure 4. Comparison of Thermotolerance in *oeTAS1a*, *rHHT1*, *rHTT2*, and *MIM480(+)* Plants.

(A) Alignment of siR480(+)/siR255 with the complementary regions of the *HTT* genes and *rHTT1* and *rHTT2* constructs.
(B) Real-time PCR showing relative expression of *HTT1* and *HTT2* in *rHTT1* and *rHTT2* seedlings. Error bars represent the SD.
(C) Hypocotyl length of *oeTAS1a*, *rHTT1*, *rHTT2*, and *MIM480(+)* seedlings after heat shock (44°C for 1 h). Error bars represent the SD (n = 60 to 100).
(D) *rHTT1*, *rHTT2*, and *MIM480(+)* seedlings after heat treatments for basal and acquired thermotolerance detection. For the same heat treatment, the wild-type and transgenic seedlings shown were treated in different plates under the same conditions. Treatment conditions are shown on the left.
(E) Survival rates of *rHTT1*, *rHTT2*, and *MIM480(+)* seedlings after heat treatments for basal and acquired thermotolerance detection (n = 60 to 100). Error bars indicate SD of >30 seedlings.
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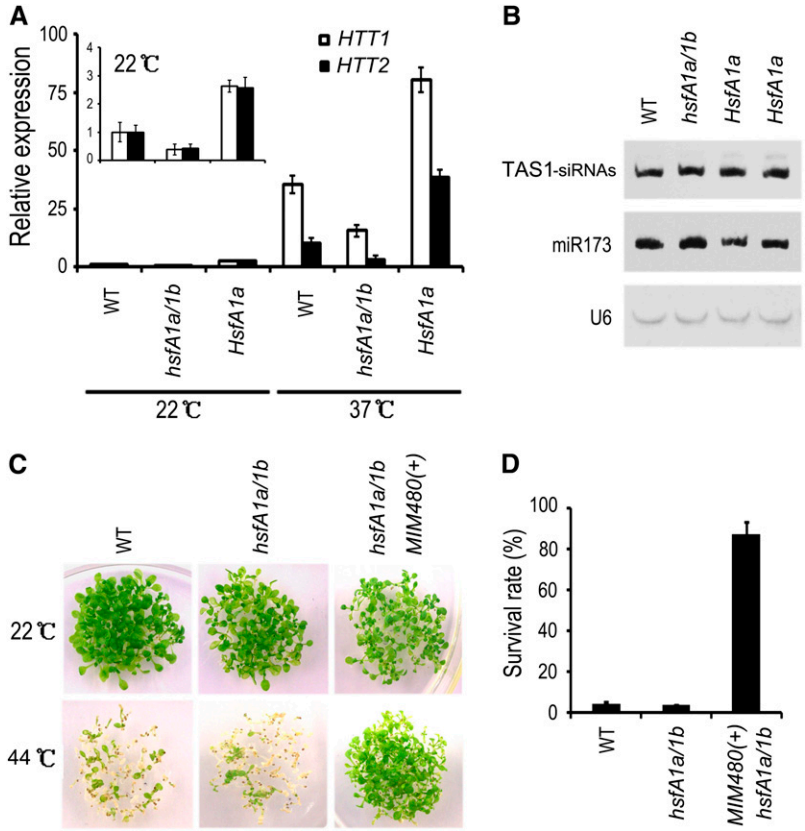

Figure 5. Genetic Relationship between *HsfA1a* and *HTT* Genes.

(A) Real-time RT-PCR showing relative expression of *HTT1* and *HTT2* in *hsfA1a/1b* and *HsfA1a* plants with and without heat shock (37°C for 1 h). The inset shows a magnification of the region below. Error bars represent the SD.

(B) RNA gel blotting showing accumulation of siR480(+)/siR255 in *hsfA1a/1b* and *HsfA1a* plants.

(C) Wild-type (Wassilewskija), *hsfA1a/1b*, and *hsfA1a/1b MIM480(+)* seedlings after heat shock (44°C for 1 h). One-week-old seedlings were exposed to 44°C for 1 h. For the same heat treatment, the wild-type and transgenic seedlings shown were treated in different plates under the same conditions.

(D) Survival rates of *hsfA1a/1b* and *MIM480(+) hsfA1a/1b* seedlings after heat shock (44°C for 1 h). Error bars represent the SD (n = 60 to 100).

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immunoprecipitated with anti-Myc antibodies, and the abundance of 100-bp regions containing the HsfA1a binding sequence (Figure 6B) was measured using quantitative PCR. There was no apparent enrichment of any segments in *35S:Myc* seedlings (Figures 6G and 6H). By contrast, segments S1-S3 of *HTT1* were significantly enriched in *Myc-HsfA1a* seedlings after pull-down with anti-Myc before or after heat treatment. This indicated that HsfA1a was able to bind to the promoter region and coding region of *HTT1* (Figure 6G). Meanwhile, a 6-fold increase in the S3 and S4 segments of *HTT2* was detected in *Myc-HsfA1a* seedlings at HT, compared with those of NT. This revealed that HsfA1a was only bound to the HSE motif of *HTT2* under heat stress (Figure 6H). These results confirmed that *HTT1* and *HTT2* are direct targets of HsfA1a. However, activation of *HTT1* by HsfA1a appeared to be more important than to *HTT2*.

The *HTT* Genes Positively Regulate the *Hsf* Genes

Although *HTT1* and *HTT2* act downstream of *HsfA1a* in the thermotolerance pathway, we wondered whether HTT proteins were able to regulate expression of *Hsf* genes. To address this question, we investigated the expression patterns of the 21 *Hsf* genes in transgenic plants overexpressing the *HTT* genes at NT and HT. The expression levels of *HsfA1a* and *HsfA1b* genes in *rHTT1*, *rHTT2*, and *MIM480(+)* plants under NT condition were remarkably higher (>1.5-fold) than in the wild type (Figure 7A), whereas those of *HsfA1d*, *HsfA1e*, *HsfA3*, *HsfA7a*, and *HsfB2b* were not. At HT, all nine *Hsf* genes examined were upregulated in *rHTT1* and *MIM480(+)* plants. These results showed that *HTT1* and *HTT2* genes positively regulate almost all *Hsf* genes.

To clarify the silencing of the HTT genes by *TAS1* in the thermotolerance pathways, we analyzed the expression levels of the nine *Hsfs* genes in *oeTAS1a* and *MIR173* plants at HT. Real-time PCR showed that the expression levels of *HsfA1a*, *HsfA1b*, *HsfA1d*, and *HsfA2* decreased in *oeTAS1a* seedlings (Figure 7B) in contrast with those in *rHTT1*, *rHTT2*, and *MIM480(+)* plants. The expression levels of *HsfA1e*, *HsfA3*, *HsfA4c*, *HsfA7a*, and *HsfB2b* were not obviously altered in *oeTAS1a* plants. These results suggested that *TAS1* genes are involved in heat stress induction of *HsfA1a*, *1b*, *1d*, and *HsfA2* (at least).

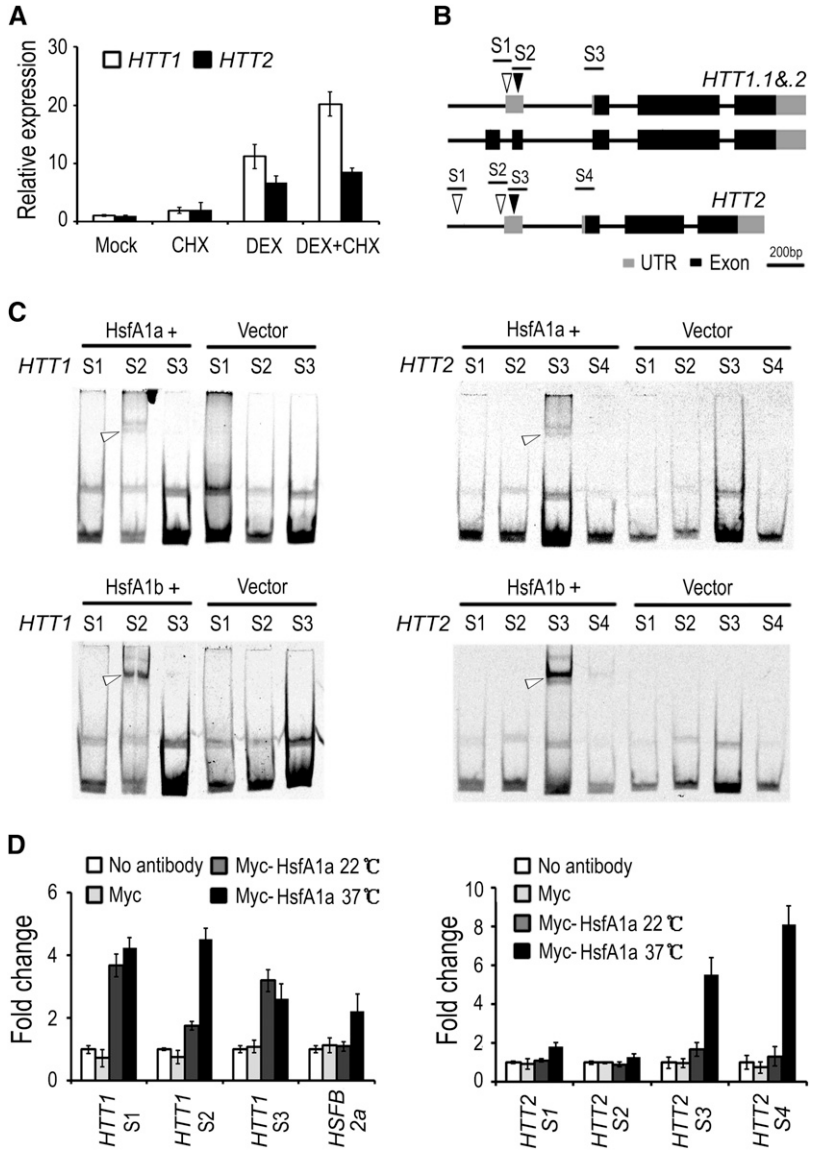

Figure 6. Binding of HsfA1a to *HTT1* and *HTT2* Promoter Regions.

(A) Real-time PCR showing the expression of *HTT1* and *HTT2* in *35S:GR-HsfA1a* seedlings treated with DEX in the absence or presence of CHX.
(B) Diagram of *HTT1.1*, *HTT1.2*, and *HTT2* genomic regions. The gray and black boxes indicate the UTR and exon regions, respectively. White and black triangles indicate the AGGGG and nnGAAnnTTCnnGAAnn motif, respectively, in the promoter and 5′ UTRs. Bar = 200 bp.
(C) EMSA showing the binding of *HTT1* and *HTT2* promoter regions by HsfA1a and HsfA1b. The position of HsfA1a/1b-DNA complexes is marked by a triangle.
(D) Real-time PCR after ChIP assays of *HTT1*, *HsfB2a* (positive control), and *HTT2* in *35S:Myc* and *35S:Myc-HsfA1a* seedlings with and without heat shock (37°C for 1 h). The seedlings used for real-time PCR and binding experiments were 7 d old. Relative enrichment of fragments was calculated by comparing samples treated with and without Myc antibodies. S1, S2, S3, and S4 represent the four segments in the promoter and 5′ UTR. Error bars indicate SD of >30 seedlings.

HTT1 Functions in the Hsp70-14 Complex

To identify the proteins that interact with HTT1 and HTT2, we used *HTT1* and *HTT2* as bait genes to query an *Arabidopsis* coexpression database using ATTED-II (Obayashi et al., 2009). Eighty-nine *Arabidopsis* genes associated with *HTT1* and *HTT2* were selected and used to identify interactions with HTT1 and HTT2 using a yeast two-hybrid assay. Considering the critical roles of Hsp proteins in heat stress and the subcellular localization of HTTs, we selected a small number of Hsps that localize to the nucleus and cytoplasm as the candidate genes for Y2H screening (Supplemental Table 1). We generated fusions of AD to HTT1 or HTT2 as bait and fused BD with the candidate genes. The two-hybrid screening showed that the NF-YC2 and Hsp70-14 proteins

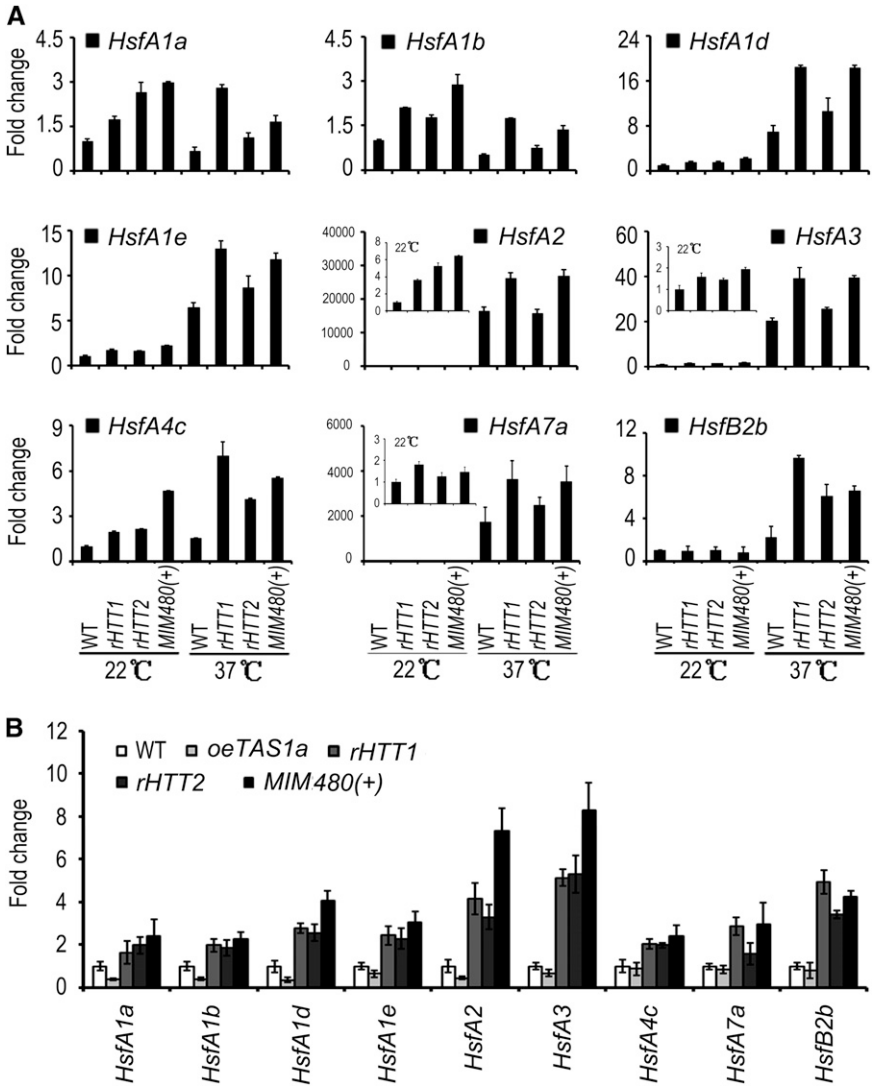

Figure 7. Expression of *Hsf* Genes in Transgenic Plants Overexpressing *HTT* Genes.

(A) Real-time PCR showing relative expression of *Hsf* genes in *rHTT1*, *rHTT2*, and *MIM480(+)* plants with and without heat shock (37°C for 1 h).
(B) Real-time-PCR showing relative expression of *Hsf* genes in *oeTAS1a*, *rHTT1*, *rHTT2*, and *35S:MIM480(+)* plants. Three biological replicates were used in these experiments. Error bars indicate SD of >30 seedlings.

could bind HTT1, but not HTT2. In addition, strong interactions were observed between Hsp70-14 and NF-YC2 and between Hsp70-14 and Hsp40 proteins (At2g33735) (Figure 8A).

Hsp70-14 belongs to the Hsp110/SSE subfamily because of its homology to yeast SSE1 and human Hsp110 (Lin et al., 2001). In yeast, SSE1 acts as an essential nucleotide exchange factor for Hsp70 activity (Raviol et al., 2006). Similarly, in plants, Hsp70 proteins do not function alone and act with cochaperones including J-proteins that stimulate the ATPase activity of Hsp70, thereby facilitating substrate capture (Mayer and Bukau, 2005). Thus, Hsp70-14 might be an important cochaperone for typical cytosolic Hsp70 proteins (Hsp70-1 through Hsp70-5). We analyzed the interaction between Hsp70-14 and Hsp70-1 through Hsp70-5 using Y2H, but no positive yeast cells grew in the selective medium.

NF-YC2 and *Hsp70*-14 show strong inducibility under heat stress (Jungkunz et al., 2011; Hackenberg et al., 2012). To identify the other factors involved in the interaction between HTT1 and Hsp70-14, we performed a bimolecular fluorescence complementation (BiFC) assay based on enhanced yellow fluorescent protein (EYFP). The full-length coding region sequences of *HTT1*, *Hsp70-14*, *Hsp40*, and *NF-YC2* were fused to the N- or C-terminal halves of EYFP. When both types of fusion proteins are transiently introduced into mesophyll protoplasts of *Arabidopsis*, protein–protein interaction between the tester proteins results in the proper folding of EYFP and its subsequent fluorescence can be detected in the coinfiltrated protoplasts. EYFP signals between HTT1, Hsp70-14, NF-YC2, and Hsp40 were observed in both the cytoplasm and nucleus,

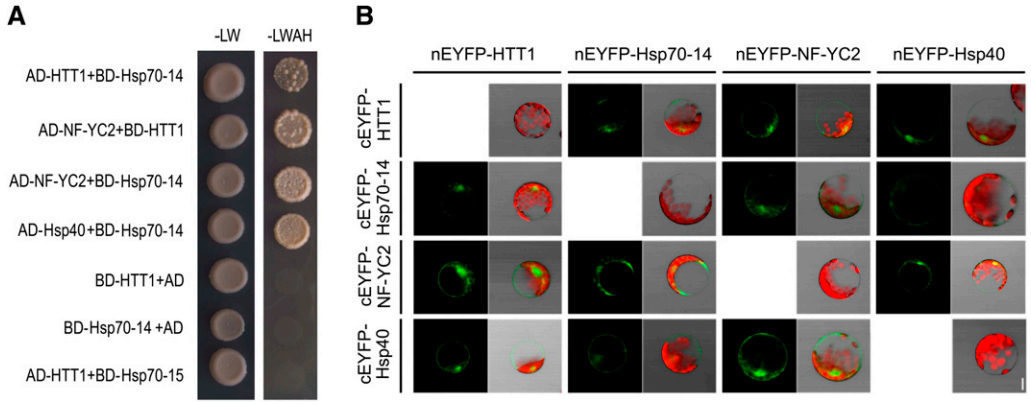

Figure 8. Direct Interaction between HTT1 and the Other Proteins.

(A) Yeast two-hybrid analysis of interactions between HTT1, NF-YC2, Hsp70-14, and Hsp40. Yeast cells containing two types of constructs were grown in medium -LW and then transferred to selective medium (-LWAH).

(B) Pairwise BiFC experiments between HTT1, Hsp70-14, NF-YC2, and Hsp40. Protein partners were fused to an N-terminal fragment or C-terminal fragment of EYFP, respectively, and cotransferred into *Arabidopsis* protoplasts. Bars = 40 μm.

whereas no BiFC signal was observed in self-interaction experiments (Figure 8B). These data suggest that HTT1 participates in the Hsp70-14-NF-YC2 complex during the heat stress response.

DISCUSSION

HTT1 and *HTT2* Are Involved in Thermotolerance

TAS1-siRNAs are heat-inhibited, whereas the *HTT* genes they target are heat inducible. This is an example of posttranscriptional silencing by ta-siRNAs in the heat response. An increasing number of reports demonstrate that miRNAs are key regulators of plant responses to nutrient homeostasis and to biotic and abiotic stresses (Sunkar and Zhu, 2004; Khraiwesh et al., 2012; Yu et al., 2012; Guan et al., 2013). The expression of miR393 is moderately downregulated by heat stress, while miR169 and miR398 are induced by heat stress (Guan et al., 2013). siR480(+)/siR255 has been reported to be responsive to various abiotic stresses, such as cold, drought, and NaCl (Sunkar and Zhu, 2004). However, the function of siR480(+)/siR255 and its targets in the heat response are unclear.

Transgenic plants overexpressing *rHTT1*and *rHTT2* and transgenic plants with *MIM480(+)* displayed enhanced basal and acquired thermotolerance, thus providing evidence for an inverse relationship between siR480(+)/siR255 and *HTT* genes, which play an important role in thermotolerance. In *Arabidopsis*, overexpression of *At5g18040* (*HTT2*) enhanced the oxidative stress response (Luhua et al., 2008), but the specific function of *HTT2* in the oxidative stress response is unclear. The signal transduction pathways of heat stress and oxidative stress are interconnected (Kotak et al., 2007). Production of H_2O_2 transiently increases after very short periods of exposure to high temperature as a result of NADPH oxidase activity (Vacca et al., 2004; Guan et al., 2013). In addition, the heat stress–induced H_2O_2 in turn increases transcripts of heat stress-responsive genes, and this process may be regulated by the sensing of H_2O_2 by Hsf proteins (Miller and Mittler, 2006; Volkov et al., 2006). In the loss-of-function mutants of *CSD1* and *CSD2*, which encode copper/zinc superoxide dismutase 1,2 and copper chaperone, respectively, altered redox status and increased resistance to heat stress are observed (Guan et al., 2013; Lu et al., 2013). Therefore, the enhanced thermotolerance in plants overexpressing *HTT2* may be related to the oxidative stress response via an unknown mechanism presumably involving altered expression of *Hsf* genes.

HsfA1a Directly Activates *HTT* Genes in a Positive Feedback Loop

HsfA1a/*1b* plays a redundant role in the heat stress response (Lohmann et al., 2004). In this work, DEX induction of *35S:GR-HsfA1a* enhanced the expression of *HTT* genes. Importantly, HsfA1a directly binds to the perfect HSE regions in the promoters of *HTT1* and *HTT2*. The difference is that HsfA1a binds to the *HTT1* promoter at either high or normal temperatures but binds to the *HTT2* promoter only at high temperatures. In heat-tolerant plants overexpressing *HsfA1a*, the *HTT* genes were upregulated, especially at high temperatures. These results suggest that HsfA1a directly activates *HTT* genes in the process of thermotolerance.

HsfA1 subfamily genes are master regulators of heat stress (Mishra et al., 2002; Liu et al., 2011a; Yoshida et al., 2011). *HsfA1* genes regulate more than 65% of heat shock protein genes, including a variety of small *Hsps*, Casein lytic proteinase B, *Hsp70*, *DnaJ* (also known as Hsp40) family, and some *Hsf* genes (Liu et al., 2011a). *HsfA2* is a secondary regulator that is in turn regulated by at least one master regulator to form a transcriptional cascade or network that covers the early and late expression of *Hsf* genes and was also shown to be involved in other environmental stress responses (Nishizawa et al., 2006; Banti et al., 2010). The question arises how *HTT* genes regulate secondary regulators such as *HsfA2*. Nine of the *Hsf* genes were expressed more strongly in plants overexpressing *HTT* genes, compared with the

wild type. They included *HsfA1a*, *HsfA1b*, *HsfA1d*, *HsfA1e*, *HsfA2*, *HsfA3*, *HsfA4c*, *HsfA7a*, and *HsfB2b*. The activation of *HSFA1* genes upregulates the transcription of *HsfA7a/7b*, *HsfA2*, *HsfB1*, and *HsfB2a* (Nishizawa-Yokoi et al., 2011; Yoshida et al., 2011). These transcription factors then act as secondary regulators of a subset of heat-induced genes (Schramm et al., 2008; Yoshida et al., 2008; Sugio et al., 2009). In this way, *HTT1* and *HTT2* induce secondary regulators of a subset of heat-induced genes by the promoting activities of *HsfA1* genes. Therefore, HsfA1 proteins activate *HTT1* genes at the transcriptional level, which in turn promotes *HsfA1* genes. This represents a typical positive feedback loop, in which the *HTT1* and *HTT2* genes are the important players.

HTT1 Function in Thermotolerance as a Cofactor of Hsp70-14 Complexes

As molecular chaperones, *Hsp70s* are upregulated during many forms of cellular stress and are essential during normal growth. Hsp70 directly interacts with HsfA1 and inhibits the binding of HsfA1 to HSE regions (Hahn et al., 2011). Constitutive overexpression of *Hsp70-1* enhanced the heat tolerance of transgenic plants (Sung and Guy, 2003; Cazalé et al., 2009). Plants expressing an antisense copy of a tobacco *Hsp70* driven by a heat-inducible promoter in *Arabidopsis* showed decreased thermotolerance (Lee and Schöffl, 1996). Disruption of the *Hsp70-2* gene causes lethality, since protein import into heat-shocked chloroplasts isolated from heat-sensitive *hsp70-2* mutants was appreciably impaired (Shi and Theg, 2010). Apparently, the members of *Hsp70s* function differentially in the plant heat stress response. Hsp70-14 and Hsp70-15 share 97% identity in amino acid sequences, with a difference of only 21 amino acids (Jungkunz et al., 2011). Hsp70-14 directly interacts with HTT1 rather than HTT2. This implies that the interaction of Hsp70-14 complexes with HTT genes is specific.

The transcription factor *NF-Y*, a CCAAT box binding factor encoded by large gene families in *Arabidopsis*, consists of the three subunits, A, B, and C, which are characterized by conserved protein domains. The C terminus of *NF-YA*, the central region of *NF-YB*, and the N terminus of *NF-YC* show sequence identities of more than 70% in stretches of 56, 90, and 84 amino acids among the *NF-Y* subunit homologs, respectively (Edwards et al., 1998; Gusmaroli et al., 2002; Hackenberg et al., 2012). Individual *NF-Y* genes are emerging as important regulators of several essential plant processes, including embryogenesis, drought resistance, maintenance of meristems in nitrogen-fixing nodules, and photoperiod-dependent flowering time (Kumimoto et al., 2010). *NF-YC2* shows strong inducibility under oxidative stress, such as photodynamic, light, oxidative, heat and drought stress, and NF-YC2-GFP fusions are localized in the nucleus (Hackenberg et al., 2012). The thermoregulated expression of soybean (*Glycine max*) *hsp17.3-B* is regulated via HSEs, but full promoter activity requires CCAAT box sequences located immediately upstream of the HSE-containing region (Rieping and Schöffl, 1992). Deletion of CCAAT box sequences reduces heat response gene expression, suggesting that CCAAT box sequences act cooperatively with HSEs to increase the heat stress promoter activity. Thus, it is possible that NF-YC2 trimmers, which bind to the CCAAT box, regulate heat stress response genes coordinately with Hsf proteins, which bind to HSE motifs. In our results, NF-YC2 and Hsp70-14 interacted in the cytoplasm and nucleus, indicating that Hsp70-14 might function as a chaperone protein and transfer NF-YC2 from the cytoplasm to nucleus, whereas HTT1 and Hsp40 possibly act as cochaperones to recruit and recognize substrates. Therefore, HTT1 may enhance thermotolerance through the Hsp70-14 and NF-YC2 complexes, although more detailed experiments are needed to confirm this.

HTT1 Mediates Thermotolerance in HsfA1a-Directed Pathways

According to the molecular relationship between TAS1-siRNAs, *HTT* and *HsfA1a* genes, and HTT1, HsfA1a, Hsf NF-YC2, and Hsp70-14 proteins, we propose a model for the function of *HTT* genes in thermotolerance pathways (Figure 9). Overexpression of a *TAS1* gene reduces thermotolerance, possibly through TAS1-siRNAs or RNA interference. This is important for genetic improvement of plant thermotolerance. Any method used to silence *TAS1* genes may trigger the production of many other ta-siRNAs as well. TAS1-siRNAs, siR396(+), siR480(+)/siR255, and siR438(+) may be involved in the silencing of the *HTT* genes. Overexpression of *HTT1* and *HTT2* enhances thermotolerance and can be utilized for crop breeding of heat-resistant varieties. Repression of TAS1-siRNAs is another approach that can be utilized. Target mimicry of siR480(+)/siR255 apparently destabilized the production of TAS1-siRNAs, upregulated *HTT* gene expression, and increased thermotolerance. Therefore, target

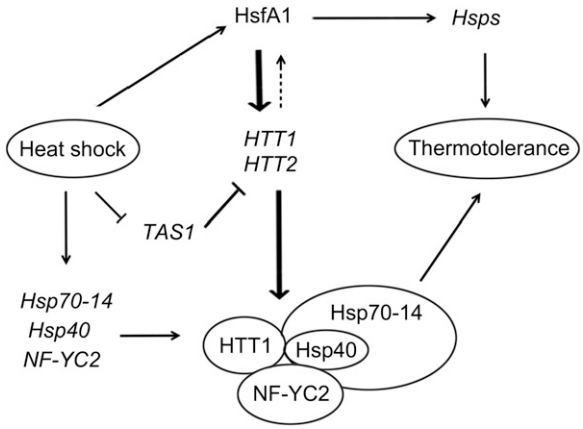

Figure 9. Schematic Model of the Function of *HTT* Genes in Thermotolerance.

Induction of *HTT* genes at high temperature is partially dependent on the activity of *TAS1* genes. On the other hand, *HTT* genes are transcriptionally activated by HsfA1a. The *HTT* genes upregulate a subset of *Hsf* genes, which in turn reinforces the strength of heat response genes such as *Hsps* for thermotolerance. During this process, HTT proteins may act as cofactors of the Hsp70-NF-YC2 complex. The dual functions of *HTT* genes provide an insight into the molecular mechanism of plant thermotolerance. The dashed line indicates an indirect effect, while the solid lines represent direct effects in which the thickness shows the relative importance of the pathways.

mimicry technology of TAS1-siRNAs has great potential. Further study of the relationship between the *HTT* genes and TAS1-siRNAs will facilitate genetic improvement of heat resistance in economically important crops.

METHODS

Plant Material and Growth Conditions

The *Arabidopsis thaliana* mutants *hsfA1a/1b* (Wassilewskija background) (Lohmann et al., 2004) and *rdr6-11* (Olmedo-Monfil et al., 2010**)**, and wild-type Wassilewskija and Columbia-0 were used in this study. The seeds of wild type and mutants were surface-sterilized in 70% ethanol for 1 min followed by 1% $NaClO_3$ for 10 min. Then, the seeds were washed four times in sterile distilled water, mixed in melted 0.1% water agar, and plated on top of solid Murashige and Skoog (MS) medium with 1% sucrose in Petri dishes. The Petri dishes were sealed with Parafilm, incubated at 4°C in darkness for 2 to 3 d, and then moved to a growth room and incubated at 22°C under16 h of light and 8 h of darkness per day. One week later, the seedlings were transplanted to peat soil in plastic pots and moved from a growth room to a growth chamber in the Phytotron at the Shanghai Institute of Plant Physiology and Ecology. In this growth chamber, the plants were grown at 22°C with 16 h of light per day under a light source of warm white fluorescent tubes. All of the seedlings were grouped randomly and grown under identical conditions for 6 weeks.

Sequence Alignments and ATTED-II Analysis

The nucleotide sequences between *HTT* genes and siR480(+)/siR255 were aligned using the GeneDoc program, version 2.6 (http://www.nrbsc.org/gfx/genedoc/index.html). For ATTED-II analysis, we chose *HTT1* and *HTT2* as query genes using the ATTED-II Network Drawer (http://atted.jp), which predicts regulatory networks based on coexpressed genes determined from microarray analyses (Obayashi et al., 2009).

Transgenic Plants

Fragments corresponding to precursor of *miR173*, *TAS1a*, full-length coding sequence of *HTT1*, *HTT2*, mutated *IPS1*, *HsfA1a*, and promoter of *TAS1a*, *TAS1b*, *TAS1c*, *HTT1*, and *HTT2* (primers shown in Supplemental Table 2) were inserted into pCAMBIA1301 or pCAMBIA2301 binary vectors (CAMBIA) and placed under the control of the cauliflower mosaic virus 35S promoter. The binary vectors were delivered into *Agrobacterium tumefaciens* strain GV3101 (pMP90RK) using the freeze-thaw method. The *Arabidopsis* plants (Columbia ecotype) were transformed using the floral dip method (Clough and Bent, 1998). For selection of transgenic plants, the seeds were sterilized and germinated on agar medium containing 25 mg/L hygromycin or 50 mg/L kanamycin. Seedlings exhibiting resistance to the herbicide hygromycin or to kanamycin were transplanted and grown in a greenhouse at 22°C under an 8-h light regime. The seeds from each transgenic plant were harvested separately for subsequent observation.

RNA Gel Blotting

Total RNA was extracted from the wild-type and transgenic plants. Antisense sequences of siR480(+)/siR255 were synthesized and end-labeled as probes with biotin (TaKaRa). A volume containing 50 to 80 μg RNA was separated on 19% polyacrylamide denaturing gels. The RNA was transferred to a Hybond membrane (Amersham Biosciences, GE Healthcare) for 2 h at 200 mA. After cross-linking by UV irradiation for 3 min, the Hybond membrane was hybridized with biotin-labeled DNA probes complementary to the predicted miRNA sequences at 42°C overnight Autoradiography of the membrane was performed using the Light Shift Chemiluminescent EMSA Kit (Pierce). RNA gel blot analysis was performed according to the methods of previous studies (Liu et al., 2011b).

GUS Staining

GUS staining was performed on the 14-d-old plants, leaves of 3-week-old plants, and inflorescences of 4-week-old plants. Leaves and inflorescences of the transgenic plants were placed in staining solution (50 mM Na_3PO_4, pH 7.0, 0.5 mM X-gluc [5-bromo-4-chloro-3-indolyl glucuronide], and 20% [v/v] methanol), vacuum infiltrated, and incubated at 37°C overnight as described previously (Yu et al., 2005). After staining, tissues were fixed in formalin–acetic acid–alcohol for further analysis.

qRT-PCR

Total RNA was extracted from seedlings or specific organs of wild-type and transgenic plants using TRIzol (Invitrogen) and treated with DNase I (TaKaRa) to remove DNA contamination. Approximately 4 μg of RNA was used for reverse transcription with oligo(dT) primers. Real-time PCR was performed with the Rotor-Gene 3000 system (Corbett Research) using SYBR Premix Ex Taq (TaKaRa) according to the manufacturer's instructions. Actin mRNA was used as an internal control. The sequences of primers are listed in Supplemental Table 2. The comparative threshold cycle (Ct) method was used to determine relative transcript levels in real-time PCR (MyiQ2 two-color real-time PCR detection system; Bio-Rad). Expression was normalized relative to that of *ACTIN.* Three biological replicates and three technical replicates were performed.

Yeast Two-Hybrid Interaction Assay

Full-length coding sequences of the candidate genes on the list were cloned into pGBKT7 bait vector (fused with Gal4 DNA binding domain [BD]). *NF-YC2* and *At2g33735* were then cloned into pGADT7 (fused with GAL4 activation domain [AD]) prey vector because of self activating in yeast cells selected in -LAH medium. Full-length coding sequences of *HTT1* and *HTT2* were amplified by PCR using KOD plus neo polymerase (TaKaRa) and cloned into pGBKT7 bait vector and pGADT7 prey vector. The following prey constructs were used: pGADT7-*AD-HTT1* (abbreviated *AD-HTT1*) and pGADT7-*AD-HTT2* (abbreviated *AD-HTT2*); the following bait construct was used: pGBKT7-BD-Candiates (abbreviated BD-candidate genes). The constructs were transformed into yeast strain AH109. For testing interactions between HTT1 and NF-YC2, At2g33735, combinations of BD-HTT1 and AD-NF-YC2, and AD-AT2G33735 constructs were coexpressed in yeast cells, respectively and selected in -LWAH medium. Each experiment was separately repeated three times.

Microarray Analysis

Wild-type Nossen ecotype seedlings were grouped randomly and grown under identical conditions for 3 weeks. Each group contained five seedlings and was designated as one biological replicate. Six biological replicates for each plant line were prepared, and three were used for array experiments. GeneChip array analysis was performed using Affymetrix ATH1 microarrays. Differentially expressed genes were identified from expression data acquired from six independent microarray hybridizations. Three replicates of wild-type reference RNA were used to calculate the expression values for each gene. For analysis of the expression profile, the fold changes in the normalized signals derived from three wild-type replicates were calculated. Only the fold changes of genes that met a significance criterion of $P < 0.05$ with a fold change of >1.5 are presented.

ChIP Assays

The *35S:6×Myc -HsfA1a* and *35S:6×Myc* transgenic plants were used in this assay. Seven-day-old seedlings with and without heat treatment (37°C 1 h) were cross-linked with 1% formaldehyde, and chromatin was isolated. ChIP was performed as described (Wang et al., 2009). Crude chromatin extract was split into three parts. One part was saved for the

input control. The other two were used for immunoprecipitation with 5 μL Myc antibodies (Sigma-Aldrich). After several washes, chromatin cross-linking was reversed, and DNA was purified. qRT-PCR analysis was performed on immunoprecipitated DNA using a MyiQ2 two-color real-time PCR detection system. Values for the Myc ChIP samples were first normalized to the input and then were divided by the normalized Myc signal to obtain fold enrichment. The sequences of the primers used to amplify different regions in the promoter are listed in Supplemental Table 2.

GR Induction and RNA Quantitation

GR induction was performed as described (Wu et al., 2009). *35S:GR-HsfA1a* seeds were plated onto MS medium. On day 7, the plates were flooded with 0.1% ethanol (mock), 10 mM DEX in 0.1% ethanol, 10 mM CHX in 0.1% ethanol, and 10 mM DEX plus 10 mM CHX in 0.1% ethanol. Seedlings were harvested after 6 h of treatment. Total RNA was isolated using Trizol (Invitrogen) and treated with RNase-free DNAase (TaKaRa). qRT-PCR was performed as described above. Primers used for qRT-PCR are described in Supplemental Table 1, and the experiment was separately repeated three times.

Thermotolerance Assays

For thermotolerance assays, seeds were sown on MS plates with 1% sucrose and incubated at 4°C for 2 d in the dark. After imbibition, the plates were moved to a growth room and incubated at 22°C under 16 h of light and 8 h of darkness per day. After 7 d, the plates were placed in a water bath (37°C) for 1 h for qRT-RCR materials. For the acquired thermotolerance assay, 7-d-old seedlings were acclimated at 37°C for 1 h, recovered at 22°C for 2 h, and then treated at 44°C for 3.5 to 4 h. For the basal thermotolerance assay, 7-d-old seedlings were directly treated at 44°C for 50 min or 1 h. After the different heat treatments, the plants were recovered at 22°C for 5 to 7 d. At the end of recovery, photographs were taken and the survival rates were measured.

EMSA

The *HsfA1a* and *HsfA1b* genes were cloned into the pET-28a(+) (Novagen) in *Escherichia coli* expression vector containing a C-terminal His-coding sequence. The fusion and empty constructs were transformed into *E. coli* cells, respectively. The fusion proteins were purified according to the manufacturer's procedure. DNA fragments from each of the *HTT1* and *HTT2* gene promoters were end-labeled with Cy5. Three micrograms of the recombinant fusion protein in binding buffer (10 mM Tris-HCl, pH 7.6, 25 mM KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 10% glycerol) was supplemented with 10 ng Cy5-labeled DNAs and incubated for 30 min at 25°C. The reaction mixtures were electrophoresed on 4.5% native polyacrylamide gels in 0.5× TBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA, pH 8.0) at 200 V for 2 h. Gels were directly exposed on Typhoon FLA 9000 (FUJIFILM FLA 9000 plus DAGE).

Stable and Transient Expression of EGFP-HTT Fusion Proteins

The whole coding sequences of *HTT1* and *HTT2* were fused with *EGFP* in the N terminus under the control of cauliflower mosaic virus 35S. The resulting constructs were used for transient transformation of onion (*Allium cepa*) epidermis via a gene gun (Bio-Rad) (Scott et al., 1999). For transient expression of fusions in *Nicotiana benthamiana* leaves, the constructs were first introduced into *Agrobacterium* strain GV3101 (pMP90RK) and then transiently transferred to *N. benthamiana* leaves mediated by *Agrobacterium* (Lavy et al., 2002). Stable transformation of *Arabidopsis* was described above. Transformed onion cells or *N. benthamiana* leaves were observed using an Olympus FV1000 confocal laser scanning microscope. Excitation was performed with an argon laser set to 488 nm, and emission was detected with a 543-nm band-path filter. Image analysis was performed with FV10-ASW 1.7 viewer and Adobe Photoshop 7.0.

BiFC Assays

The BiFC assay was performed according to Walter et al. (2004). Full-length coding regions of *HTT1*, *Hsp70-14*, *NF-YC2*, and *Hsp40* genes were amplified from *Arabidopsis* cDNA by PCR and cloned into BiFC vector pSET-n/cEYFP-c1 (Tzfira et al., 2005). The constructs were introduced into *Arabidopsis* protoplasts via polyethylene glycol–mediated transformation (Marion et al., 2008). After 12 h of incubation in the dark, the cells were observed using an Olympus FV1000 confocal laser scanning microscope described above.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: *HTT1*, *AT4G29770*; *HTT2*, *AT5G18040*; *HTT3*, *AT5G18065*; *HTT4*, *AT4G29760*; *HTT5*, *AT1G51670*; *Hsp70-14*, *AT1G79930*; *NF-YC2*, *AT1G56170*; and *Hsp40*, *AT2G33735*.

Supplemental Data

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

Supplemental Figure 1. Relative Expression of *HTT3*, *HTT4*, and *HTT5* in Different Organs of Plants.

Supplemental Figure 2. Temporal and Spatial Expression Patterns of the *TAS1* and *HTT* Genes in 1-Week-Old Seedlings.

Supplemental Figure 3. Subcellular Localization of HTT1 and HTT2 Proteins.

Supplemental Table 1. List of Genes Coexpressed with *HTT1* and *HTT2* (Predicted by ATTED-II).

Supplemental Table 2. Primer Sequences Used in This Study.

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AUTHOR CONTRIBUTIONS

S.L. and Y.H. designed and performed research. J.L., Z.L., X.L., and F.W. performed research. S.L. and Y.H. wrote the article.

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