ABSTRACT

Although much is known about plant responses to heat shock (HS), how plants sense high temperature and the primary HS signal transduction pathway leading to HS-regulated gene expression are still poorly understood. To identify primary transcription factors that mediate HS-regulated gene expression and their target genes, RNA-seq was performed to detect genes whose expression is rapidly altered by HS in *Arabidopsis thaliana*. The results showed several genes were induced after only 5 min of HS treatment, suggesting that HS signaling occurs very rapidly. Analysis of the cis-elements in the promoters of genes upregulated by 10 min of HS treatment identified HEAT SHOCK FACTOR A1s (HSFA1s) and circadian clock proteins REVEILLE4/8 (RVE4/8) as essential transcription factors that independently mediate early HS-induced gene expression. Using *hsfa1a/b/d/e* and *rve4/8* mutants, we identified subsets of HSFA1s- or RVE4/8-dependent early HS-induced genes, and showed RVE4/8 regulate plant thermotolerance partially by regulating the expression of downstream transcription factors ETHYLENE RESPONSIVE FACTOR53 (ERF53)
and ERF54, specifically around noon. These findings reveal a potential transcriptional regulatory hierarchy governing the first wave of HS-induced gene expression. They also provided important insight into the mechanism by which the circadian clock gates thermotolerance and prepares plants for exposure to high temperatures during the day.

INTRODUCTION

Temperature is one of the most important environmental factors affecting the diurnal and seasonal growth of plants, as well as their geographical distribution. Moderate increases in temperature above the optimum for plant growth (~28 °C for Arabidopsis thaliana) stimulate thermomorphogenesis, which is characterized by hypocotyl and petiole elongation, hyponastic leaf growth, and reduced leaf thickness (Koini et al., 2009; Quint et al., 2016). When the environmental temperature rises above the thermomorphogenesis-inducing temperature for extended periods, plants experience heat shock (HS) stress (Liu et al., 2015). HS stress has a profound impact on the plant life cycle, including suppressed seed germination (Hasanuzzaman et al., 2013), reduced fertility (Zinn et al., 2010), decreased photosynthetic efficiency (Bita et al., 2013), inhibited grain filling (Wahid et al., 2007), and, in extreme cases, cell or plant death (Balk et al., 1999). HS significantly reduces global crop yields (Lobell et al., 2011). Therefore, understanding the molecular mechanisms by which plants sense and respond to HS stress will profoundly affect the future security of global agricultural productivity.

Pre-exposure to nonlethal high temperatures (generally ~37 °C for Arabidopsis thaliana) for certain periods of time can increase plant tolerance to lethal high temperatures (generally 42 °C or higher), a process known as acquired thermotolerance (Mittler et al., 2012). During this process, exposure to nonlethal high temperature induces intracellular signaling pathways that alter gene expression, protein accumulation, and cellular metabolism to enhance plant tolerance to subsequent exposure to lethal high temperatures. In contrast to traditional chemical signals, which are kept outside the cell by the plasma membrane and usually require surface receptors to produce a cellular response, temperature is a physical signal; it reaches the cell surface and interior almost simultaneously. Therefore, cells can sense
changes in environmental temperature at multiple sites, including the plasma membrane and intracellular compartments, and initiate cellular responses through a variety of mechanisms. Accordingly, several signaling pathways have been proposed to regulate cellular responses to elevated environmental temperatures, including a PHYTOCHROME B- and PHYTOCHROME-INTERACTING FACTOR4 (PIF4)-dependent thermomorphogenesis pathway (Jung et al. 2016, Legris et al. 2016), a CNGC-calcium-calmodulin pathway (Liu et al., 2008; Saidi et al., 2009; Finka et al., 2012), a proteolytic cleavage-activated transcriptional activation pathway (Liu et al., 2007; Gao et al., 2008), a reactive oxygen species (ROS)-induced pathway (Mittler et al., 2011, 2012), and a H2A.Z-related pathway (Kumar and Wigge, 2010). Elevated temperatures also modulate cellular responses via phytohormone signaling and epigenetic mechanisms (Liu et al., 2015). However, because different studies used different high temperature and treatment durations, it is unclear whether high temperatures initiate these signaling pathways simultaneously, or whether there is a regulatory hierarchy in which a primary signaling pathway is activated by high temperature and subsequently affects other signaling pathways through crosstalk or transcriptional activation.

In general, signal transduction occurs very quickly. For example, plant exposure to brassinosteroids (BRs) for 5 min induced significant dephosphorylation of the transcription factor BRASSINAZOLE RESISTANT1 (BZR1) (Wang et al., 2016). HS-induced cellular responses in plants can also occur quickly. For example, treatment at 37 °C for 60 s increased the intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) in wheat sheath cells; [Ca\(^{2+}\)]\(_i\) continued to increase with increasing HS period until it reached a plateau at ~5 min (Liu et al., 2003). Similar results were also obtained in Arabidopsis (Gao et al., 2012) and moss (Saidi et al., 2009). Using two-dimensional difference gel electrophoresis, it was found that 5 min of treatment at 37 °C altered the abundance of at least 19 microsomal proteins in Arabidopsis seedlings (Wang et al. 2015). These HS-induced cellular responses occurred much more rapidly than those reported in many HS studies, in which plants were generally exposed to high temperatures for 30 min or longer. Meanwhile, 2 min of treatment at 37 °C induced the formation of a chromatin puff in Drosophila larvae; the size of the puff peaked within 5 min of heat treatment (Belyaeva and Zhimulev, 1976). Moreover, HS treatment at 42 °C for 2.5 min led to the up- or downregulation of numerous genes in
cultured mouse cells (Mahat et al., 2016). These findings indicate that HS can induce rapid transcriptional responses in various organisms. Exploring how quickly heat treatment regulates the expression of HS-responsive genes (HSRs) in plants would help identify the major transcription factors that mediate the first wave of HS-regulated gene expression and the cellular responses that are directly regulated by the primary HS signal transduction pathway.

In this study, we systematically analyzed the transcriptional regulatory network in Arabidopsis plants after exposure to HS at 37 °C using RNA sequencing (RNA-seq). We determined that HEAT SHOCK FACTOR A1 family proteins (HSFA1s), and the circadian clock proteins REVEILLE4 (RVE4) and RVE8, are among the primary transcription factors that mediate the first wave of HS-induced gene expression. In addition, we found that the mechanism by which RVE4/8 regulate thermotolerance in Arabidopsis around noon time involves the direct regulation of the expression of two downstream target transcription factors genes, ETHYLENE RESPONSIVE FACTOR53 (ERF53) and ERF54. Based on these results, a transcription regulatory hierarchy governing early HS-induced gene expression is proposed. Our study not only identified the transcription factors that regulate first wave of HS regulated gene expression, but also provide insight into how the circadian clock helps plants adapt to high temperatures during the day.

RESULTS

Identification of rapidly responding HS-sensitive genes by RNA-seq

To determine how quickly HS treatment alters gene expression and to identify genes that are directly regulated by the primary HS signaling pathway, Arabidopsis seedlings (ecotype Columbia [Col-0]) were grown in agar plates for one week. With lids on to prevent withering of the seedlings due to sudden humidity and temperature changes, the seedlings were exposed to 37 °C for 2.5, 5, 7.5, 10, 15, or 30 min and then snap-frozen in liquid nitrogen for total RNA extraction and RNA-seq. As shown in Supplemental Figure 1, under our treatment conditions, the temperature inside the plates reached 28.7±0.9 °C, 31±0.6 °C, 32.7±0.4 °C, 33.9±0.3 °C, 35.5±0.2 °C and 37.2±0.4 °C after HS treatment for 2.5, 5, 7.5, 10, 15, and 30 min, respectively.

Using a false discovery rate (FDR) < 0.05 and fold change of > 2 or < 0.5 as cut-offs,
we identified 3259 early HS-regulated genes (eHSRs; Supplemental Dataset 1),
including 938 upregulated and 2326 downregulated genes, whose expression was
differentially affected by HS treatment during at least one exposure period. Five genes
were initially downregulated by HS but subsequently upregulated as HS treatment
time increased to 30 min. These genes are included in both HS downregulated and
upregulated gene lists (Supplemental Dataset 1). Surprisingly, brief exposure to HS
(2.5 min) reduced the expression of 877 genes but increased the expression of only 16
genes (Figure 1A and Supplemental Dataset 1). However, the upregulation of these
genes was not detected in plants treated with HS for 5 min or longer. HS treatment for
5 min downregulated 722 genes and upregulated 6 genes. (Supplemental Dataset 1).

As the HS period increased, the number of HS-downregulated genes gradually
decreased while the number of HS-upregulated genes continue to increase (Figure 1A
and Supplemental Dataset 1). HS treatment for 10 min appeared to be a threshold
point; at 10 min, the number of HS-downregulated genes reached its lowest level,
while the numbers of HS-downregulated and -upregulated genes increased rapidly
beyond this point, possibly due to the activation of secondary or higher-order
transcriptional regulation. We then performed analysis using Dynamic Regulatory
Events Miner (DREM), which summarizes the time-dependent emergence of new
transcriptional regulatory events for groups of genes (Schulz et al., 2012). We
detected 13 dynamic paths of differential gene expression within 30 min of HS
treatment. Beginning at 10 min, the splitting of these nodes increased, most likely due
to sequential transcriptional regulatory events (Figure 1B, Supplemental Dataset 2).

Gene Ontology (GO) enrichment analysis of genes in these paths showed that paths
1–5, which included HS-upregulated genes, were enriched for the categories related to
abiotic stress responses, including responses to heat, temperature stimuli, high light,
reactive oxygen species (ROS), radiation, and protein folding. By contrast, paths 6–13,
which included HS-downregulated genes, were enriched for categories involved in the
regulation of plant growth and development, including cell surface receptor signaling,
floral development, protein phosphorylation, plant organ morphogenesis, and so on
(Figure 1C). These results suggest that HS induces the expression of stress-related
genes to increase plant resistance to elevated environmental temperatures while
suppressing the expression of growth- and development-related genes to ensure the
appropriate energy and metabolite allocation, thereby increasing the likelihood of
To further explore the potential transcriptional regulatory hierarchy that governs HS responses in plants, we constructed a gene co-expression network focusing on transcription factor-related eHSRs in DREM paths 1–5 as previously described (You et al., 2016). As observed in the network, as the HS treatment time increased, the number of gene clusters with similar HS-upregulated co-expression patterns increased, indicating that additional transcriptional events were activated (Supplemental Dataset 3 and Supplemental Figure 2). Correspondingly, the number of HS-upregulated transcription factor genes increased with increasing treatment time. For example, MBF1c was the only transcriptional regulator among the eHSRs that was upregulated by 5 min of HS treatment, whereas at 7.5 min of HS treatment, the expression of MBF1c, HSF2A, HSF7a, HSF2b, and ZAT7 increased. When HS treatment was extended to 10 min, 16 transcription factor genes were identified among the HS-upregulated eHSRs (Supplemental Dataset 1). Taken together, these results indicate that increasing numbers of transcription factors are sequentially activated in response to longer HS treatment times.

Housekeeping genes are generally used as internal controls to ensure equal loading of samples in gene expression studies. To identify good internal control genes for future HS-regulated gene expression studies, we examined the expression patterns of ACTIN2 (AT3G18780), UBC30 (AT5G56150), TUBULIN4 (AT1G04820), PP2A A3 (AT1G13320), and UBQ5 (AT3G62250), which have been used as internal controls in previous studies (Franklin and Whitelam, 2007; Baron et al., 2012; Mu et al., 2013; Im et al., 2014; Zhang et al., 2016), based on our RNA-seq data. Although none of these genes were on our list of eHSRs, UBQ5 and PP2A A3 expression increased ≥1.5 fold following at least one HS exposure period, whereas the expression of UBC30, ACTIN2, and TUBULIN4 was relatively stable (Supplemental Figure 3). To validate these results, we measured the relative expression levels of UBC30, ACTIN2, and TUBULIN4 by RT-qPCR. As shown in Supplemental Figure 3, ACTIN2 and UBC30 expression was relatively stable in HS-treated plants, indicating that these genes could be used as internal controls in early HS-regulated gene expression studies in Arabidopsis. We chose UBC30 as an internal control for subsequent RT-qPCR.

The first wave of HS-upregulated gene expression is partially mediated by HSFA1s.
Transcription factors recognize the cis-regulatory elements in the promoter regions of their target genes to regulate their spatial and temporal expression patterns. To identify the primary transcription factors that mediate eHSR expression under HS conditions, we identified 1000-bp genomic sequences upstream of the translation start codons (ATG) of the 3259 eHSRs and subjected them to MEME (Multiple Em for Motif Elicitation) analysis. Cis-elements recognized by multiple transcription factor family proteins, including WRKY, MYB, and NAC transcription factors, were highly enriched among HS-downregulated eHSRs, suggesting that these transcription factors are involved in the suppression of early HS-regulated gene expression. In addition, the WRKY binding motif was the most highly enriched motif among genes downregulated by HS treatment for 2.5, 5, 7.5, and 15 min and the second-most highly enriched motif among genes downregulated by 10 min of HS treatment. These findings suggest that WRKY family transcription factors might play critical roles in regulating the expression of early HS-downregulated genes (Supplemental Figure 4).

However, it is also possible that transcriptional complexes containing WRKY, MYB, or NAC might be heat labile; thus, these complexes could be quickly inactivated by HS, resulting in the downregulation of their target genes. As the HS-induced expression of HSF and HEAT SHOCK PROTEIN (HSP) genes is generally considered a critical cellular response that increases plant thermotolerance, we focused our attention on the early HS-upregulated genes.

Few genes were upregulated following 2.5, 5, or 7.5 min of HS treatment (Figure 1A); therefore, our MEME analysis did not identify any enrichment of cis-elements from these groups of eHSRs. Interestingly, 59% of genes that were up-regulated following 10 min of HS treatment contained the HSF-binding cis-element, HSE (heat shock element), in their promoters or 5'-untranslated regions (UTRs; Figure 2A). As the HS period increased to 15 or 30 min, the number of HS-upregulated genes increased dramatically (Figure 1A), whereas the percentage of eHSRs containing HSEs decreased (Figure 2A), supporting our hypothesis that a secondary or higher-order transcriptional regulatory mechanism emerged after 10 min of HS treatment. For eHSRs that were upregulated by 10 min of HS, many are likely the direct targets of the primary HS signal transduction pathway; these genes included 6 HSFs, 14 HSPs (most encoding small HSPs), and other genes involved in plant stress responses (e.g., heat stress, high light, wounding, salt stress), transcriptional regulation, protein
stability, ROS responses, or hormonal signaling (Figure 2B, Supplemental Dataset 1).

HSFs are highly conserved transcription factors that regulate HS responses in
eukaryotes (Scharf et al., 2012). The Arabidopsis genome contains 21 HSF genes, and
HSFA1 family transcription factors (HSFA1s) are master transcription factors that
regulate HS-induced gene expression (Liu et al., 2011; Yoshida et al., 2011). Based on
RNA-seq, we identified several eHSRs that were regulated by 10 min of HS
(including HSFA2, HSFA7a, HSFA7b, HSFB1, HSFB2a and HSFB2b, MBF1c, and
multiple small HSPs) that were previously shown to be directly regulated by HSFA1s
in plants treated with HS for 60 min (Liu et al. 2011; Yoshida et al., 2011). These
results suggest that HSFA1s are also master regulators of genes that are rapidly
upregulated by 10 min of HS treatment. However, MEME analysis showed that
approximately 40% of the eHSRs that are upregulated by 10 min of HS treatment do
not contain HSEs (Figure 2A), implying that additional primary transcription factors
(other than HSFA1s) are involved in regulating HS-induced gene expression.

To verify this, we examined the basal and acquired thermotolerance of hsfa1-qk
mutant, in which the expression of all four HSFA1 family transcription factors are
knocked out by transfer-DNA (T-DNA) insertions (Liu et al. 2011). Because the
hsfa1-qk mutant was generated by crossing mutants produced in the Col-0 and
Wassilewskija (Ws-2) backgrounds, we used both Col-0 and Ws-2 plants as controls
(Liu et al. 2011). Similar to previous findings (Liu et al. 2011), the hsfa1-qk mutant is
less resistant to HS treatment than the wild type. However, when the mutant was pre-
treated at 37 °C for 30 min and subjected to the same HS conditions as in the basal
thermotolerance assay, the survival rates of the seedlings greatly improved
(Supplemental Figure 5). These results demonstrate that in the absence of HSFA1s,
Arabidopsis plants can still sense elevated temperatures, regulate the expression of
HS-related genes, and boost their tolerance to high environmental temperatures.

Identification of HSFA1-dependent and -independent eHSRs by RNA-seq

To explore the HSFA1-dependent and -independent mechanisms that regulate early
HS-induced gene expression, we compared the HS-regulated transcriptomes of wild-
type vs. hsfa1-qk plants. Meanwhile, because 1-week-old hsfa1-qk seedlings grown
on agar plates were tiny and almost completely lacked expanded cotyledons
(Supplemental Figure 6), we used 3-week-old soil-grown seedlings, as the difference
in vegetative growth between wild-type and *hsfa1-qk* plants was less dramatic (Supplemental Figure 6). We treated the seedlings at 37 °C for 10 min before harvesting the leaves for RNA-seq. Using an FDR < 0.05 and fold change of > 2 or < 0.5 as cut-off values, we determined that 10 min of HS treatment differentially regulated the expression of 1790 genes (842 up- and 948 downregulated) in Col-0, 1049 genes (575 up- and 474 downregulated) in Ws-2, and 1495 genes (768 up- and 727 downregulated) in *hsfa1-qk* (Supplemental Dataset 4 and Figure 3A). Therefore, many more HS-regulated genes were identified in soil-grown plants than in seedlings grown on agar plates, perhaps because the soil-grown plants were directly exposed to high temperature. Under this condition, the surfaces of the soil-grown plants reached the designated temperature much more rapidly than seedlings grown in agar plates (Supplemental Figure 1).

We identified 566 genes (331 up- and 235 downregulated) with similar changes in expression in response to HS in three-week-old Col-0 and Ws-2 plants (Figure 3A). The small amount of overlap in eHSRs between Col-0 and Ws-2 indicates that different Arabidopsis ecotypes have different levels of sensitivity to HS or have evolved different mechanisms to adapt to increasing environmental temperatures. A comparison of these 566 genes with the eHSRs identified from 1-week-old seedlings listed in Supplemental Dataset 1 revealed that 40.46% (44.11% up- and 35.32% downregulated) of the genes were also identified as HS-regulated genes in 1-week-old Col-0 seedlings. This group of overlapping eHSRs might represent core HSRs with essential roles in early HS responses in Arabidopsis.

Of the 566 eHSRs that were similarly regulated by HS in Col-0 and Ws-2, 158 (47.73%) and 181 (67.23%) genes were up- or down-regulated, respectively, to similar levels in *hsfa1-qk*, suggesting that these genes are regulated by HS in an HSFA1-independent manner. By contrast, 202 genes (155 up- and 47 down-regulated, including *HSFA2*, *HSFA7a*, and *HSFB2a*) were not regulated by heat treatment in *hsfa1-qk* (or the fold change did not meet the selection criteria); these genes were considered to be HSFA1-dependent eHSRs. Additionally, 25 of the 566 eHSRs (18 up- and 7 downregulated) were up- or downregulated at least 2-fold (FDR < 0.05) in *hsfa1-qk*, but the changes in expression were much smaller in *hsfa1-qk* than in Col-0 or Ws-2. Thus, the HS-regulated expression of this small group of genes might be controlled by crosstalk between HSFA1s and (currently) unidentified transcription
factors (Figure 3B, Supplemental Dataset 5).

GO enrichment analysis indicated that genes involved in plant growth and development were highly enriched among the HS-downregulated eHSRs identified in three-week-old plants, regardless of whether these genes were regulated in an HSFA1-dependent or -independent manner (Figure 3C). Interestingly, the HSFA1-dependent HS-downregulated eHSRs from 3-week-old plants were more likely to be involved in plant growth processes (such as the response to auxin or gibberellin and organism development), whereas genes involved in cell differentiation processes (including the response to cytokinin, cell differentiation, and leaf morphogenesis) were more highly enriched among the HSFA1-independent HS-downregulated eHSRs from 3-week-old plants (Figure 3C).

Similar to the findings for 1-week-old seedlings, most HS-upregulated eHSRs from 3-week-old plants were related to plant stress responses. However, the HSFA1-dependent HS-upregulated eHSRs from 3-week-old plants were mainly involved in heat stress, chaperone binding, protein folding, and stress responses, whereas the HSFA1-independent HS-upregulated eHSRs from 3-week-old plants were more likely to be involved in drought, cold, salt, and abscisic acid responses (Figure 3C). Taken together, these results suggest that HSFA1s play essential roles in regulating the expression of genes that function in plant adaptation to HS, whereas other primary transcription factors involved in HS signaling mediate crosstalk with other abiotic stress signaling pathways.

RVE4 and RVE8 regulate the expression of HSFA1-independent eHSRs

To validate our RNA-seq results, we performed RT-qPCR analysis of select eHSRs (mostly encoding transcription factors) from 3-week-old plants grown in soil under long-day (LD) conditions (16 h light/8 h dark) to investigate the effects of mutations in HSFA1s on their early HS-induced expression patterns. Consistent with our RNA-seq results, the HS-induced expression (after 10 min of treatment) of DREB2A, NFERYC2, WIND1, SAP3, ZAT6 was abolished in hsfa1-qk, suggesting that the HS-induced expression of these genes is HSFA1-dependent. By contrast, ERF53, ERF54, BBX17, ABCG36, COR47, MAGL4, SCL13, RD26, MYB44, and At1g22190 were upregulated to similar levels under HS conditions in hsfa1-qk and wild-type plants, suggesting that the HS-induced expression of these genes is HSFA1-independent. Together, these
results indicate that our RNA-seq data are reliable (Figure 4).

To discover new primary transcription factors that mediate the transcriptional activation of HSF1-independent eHSRs following HS exposure, we identified 1000 bp of genomic sequence located upstream of the translation initiation codon (ATG) of the 331 HS-upregulated eHSRs shown in Supplemental Dataset 5 and subjected them to MEME analysis. The analysis suggested that these eHSRs might be regulated by HSF, bZIP, NAC, bHLH, BZR, and MYB transcription factors (Figure 5A). Not surprisingly, HSEs were highly enriched among the HSF1-dependent eHSRs, but not among the HSF1-independent eHSRs (Figure 5A–C). Interestingly, in contrast to bZIP, NAC, bHLH, and BZR transcription factors, which regulate both HSF-dependent and -independent eHSRs, a cis-element directly targeted by the MYB transcription factor, RVE4, and its homologs, RVE8, RVE7, and LHY, was only enriched among HSF1-independent eHSRs, suggesting that RVE4 family transcription factors specifically regulate HSF1-independent eHSRs (Figure 5A–C).

RVE4 and RVE8 are components of the central oscillator of the circadian clock. RVE4 and RVE8 transcription is regulated by the circadian clock under both long-day (Supplemental Figure 7) and short-day conditions (Hsu et al. 2013). RVE8 accumulates to its highest levels around Zeitgeber time (ZT) 3–6, i.e., 3–6 hours after turning on the light, under 12 light/12 dark growth conditions, when it binds to the evening elements (EE) on the promoters of evening circadian clock genes, such as PSEUDO RESPONSE REGULATOR5 (PRR5), and activates their expression (Hsu et al. 2013; Rawat et al. 2011). RT-qPCR indicated that the expression of RVE4 and RVE8 was not significantly regulated by brief HS treatment (within 10 min, Supplemental Figure 8). Knocking out or knocking down the expression of RVE4, RVE8, or LHY alone slightly decreased the basal thermotolerance of the mutants, whereas simultaneously knocking out/down the expression of RVE4 and RVE8 significantly increased susceptibility of the rve4 rve8 mutant (rve4/8) to HS treatment (Supplemental Figure 9). To minimize the influence of the circadian phase changes of rve4/8 on its HS responses, we also grew the seedlings under 24 h of continuous light at 22 °C for 7 days before subjecting them to HS treatment. Both basal and acquired thermotolerance were significantly reduced in rve4/8 vs. the wild type (Figure 5D–F). Taken together, these results suggest that RVE4 and RVE8 play redundant, critical roles in regulating thermotolerance in Arabidopsis.
To investigate whether RVE4 and RVE8 are the primary transcription factors that mediate HS-induced, HSFA1-independent eHSRs expression, we performed RT-qPCR to test for the HS-induced expression of the HSFA1-independent genes listed in Figure 4 in rve4/8 double mutant plants. Under LD conditions, the HS-induced expression of ERF53, ERF54, ABCG36, BBX17, COR47, and MAGL4 was not significantly altered in rve4/8 plants after 10 min of heat exposure, demonstrating that the HS-induced expression of these eHSRs is dependent on RVE4/8 (Figure 6). By contrast, the expression of SCL13, RD26, MYB44, At1g22190, DREB2A, NF-YC2, WIND1, SAP3, ZAT6, HSFA2, HSFA7a, HSFA7b, HSFB1, HSFB2a and HSFB2b in rve4/8 plants was induced by HS treatment to a level similar to that of wild-type plants. These results suggest that other primary transcription factors (e.g., bZIP, NAC, and bHLH transcription factors) are involved in mediating the HS-induced expression of SCL13, RD26, MYB44, and At1g22190.

To compare the contributions of RVE4/8 and HSFA1s to plant thermotolerance, we generated additional hsf1a or hsf1b mutations in the hsf1a/1b T-DNA insertion double mutant using CRISPR/Cas9. Among the mutants generated, hsf1a-crispr contained an extra base in the coding sequence, while hsf1b-crispr had a missing base in the coding sequence, which resulted in a frameshift at codon 126 and codon 70 of HSFA1a and HSFA1b, respectively. These two CRISPR/Cas9 mutants were crossed with each other to generate the quadruple mutant (hsfa1-cq) for HSFA1s in the Columbia ecotype background (Supplemental Figure 10).

The HS-regulated expression patterns of the eHSRs in the new hsf1a-cq mutant (shown in Supplemental Figure 11) and the hsf1a-qk mutant (Figure 4) were similar. When hsf1a-cq plants were grown side-by-side with rve4/8 mutants and subjected to HS treatment, both basal and acquired thermotolerance were reduced in hsf1a-cq and rve4/8. However, hsf1a-cq was much more sensitive to HS treatment than rve4/8 (Supplemental Figure 12), suggesting that HSFA1s are more important than RVE4/8 for regulating thermotolerance in Arabidopsis.

**ERF53 and ERF54 are direct targets of RVE8**

Among the RVE4/8-dependent HS-upregulated eHSRs were two homologous transcription factor genes: ERF53 and ERF54. Given that ERF53 overexpression increases thermotolerance in Arabidopsis (Hsieh et al., 2013) and that RVE4, RVE8,
ERF53, and ERF54 are universally expressed genes (Supplemental Figure 13), we investigated whether the heat-induced expression of ERF53 and ERF54 is directly regulated by RVE4/8. We examined the promoter sequences of ERF53 and ERF54 and found two and three EE-like cis-elements located within 1000 bp upstream of the translation initiation codons (ATG) of ERF53 and ERF54, respectively (Figure 7A). We therefore performed a chromatin immunoprecipitation (ChIP) assay using pRVE8:RVE8-YFP transgenic plants to test for the direct binding of RVE8 to the promoter regions of ERF53 and ERF54. Because we determined that RVE8 regulates ERF53 and ERF54 under HS condition in an HSFA1-independent manner, pHSFA1d:HSFA1d-GFP transgenic plants were used as an additional negative control. When expressed in rve4/8 or hsfa1-qk plants, the pRVE8:RVE8-YFP transgene recovered the HS-induced expression of ERF53 and ERF54 to wild-type levels in rve4/8 (Supplemental Figure 14), and the pHSFA1d:HSFA1d-GFP construct rescued the dwarf phenotype of hsfa1-qk (Supplemental Figure 15), demonstrating that the RVE8-YFP and HSFA1d-GFP fusion proteins used in the ChIP assay were functional. No significant enrichment of EEs in the promoters of ERF53 and ERF54 was observed at room temperature, as determined by RT-qPCR. In plants treated at 37°C for 10 min, we detected a significant increase in the binding of RVE8-YFP to the EE motif located -169 to -161 bp upstream of the ATG in ERF53 and to the EE motifs located -479 to -471, -551 to -543, and -756 to -748 bp upstream of the ATG in ERF54 (Figure 7B). By contrast, RVE8-YFP bound strongly to the EE located at -389 to -381 bp in the PRR5 promoter; however, this binding was not regulated by HS treatment (Figure 7A and 7B). As a negative control, HSFA1d-GFP bound directly to the HSE motif in the HSP70 promoter (a direct target of HSFA1d) under HS conditions, where the binding of HSFA1d-GFP to the EE motifs in the ERF53 and ERF54 promoters was not detected (Figure 7B).

To obtain additional evidence that RVE8 is a transcriptional activator of ERF53 and ERF54, we performed a transient transcriptional reporter assay using Nicotiana benthamiana leaves. At room temperature, firefly luciferase activity in N. benthamiana leaves co-transformed with the p35S:YFP-RVE8 and pERF53:luciferase (or pERF54:luciferase) expression vector pair was similar to that of leaves co-transformed with p35S:CFP and pERF53:luciferase (or pERF54:luciferase). A 10 min HS treatment significantly increased the luciferase signal in leaves co-
transformed with p35S:YFP-RVE8 and pERF53: luciferase (or pERF54: luciferase) vs. leaves co-transformed with p35S:CFP and pERF53: luciferase (or pERF54: luciferase) (Figure 7C). These results help confirm the ChIP-qPCR data and suggest that RVE8 activates the expression of ERF53 and ERF54 only under HS conditions. Furthermore, overexpressing ERF53 increased the thermotolerance of both wild-type and rve4/8 plants, which corresponded with the increased expression level of ERF53 (Supplemental Figure 16, Figure 7D and 7E). Taken together, these results demonstrate that ERF53 and ERF54 are direct targets of RVE8. In addition, HS stimulates the binding of RVE8 to the promoters of ERF53 and ERF54 and induces their expression to increase plant tolerance to HS treatment.

**RVE8-regulated plant thermotolerance is gated by the circadian clock**

RVE8 protein accumulated to its highest level at ZT 3–6 (Supplemental Figure 17) under our growth conditions (16 h light/8 h dark), and this is the time point at which we harvested seedlings for RNAseq and HS treatment. To investigate whether RVE4/8 play a general role in regulating plant HS responses, or whether they regulate plant thermotolerance specifically around the time when their expression reaches its highest level, we subjected 7-day-old seedlings grown under long-day conditions to 45°C HS treatment at ZT 0–1, ZT 6–7, ZT 15–16 and ZT 19–20. The thermotolerance of wild-type plants increased at ZT 6–7 and ZT 15–16 but returned to a level similar to that at the beginning of the experiment (ZT 0–1) at ZT 19–20. By contrast, the thermotolerance of rve4/8 was similar to that of the wild type at ZT 0–1, ZT 15–16, and ZT 19–20 but significantly lower at ZT 6–7 (Figure 8A and 8B). Similarly, RT-qPCR analysis showed that HS-induced expression of ERF53, ERF54 and BBX17 (all RVE4/8-dependent eHSRs) was significantly lower in rve4/8 at ZT 6–7 but not at any other time point compared to the wild type (Figure 8C). By contrast, the HS-induced expression of HSF A2 was not altered in rve4/8 compared to the wild type at any time point (Figure 8D). Finally, the expression of the RVE8 target clock gene, PRR5, was reduced in rve4/8 at ZT 6–7 compared to the wild type in both HS treated and untreated conditions (Figure 8D). Taken together, these results demonstrate that circadian clock play important roles in regulating plant tolerance to high temperatures and that RVE4/8 regulate plant thermotolerance specifically around ZT 6–7 under LD conditions.
DISCUSSION

In recent decades, significant progress has been made in elucidating the responses of plants to environmental HS at the molecular level. However, how plants sense HS and the primary HS signal transduction pathway leading to HS-regulated gene expression are not well understood. One reason for this situation is the lack of appropriate standards to determine whether the primary HS signal transduction pathway is affected in mutant or transgenic plants. Traditionally, differences in plant survival after HS treatment, and differences in the transcript levels of HSFs and HSPs between HS-treated and -untreated plants, have been used to evaluate whether specific genes play important roles in regulating plant HS responses. In these studies, the HS treatment time used was generally 30 min or longer. Under these conditions, genes whose expression is directly regulated via the primary HS signal transduction pathway are not usually separated from genes whose expression is indirectly regulated by a transcriptional cascade or signaling crosstalk. The cellular abundance of HSFs and HSPs in plants generally corresponds to their HS tolerance. Therefore, genes or signaling pathways that directly or indirectly affect HSF and/or HSP expression could alter plant thermotolerance (Swindell et al., 2007), which could lead to the incorrect conclusion that certain genes or pathways regulate HS signaling. However, this confusion could be avoided by reducing the HS treatment time to as short as possible.

In this study, we obtained a comprehensive view of how brief HS exposure affects the Arabidopsis transcriptome, including an assortment of eHSRs whose expression is rapidly regulated by HS. We determined that under HS conditions, transcriptional reprogramming occurs quickly; a 2.5 min HS treatment inhibited the expression of 877 genes (Figure 1A). This rapid heat-induced global gene suppression, which has also been reported previously (Duarte et al., 2016), could be caused by the rapid HS-induced dissociation of RNA polymerase II from DNA (Hieda et al., 2005). We also detected a second round of global HS-induced gene suppression, which occurred after 15 min of HS treatment. This second round of transcriptional suppression occurred after the HS-induced expression of a number of HSPs. HSPs are molecular chaperones that prevent high temperature-induced protein aggregation by facilitating the correct folding of cellular proteins (Figure 1A, 2B). Thus, the molecular mechanism underlying the second round of HS-induced gene suppression is likely different from that during the first round.
RNA-seq showed that 5 min of HS treatment at 37 °C upregulated the expression of MBF1c, HSFA2, HSFA7a, HSFA7b, and HSFB2b in one-week-old seedlings grown on agar plates by at least 2-fold; the HS-induced expression of these genes continued to increase with prolongation of HS treatment time. Therefore, it appears that 5 min is the minimum amount of time required for the primary HS signal transduction pathway in plants, from HS sensing to alterations in nuclear gene expression. However, under our HS treatment conditions, the temperature inside the agar plates only reached an average of approximately 28.7±0.9 °C after 2.5 min of HS treatment and 31±0.6 °C when HS treatment was extended to 5 min (Supplemental Figure 1), suggesting that plant HS signal transduction is accomplished even more rapidly and is evoked at a lower temperature than expected. In our RT-qPCR experiments, the 2-fold or higher HS-induced expression of a number of HSFs and many other eHSRs was stably reproduced in plants treated with HS for 10 min or longer. Therefore, we used 10 min of HS exposure in all subsequent early HS-induced gene expression experiments.

Increased HSF expression is generally used as an indicator of the turning on of plant HS responses. Activated HSFs bind to HSEs in the promoters of HSRs (e.g., HSPs and genes encoding ROS-producing and/or scavenging enzymes) and regulate their expression, thereby increasing plant thermotolerance. By analyzing the cis-elements present in the 1000-bp promoter region or 5’-UTR of each eHSR that was upregulated in response to 10 min of HS treatment, we found that HSEs (which are bound by HSFs) were highly enriched. Using hsfa1-qk plants, we demonstrated that HSFA1s are master regulators of rapid HS-induced gene expression; approximately 50% of the eHSRs that are upregulated after 10 min of HS treatment in 3-week-old plants are regulated by HSFA1s (Figure 3). Interestingly, the expression of HSP90 and many other HSFA1-dependent HS-upregulated genes did not change within 10 min of HS treatment, but they were upregulated after 15 or 30 min of HS treatment (Supplemental Dataset 1). The HSFA1-dependent temporal regulation of HS-upregulated genes suggests that chromosome structure might be sequentially regulated by HS; for example, the HSFA1-dependent depletion of H2A.Z from nucleosomes is regulated by HS conditions (Cortijo et al., 2017). Alternatively, the binding of additional transcription factors might be responsible for upregulating the HSFA1-dependent eHSRs that require a longer HS treatment period.
The circadian clock regulates plant responses to daily temperature and light cycles. For example, in green and ripening grapevine (*Vitis vinifera*) fruit, HS triggers different transcriptomic responses depending on whether heat exposure occurs during the day or night, suggesting that the responses of plants to HS at different times of the day are not the same (Rienth et al., 2014). Plants also differ in their ability to withstand high temperatures at different times of day (Dickinson et al., 2018). The current results reinforce the notion that the thermotolerance of plants varies at different times of day (Figure 8A and 8B).

Recent studies have shown that various light receptors and the circadian clock protein, TIMING OF CAB EXPRESSION 1 (TOC1), may gate daily plant thermomorphogenesis (Li et al., 2018; Zhu et al., 2016); however, the molecular mechanism underlying circadian clock-dependent regulation of plant thermotolerance is largely unknown. Among the principal clock-regulating genes, the expression of *RVE8* peaks 3–6 h after turning on the light (Rawat et al., 2011; and Supplemental Figure 19), that is, around noon or in the early afternoon, when HS is most likely to occur. Temperature compensation of the circadian rhythm was disrupted in *rve8* and *RVE8*-overexpressing plants (Rawat et al. 2011), suggesting that RVE8 plays an important role in integrating the temperature and circadian responses of plants.

In the current study, we provided several lines of evidence demonstrating that RVE8 and its close homolog, RVE4, play important roles in regulating plant thermotolerance and the HS-induced expression of HSFA1-independent *eHSRs*. First, analysis of the *cis*-elements of the HS-upregulated *eHSRs* in three-week-old plants revealed strong enrichment of the RVE4/8-recognizing EE motif only in HSFA1-independent *eHSRs* (Figure 5A-C). Second, knocking out/down the expression of *RVE4* and/or *RVE8* impaired both basal and acquired thermotolerance in plants grown under LD or continuous light conditions (Figure 5D-F, Supplemental Figure 9). Third, RT-qPCR showed that after 10 min of heat treatment, the HS-induced expression of *ERF53*, *ERF54*, *ABCG36*, *BBX17*, *COR47*, and *MAGL4* was similar in wild type and *hsfa1-qk* plants but impaired in *rve4/8* plants (Figure 4, 6, Supplemental Figure 11). Of these RVE4/8-dependent HS-upregulated *eHSRs*, the overexpression of *ERF53* increases plant resistance to HS (Figure 7D-E, and Hsieh et al., 2013), suggesting that these RVE4/8-dependent *eHSRs* play critical roles in regulating plant thermoresponses. Fourth, chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) and transient
transcription assays showed that RVE8 binds directly to the promoter regions of
*ERF53* and *ERF54* and activates their expression only under HS conditions.
Interestingly, the binding of RVE8 to the promoter of its circadian clock target gene,
*PRR5*, is not regulated by HS (Figure 7A-C). It was showed that overexpressing
*TOC1*, another circadian clock gene directly targeted by RVE8, reduced acquired
thermotolerance in plants, whereas the *toc1 prr5* double mutant showed enhanced
acquired thermotolerance (Rawat et al., 2011; Zhu et al., 2016). These findings,
combined with the current results, suggest that the molecular mechanisms by which
RVE8 regulates plant thermotolerance and circadian rhythms are not the same.
Together, our results demonstrate RVE4/8 are primary transcription factors that
function in parallel with HSFA1s to regulate the first wave of HS-induced gene
expression, and prepare plants for exposure to high temperatures during the day.

HSFA1s and RVE4/8 are constitutively expressed transcription factors and their
expression is not significantly regulated by brief HS exposure, suggesting that HS
signal transduction might activate HSFA1s and RVE4/8 post-transcriptionally. The
DNA binding or transcriptional activity of HSFA1s can be altered by post-
translational modifications such as protein phosphorylation (Reindl et al., 1997; Liu et
al., 2008), protein sumoylation (Miller et al. 2010), and/or oxidative damage caused
by ROS (Jung et al., 2013). However, direct evidence that these post-translational
modifications are induced by HS *in vivo* is currently lacking. Additionally, HSFA1s
are thought to be activated by the cytoplasmic protein response (CPR) (Ohama et al.,
2016). According to this model, HSP70 and HSP90 bind to HSFA1s under normal
conditions and prevent their binding to DNA to influence gene expression. HS
induces cytosolic protein misfolding and denaturation, recruit molecular chaperones,
including HSP70 and HSP90, to help refold the denatured proteins. As a result,
HSFA1s are released and translocated into the nucleus where they regulate the
expression of HS-related genes. Because the HS condition used in the current study
was relatively mild (37 °C) and the treatment time was relatively short (10 min or
less), whether this treatment is sufficient to trigger the CPR has yet to be determined.

By contrast, limited information is available about how RVE4/8 transcriptional
activity is regulated. Given that RVE4/8 and HSFA1s might simultaneously regulate
the first wave of HS-upregulated gene expression, it is likely that RVE4/8 and
HSFA1s are activated via similar mechanisms under HS conditions. The identification
of proteins that can bind to both RVE4/8 and HSFA1s under HS conditions would help decode the mechanisms leading to HS-induced gene expression.

*SCL13, RD26, MYB44, and At1g22190* were induced to approximately wild-type levels in *hsfa1-qk* and *rve4/8* plants following 10 min of HS treatment (Figures 4 and 6), as revealed by RT-qPCR, suggesting that additional primary transcription factors regulate the first wave of HS-upregulated gene expression. Indeed, our MEME analysis showed that *cis*-elements that are recognized by bZIP, NAC, bHLH, and BZR transcription factors were highly enriched among *eHSRs* upregulated after 10 min of HS treatment (Figure 5A). Of these transcription factors, BZR transcription factors are key regulators of BR signaling. Exogenously applied BR increases plant tolerance to HS (Vardhini et al. 2010). In addition, BZR1 regulates thermotolerance in tomato by regulating the expression of its downstream target genes, the homologous receptor-like kinase genes *FERONIA2 (FER2)* and *FER3* (Yin et al. 2018). The enrichment of BZR-binding *cis*-elements in HS-upregulated *eHSRs* suggests that HS might rapidly modulate BR signaling to coordinate plant growth and stress responses.

In the current study, we identified numerous *eHSRs*, including many previously confirmed HS-regulated genes such as *MBF1c, HSFA2, HSFA7a, HSFA7b, HSFB1, HSFB2a, HSFB2b, HSPs, GALACTINOL SYNTHASE 1 (Gols1), Gols2, FKBP 62, DREB2A, DREB2B, bZIP28*, genes related to superoxide production or inactivation, and so on. These findings suggest that the RNA-seq database generated in this study will be highly valuable for future in-depth data mining to uncover mechanisms that govern plant responses to HS. Through RT-qPCR, we demonstrated that the early upregulation of a select group of *eHSRs* (primarily encoding transcription factors) by HS is dependent on HSFA1s, RVE4/8, and currently unidentified transcription factors.

Based on these findings, we propose a model describing how a transcriptional regulatory hierarchy governs the first wave of HS-regulated gene expression (Figure 9). According to this model, increased environmental temperatures initiate a signal transduction pathway that activates primary transcription factors such as HSFA1s and RVE4/8 to initiate the first wave of HS-regulated gene expression and to upregulate the expression of genes encoding a number of transcription factors, including *HSFA2, HSFA7a, HSFA7b, HSFB1, HSFB2a, HSFB2b, DREB2A, ZAT6, ERF53, ERF54*, and so on. These transcription factors, in turn, activate the expression of their own set of downstream target genes to fine-tune cellular protein or metabolic dynamics to help...
plants adapt to increased environmental temperatures. However, it appears that RVE4/8 regulate plant thermotolerance specifically at ~ZT 6–7, but not at ZT 15–16 (Figure 8). As the plants showed the highest thermotolerance at ZT 15–16 under our growth conditions, it would be interesting to investigate whether additional clock-related gene are activated at ZT 15–16 and increase the tolerance of Arabidopsis plants to HS. Finally, the 10 min HS-upregulated eHSRs identified in this study and the HS treatment conditions (10 min of HS treatment of plants grown in agar plates or soil) used in this study could be used as references for future HS signal transduction studies. Such studies could help uncover novel signaling mechanisms by which plants sense and adapt to increasing environmental temperatures.

METHODS

Plant growth and HS treatment

*Arabidopsis thaliana* seeds (40–50 per genotype) were surface-sterilized and sown on glass plates containing 30 ml of growth medium (0.6% Phytagel, 0.5x Murashige and Skoog [MS] basal salt mixture, and 1% sucrose, pH 5.7). The seeds were stratified at 4 °C in the dark for 3 days and grown at 22 °C under LD conditions (16 h light [~100 μmol m$^{-2}$ s$^{-1}$]/8 h dark) or under constant light for 7 days in a growth chamber (Percival Scientific, cool white fluorescent bulb). For the basal thermotolerance assay, at ZT 3–6, the plates were wrapped in plastic wrap, divided into two groups, and submerged directly in a water bath at 45 °C (HS) or 22 °C (control) for 12–13 min. For the acquired thermotolerance assay, at ZT 3–6, the plates were incubated in a water bath at 37 °C for 30 min and transferred to a growth chamber at 22 °C for 2 h of recovery before being submerged in a 45 °C or 22 °C water bath for 15–16 min. For the diurnal thermotolerance assay, the plates were divided into five groups: HS-treated plates placed in a 45°C water bath for 13 min at ZT 0–1, ZT 6–7, ZT 15–16, and ZT 19–20, and the control. The heat-treated and control plants were allowed to recover at 22 °C under LD conditions for 7 days before being assessed for survival.

The temperature inside and outside the plates during the HS treatment was measured simultaneously using electronic thermometers with microprobe. The temperature was automatically recorded every 30 seconds.

Isolation of Arabidopsis mutants
The T-DNA insertion mutants used in this study, SALK_051728 (rve4), SALK_053482 (rve8), SALK_031092 (lhy), SAIL_410_E01 (hsfa1d), and SALK_094943 (hsfa1e), were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org). The confirmed mutants were backcrossed once with the corresponding wild-type plants to clean up the background. The rve4/8 double mutant was generated by crossing SALK_051728 with SALK_053482. F3 segregated homozygous single or double mutants were used in this study. The gene-specific primers used to genotype the mutants are listed in Supplemental Table 1.

To generate CRISPR/Cas9 mutants for HSFA1a and HSFA1b, guide RNA sequences were designed (CTAATGAAGGTTTCTTAAG for HSFA1a and AAGTATTTCAAGCACAACA for HSFA1b) and cloned into the CRISPR/Cas9 vector pHEC-401 as described (Xing et al., 2014). The CRISPR/Cas9 vectors were transformed into the hsfa1 dhsfa1e T-DNA double homozygous mutant using the Agrobacterium tumefaciens (strain GV3101)-mediated floral-dip method (Clough and Bent, 1998). T1 transgenic plants were selected on 1/2 MS agar medium containing 50 µM hygromycin. The genomic DNA sequence containing the CRISPR/Cas9 target sites was amplified and sequenced to check for mutations. hsfa1a-cirspr and hsfa1b-cirspr T1 heterozygous plants were crossed with each other to generate the hsfa1-cq quadruple mutant, which was crossed once with hsfa1d hsfa1e to clean up potential background mutations. All primers used for construct generation and genotyping are listed in Supplemental Table 1.

RNAseq and gene expression analysis

Seven-day-old seedlings grown on agar plates (covered with lids and sealed) or 3-week-old plants grown in soil at 22 °C in a growth chamber under LD conditions were transferred to a new growth chamber with an inside temperature pre-set to 37 °C for heat treatment for the designated time or kept in the same growth chamber without heat stress (control). Immediately after HS treatment (at ZT 3–6), the plates containing 7-day-old seedlings were snap-frozen in liquid nitrogen, and the seedlings (40–50 individual plants) were harvested by scraping the agar surface with a spatula under liquid nitrogen. For 3-week-old plants, 1–2 rosette leaves each from 5–6 independent plants per genotype were harvested, pooled together as one biological
sample, and used for RNAseq or RT-qPCR.

For RNAseq, plant tissue was ground to a fine powder in liquid nitrogen, and total RNA was extracted from the tissue using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Three biological replicates were used for RNA-seq and subsequent bioinformatic analyses. RNA-seq was performed on the Illumina HiSeq X platform (Illumina Inc., San Diego, CA) using 150-bp double-ended reads. The reads were aligned to the Arabidopsis reference genome (version TAIR10) using TopHat (version 2.0.12) (Trapnell et al., 2009; Kim et al., 2013). After filtering out low-quality reads with FASTX-Toolkit (version 0.0.13), uniquely aligned reads were counted for each annotated gene using the program htseq-count from the python package HTSeq (version 0.6.1) (Anders et al., 2015). Differential gene expression was evaluated using the R package DESeq (Anders and Huber, 2010); P-values were adjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) to control the FDR. Differentially expressed genes were defined based on the following criteria: FDR < 0.05 and at least a 2-fold change in expression compared to the control samples.

For RT-qPCR, total RNA was extracted from the tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. First-strand cDNA was synthesized using a PrimeScript RT Reagent Kit with Genomic DNA Eraser (Takara Bio Inc., Kusatsu, Japan). HS-regulated gene expression was analyzed by RT-qPCR using SYBR Premix Ex Taq (Takara Bio Inc.) and a C1000 Touch Thermal Cycler CFX384 (Bio-Rad, Hercules, CA). The gene-specific primers used for gene amplification are listed in Supplemental Table 1. The relative abundance of the target transcripts was determined by the comparative Ct method (Schmittgen and Livak, 2008), using UBC30 as an internal reference.

**Bioinformatic analysis**

RNA expression DREM paths were constructed using DREM (version 2.0) as described (Ernst et al., 2007). The log_{2}(fold change) values of the eHSRs for each HS treatment period were used for analysis. GO enrichment of genes in each DREM path was calculated using DREM (version 2.0) as follows: genes enriched = genes assigned − genes expected (Schulz et al., 2012). The remaining GO enrichment analysis of the eHSRs was performed using the online tool Database for Annotation,
Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) (Huang et al., 2009), using -log2(p-value) to evaluate enrichment levels. Heat maps of the differentially expressed genes were generated using HemI (version 1.0) (Deng et al., 2014). Enrichment of cis-elements in the -1000 to -1 promoter sequences of the eHSRs (downloaded from TAIR10, http://www.arabidopsis.org/) was performed using the online tool MEME (version 4.12.0, http://meme-suite.org/tools/centrimo) (Bailey et al., 2009, 2015; Bailey and Machanick, 2012; O’Malley et al., 2016). Transcription factors were identified according to the Arabidopsis thaliana transcription factor database (http://planttfdb.cbi.pku.edu.cn/). eHSRs in DREM paths 1–5 were used to generate a gene co-expression network as described (You et al. 2016). The network was visualized using Cytoscape (version 3.7.1, Shannon et al., 2003).

**Cloning and generation of transgenic plants**

The 1508 bp (ERF53) and 1502 bp (ERF54) promoter sequences (relative to the translation start site), the coding sequences of ERF53 and RVE8, and 2754 bp (RVE8), and 4547 bp (HSFA1d) genomic DNA sequences upstream of the stop codon (stop codon not included) were cloned into the pENTR/SD/D-TOPO vector (Invitrogen, Carlsbad, CA) and subcloned into Gateway-compatible binary vectors pHGWL7.0, pCAMBIA1390, pEarleyGate 104, pEarleyGate TW1 (a modified pEarleyGate 101 vector), and pGWB4 to generate the pHGWL-ERF53 (pERF53:Luciferase), pHGWL-ERF54 (pERF54:Luciferase), p1390-ERF53 (p35S:ERF53-myc), p104-RVE8 (p35S:YFP-RVE8), pTW1-RVE8 (pRVE8:RVE8-YFP), and pGWB4-HSF1a (pHSFA1d:HSFA1d-GFP) expression vectors by LR cloning (Invitrogen). The expression vectors were incorporated into Agrobacterium tumefaciens (strain GV3101). Transgenic Arabidopsis plants were generated using the floral dip method. T3 transgenic homozygous plants were used for functional analysis unless otherwise indicated. Levels of the proteins in the transgenic plants were analyzed by immunoblots using anti-GFP antibody (Roche, Cat. No. 11814460001, dissolved in 500 μL ddH2O to create the stock, used as 1:1000 dilution) or anti-myc antibody (Millipore, Cat. No. M4439, 1:5000 dilution), anti-tubulin antibody (Sigma-Aldrich, Cat. No. T5168, 1:5000 dilution) was used for equal loading control.
ChIP-qPCR

Seedlings expressing pHSFA1d:HSFA1d-GFP (hsfa1d background) and pRVE8:RVE8-YFP (Col-0 background) were grown in soil at 22 °C under LD conditions for 3 weeks. At ZT 3-6, the seedlings were exposed to 37 °C (HS) or kept at 22 °C (control) for 10 min. Freshly harvested plant tissues were immediately cross-linked by vacuum infiltration with 1% formaldehyde for 5 min and held for 5 min after releasing the vacuum. Cross-linking was quenched by submerging the tissues in 0.25 M glycine for 10 min. The tissues were ground into a fine powder in liquid nitrogen. ChIP was performed as described by Zhu et al. (2012). In brief, the isolated chromatin was fragmented (average length: 0.5–1 kb) by sonication at 4 °C and immunoprecipitated using 10 μl of GFP-Trap agarose (ChromoTek, Planegg-Martinsried, Germany) or anti-myc agarose beads for the control (Millipore, Cat. No. A7470). The primers used for ChIP-qPCR are listed in Supplemental Table 1. Relative enrichment was calculated based on the fold change value compared to the UBC30 control using the comparative Ct method (Schmittgen and Livak, 2008).

Transient transcriptional activation assay

Agrobacterium tumefaciens (strain GV3101) cells harboring the p35S:CFP, p104:RVE8, pHGWL-ERF53, and pHGWL-ERF54 expression vectors were mixed in pairs and co-infiltrated into leaf epidermal cells of 4-week-old N. benthamiana grown in the soil at 22 °C under LD white light (~100 μmol m⁻² s⁻¹, cool white fluorescent bulb) in a growth room. 36 h after infiltration, the leaves were sprayed with firefly luciferase substrate, incubated in completely darkness for 5 min, and images captured using FUSION FX5 (Vilber Lourmat, France). After recording the bioluminescence signal from a leaf, the leaf was surface dried with Kimwipes (Sigma-Aldrich) and incubated in a growth chamber with the internal temperature preset to 37°C for 10 min. The leaves were sprayed with new luciferase substrate, incubated in the dark for 5 min, and the images recorded using the same settings as before HS treatment. ImageJ software was used to quantify the bioluminescence signal. Relative luciferase activity was determined based on the ratio between YFP-RVE8 and CFP-upregulated luciferase activity.
Experimental replicates and statistical analysis

All RT-qPCR and thermoresponsive assay results in this article represent the average value from at least three biological replicates, and error bars represent ± standard error (± SE). The term “different biological replicates” refers to plants grown and HS treated on different days and harvested for analysis. Unless otherwise indicated, two-way ANOVA with Tukey’s honestly significant differences test were used to evaluate the differences across multiple data sets, and statistically significant differences are indicated by different lowercase letters, p<0.05. ANOVA results are listed in Supplemental Dataset 6.

Data availability

The RNAseq data of this article have been submitted to the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra/), and was assigned with the following identification accession number: SRP187923 and SRP187905.

Accession numbers

Supplemental Data

Supplemental Figure 1. Time-dependent increases of the temperature inside or outside the plates during HS treatment at 37 ℃.

Supplemental Figure 2. A co-expression network of eHSRs.

Supplemental Figure 3. The expression of various reference genes in response to HS.

Supplemental Figure 4. Enrichment of cis-elements recognized by various transcription factors in HS down-regulated eHSRs.

Supplemental Figure 5. Acquired thermotolerance in hsfa1-qk.

Supplemental Figure 6. The vegetative growth of hsfa1-qk is slow.

Supplemental Figure 7. The circadian-dependent expression of RVE4 and RVE8 under long-day conditions.

Supplemental Figure 8. The expression of RVE4 and RVE8 is not regulated rapidly by HS.

Supplemental Figure 9. RVE4 and RVE8 redundantly regulate plant basal thermotolerance.

Supplemental Figure 10. Generation of the hsfa1-cq quadruple mutant.

Supplemental Figure 11. RT-qPCR validation of RVE4/8 and HSFA1s dependent and independent eHSRs using rve4/8 and hsfa1-cq mutants.

Supplemental Figure 12. HSFA1s are more important than RVE4/8 in regulating plant thermotolerance.

Supplemental Figure 13. RT-qPCR analysis of tissue-specific expression patterns of RVE4, RVE8, ERF53 and ERF54.

Supplemental Figure 14. Recovery of the HS-induced expression of ERF53 and ERF54 in rve4/8 mutants by the pRVE8:RVE8-YFP transgene.

Supplemental Figure 15. Complementation of the growth phenotypes of hsfa1-qk by the pHSFA1d:HSFA1d-GFP transgene.

Supplemental Figure 16. The level of ERF53 protein in ERF53 overexpression transgenic plants.
Supplemental Figure 17. Circadian-dependent accumulation of RVE8 proteins under long-day growth conditions.

Supplemental Table 1. Primers used in this study.

Supplemental Dataset 1. Rapid HS regulated genes identified by RNAseq.

Supplemental Dataset 2. Classification of eHSRs in different DREM paths.

Supplemental Dataset 3. Co-expression analysis of eHSRs.

Supplemental Dataset 4. eHSRs regulated by 10 min of HS treatment in three-week-old soil grown Col, Ws and hsfa1-qk.

Supplemental Dataset 5. List of HSF-dependent and independent eHSRs identified from three-week-old soil grown plants.

Supplemental Dataset 6. ANOVA tables.

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AUTHOR CONTRIBUTIONS

W.T. and D.S. designed the research. B.L. performed research under the supervisions of W.T. and D.S., Z.G. analyzed the RNAseq data. B.L. and X.L did the bioinformatics analysis. B.L. and W.T. wrote the manuscript together.
**FIGURE LEGENDS**

**Figure 1. RNA-seq analysis of rapidly responding HS-regulated genes.** (A) Number of differentially expressed genes following HS treatment at 37°C for the indicated time period. (B) Gene expression paths within 30 min of HS treatment constructed using DREM. Each path represents a set of co-expressed genes. Split nodes (green dots) represent the divergence of a set of co-expressed genes, most likely due to the emergence of new regulatory events. A relatively small node implies that the expression levels of the genes going through that node are tightly centered around the node. (C) GO classification of the genes in each DREM path.

**Figure 2. The rapid upregulation of genes by HS is partially mediated by HSFs.** (A) The percentages of eHSRs upregulated by 10, 15, or 30 min of HS treatment containing cis-elements in their 1000-bp promoter regions recognized by the indicated transcription factors. HSF: heat shock factors; PLATZ: PLATZ transcription factor; ND: AGAMOUS-like gene; S1Falike: S1FA-like DNA-binding protein; Orphan: paired amphipathic helix (PAH2) superfamily protein; Homeobox: homeodomain leucine zipper class I (HD-Zip I) protein; CPP: CXC domain-containing TSO1-like protein; NLP: plant regulator RWP-RK family protein; C2H2dof: Dof-type zinc finger DNA-binding family protein. (B) GO analysis of the eHSRs that were upregulated by 10 min of HS treatment.

**Figure 3. Identification of HSFA1-dependent and -independent eHSRs.** (A) Venn diagram showing the number of eHSRs that were up- or down-regulated in wild-type (Col-0 and Ws-2) and hsfa1-qk plants following HS treatment (37°C for 10 min; FDR < 0.05, fold change > 2 or <0.5). (B) Heat maps showing the expression patterns of HSF-dependent and -independent HS-regulated genes from (A). Log₂(FPKM) values were used to generate the heat map. (C) GO classifications of the HSF-dependent and -independent HS-regulated genes shown in (B).

**Figure 4. Validation of the HSFA1-dependent and -independent eHSRs by RT-
Quantitative PCR (qPCR). Relative expression levels of the indicated genes in 3-week-old HS-treated plants (37°C for 10 min). The gene expression levels are expressed as relative values compared to the value in non-heat-treated Col-0 seedlings, and were analyzed by two-way ANOVA with Tukey’s significant difference test. Error bars indicate mean ± standard error (SE) of n ≥ 3, significant differences are indicated by different lowercase letters (p<0.05).

Figure 5. RVE4/8 play an important role in regulating thermotolerance in Arabidopsis. (A) Enrichment of the cis-elements recognized by various transcription factors in the 1000-bp promoter regions of HSFA1-dependent and -independent HS-upregulated genes. (B-C) The top nine most highly enriched cis-elements for HSFA1-dependent (B) and -independent (C) HS-upregulated genes are shown. (D-F) A comparison of basal and acquired thermotolerance in rve4, rve8, and rve4/8 plants. Seven-day-old seedlings grown at 22°C under continuous light were used. Seedlings recovered 7 days after HS were photographed (D), and the survival rates of the plants were calculated (E and F). Each dot represents the result from one biological replicate, error bars indicate mean ± standard error (SE). Statistically significant differences are indicated by different lowercase letters (p<0.05, two-way ANOVA with Tukey’s significant difference test).

Figure 6. RT-qPCR validation of the RVE4/8-dependent and -independent eHSRs. Relative expression of the indicated genes in 3-week-old plants exposed to 37°C for 10 min. Gene expression levels are expressed relative to the value in non-heat-treated Col-0 seedlings. Error bars indicate mean ± standard error (SE) of n ≥ 3. Statistically significant differences are indicated by different lowercase letters (p<0.05, two-way ANOVA with Tukey’s significant difference test).

Figure 7. RVE8 regulates plant thermotolerance by regulating the expression of ERF53 and ERF54. (A) Structural diagrams of the ERF53, ERF54, PRR5, and HSP70 promoters. The locations and sequences of putative EEs (a1-2, b1-3, c) in the ERF53, ERF54 and PRR5 promoters or HSE in the HSP70 promoters are shown. (B) ChIP-qPCR assays of select EE- or HSE-containing regions from the promoters of
ERF53, ERF54, PRR5 or HSP70 in pRVE8:RVE8-YFP or pHSFA1d:HSFA1d-GFP transgenic plants. GFP-trap agarose (GFP) or anti-myc agarose (Control) beads were used for the pull down assay. (C) RVE8 activates the expression of ERF53 and ERF54 under HS conditions. The pERF53:luciferase or pERF54:luciferase expression vector was co-transformed with the p35S:YFP-RVE8 or p35S:CFP (control) expression vector into N. benthamiana leaves. Relative luciferase activity represents the ratio between YFP-RVE8- and CFP-upregulated luciferase activity. (D-E) Overexpression of ERF53 increases basal thermotolerance of both wild type and rve4/8 plants. Each dot represents the result from one biological replicate. Error bars indicate mean ± standard error (SE) of n ≥ 3. Statistically significant differences are indicated by different lowercase letters (p<0.05, two-way ANOVA with Tukey’s significant difference test).

Figure 8. Circadian clock-dependent regulation of plant thermotolerance by RVE4/8. (A-B) Basal thermotolerance analysis of Col-0 and rve4/8 plants at ZT 0–1, ZT 6–7, ZT 15–16, and ZT19–20. Seven-day-old seedlings grown at 22°C under LD conditions were transferred to a 45°C water bath. The plants were allowed to recover at 22°C for 7 days before being photographed (A) and assessed for survival (B). Each dot represents the result from one biological replicate. (C–D) The effect of the circadian clock on the HS-induced upregulation of HSFA2, ERF53, ERF54, and BBX17. PRR5 was used as a negative control. Three-week-old soil-grown Col-0 and rve4/8 plants were treated with 37 °C for 10 min at different times (ZT). Error bars indicate mean ± standard error (SE) of n ≥ 3. Statistically significant differences are indicated by different lowercase letters (p<0.05, two-way ANOVA with Tukey’s significant difference test).

Figure 9. Proposed model of the transcriptional regulatory hierarchy governing HS-induced gene expression. According to this model, HSFA1s and RVE4/8 serve as the primary transcription factors that are directly activated by HS signaling and initiate the expression of their downstream target genes, including a number of transcription factor genes, to regulate plant thermotolerance.
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Figure 1. RNA-seq analysis of rapidly responding HS-regulated genes. (A) Number of differentially expressed genes following HS treatment at 37°C for the indicated time period. (B) Gene expression paths within 30 min of HS treatment constructed using DREM. Each path represents a set of co-expressed genes. Split nodes (green dots) represent the divergence of a set of co-expressed genes, most likely due to the emergence of new regulatory events. A relatively small node implies that the expression levels of the genes going through that node are tightly centered around the node. (C) GO classification of the genes in each DREM path.
Figure 2. The rapid upregulation of genes by HS is partially mediated by HSFs. (A) The percentages of eHSRs upregulated by 10, 15, or 30 min of HS treatment containing cis-elements in their 1000-bp promoter regions recognized by the indicated transcription factors. HSF: heat shock factors; PLATZ: PLATZ transcription factor; ND: AGAMOUS-like gene; S1Falike: S1FA-like DNA-binding protein; Orphan: paired amphipathic helix (PAH2) superfamily protein; Homeobox: homeodomain leucine zipper class I (HD-Zip I) protein; CPP: CXC domain-containing TSO1-like protein; NLP: plant regulator RWP-RK family protein; C2H2dof: Dof-type zinc finger DNA-binding family protein. (B) GO analysis of the eHSRs that were upregulated by 10 min of HS treatment.
Figure 3. Identification of HSFA1-dependent and -independent eHSRs. (A) Venn diagram showing the number of eHSRs that were up- or down-regulated in wild-type (Col-0 and Ws-2) and hsfa1-qk plants following HS treatment (37°C for 10 min; FDR < 0.05, fold change > 2 or <0.5). (B) Heat maps showing the expression patterns of HSF-dependent and -independent HS-regulated genes from (A). Log₂(FPKM) values were used to generate the heat map. (C) GO classifications of the HSF-dependent and -independent HS-regulated genes shown in (B).
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Transcriptional Profiling Reveals a Time-of-day Specific Role of REVEILLE 4/8 in Regulating the First Wave of Heat Shock-induced Gene Expression in Arabidopsis
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