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A hit-and-run heat shock factor governs sustained histone methylation and transcriptional stress memory

Jörn Lämke, Krzysztof Brzezinka, Simone Altmann† & Isabel Bäurle*

Abstract

In nature, plants often encounter chronic or recurring stressful conditions. Recent results indicate that plants can remember a past exposure to stress to be better prepared for a future stress incident. However, the molecular basis of this is poorly understood. Here, we report the involvement of chromatin modifications in the maintenance of acquired thermotolerance (heat stress [HS] memory). HS memory is associated with the accumulation of histone H3 lysine 4 di- and trimethylation at memory-related loci. This accumulation outlasts their transcriptional activity and marks them as recently transcriptionally active. High accumulation of H3K4 methylation is associated with hyper-induction of gene expression upon a recurring HS. This transcriptional memory and the sustained accumulation of H3K4 methylation depend on HSFA2, a transcription factor that is required for HS memory, but not initial heat responses. Interestingly, HSFA2 associates with memory-related loci transiently during the early stages following HS. In summary, we show that transcriptional memory after HS is associated with sustained H3K4 hyper-methylation and depends on a hit-and-run transcription factor, thus providing a molecular framework for HS memory.

Keywords chromatin; H3K4 methylation; heat shock transcription factor; priming; transcriptional memory

Subject Categories Chromatin, Epigenetics, Genomics & Functional Genomics; Physiology; Plant Biology

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Introduction

Plants are sessile organisms that gauge and react to stressful conditions in order to ensure survival and reproductive success. Such stressful conditions include extreme temperatures, drought, salinity as well as pathogen and herbivore attacks. In nature, these are often chronic or recurring. Thus, plants have evolved strategies to remember a past exposure to stress to be better prepared for the next incident (Jaskiewicz *et al*, 2011; Ding *et al*, 2012a; Sani *et al*, 2013; Stief *et al*, 2014a). This area of research has only recently received increasing attention (Bruce *et al*, 2007; Conrath, 2011; Avramova, 2015; Hilker *et al*, 2015; Kim *et al*, 2015; Vriet *et al*, 2015), and the molecular basis of plant stress memory is still largely unknown. In this context, the term memory refers to the phenomenon where a signal of limited duration is perceived, stored and later retrieved, as evidenced by a modified response (Stief *et al*, 2014b). A related phenomenon in animals is hormesis, where a low-level stress treatment enhances resistance against the same and other stresses and increases growth, fecundity, and longevity (Gems & Partridge, 2008). Mechanistically, stress memory may be regulated at different levels ranging from metabolites to chromatin structure. In cases where stress memory involves modified gene expression patterns, a plausible hypothesis is that modifications of chromatin structure mediate such a memory.

Chromatin structure is an important determinant of the regulation of gene expression. Chromatin structure is modified through nucleosome positioning, histone variants, and posttranslational modification of histones (Struhl & Segal, 2013; Zentner & Henikoff, 2013). Depending on their nature and position, these modifications can promote or repress transcription by altering chromatin accessibility or interaction with specific protein complexes. For example, histone acetylation is associated with active transcription and correlates closely with the rate of transcription (Zentner & Henikoff, 2013). Histone H3 lysine 4 (H3K4) can be mono-, di-, or trimethylated, and its functions are largely conserved between yeast, animals, and plants (Santos-Rosa *et al*, 2002; Zhang *et al*, 2009; Shilatifard, 2012). In plants and animals, H3K4 trimethylation (H3K4me3) is highly correlated with active transcription (Guenther *et al*, 2007; Zhang *et al*, 2009) and is thought to be required for efficient RNA polymerase II elongation (Ding *et al*, 2012b; Kwak & Lis, 2013). Similar to H3K4me3, H3K4 dimethylation (H3K4me2) is associated with the 5′-region of genes, but it does not correlate with active transcription (Guenther *et al*, 2007; Zhang *et al*, 2009). H3K4 monomethylation (H3K4me1) is not correlated with gene expression and accumulates within transcribed regions (Zhang *et al*, 2009). In mammals, H3K4me1 is enriched at promoters and enhancers (Cheng *et al*, 2014).

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It has been hypothesized that recent transcriptional activity of a locus may be marked to mediate a modified response following a second stimulus. H3K4me3 and H3K4me2 have been discussed as such chromatin marks. In yeast, H3K4me3 hyper-methylation was proposed to act as a memory of recent transcriptional activity (Ng *et al*, 2003). It was suggested that elevated H3K4me3 is important for genes to be rapidly switched on and off by environmental stimuli and that it acts to prevent the associated genes from being silenced (Ng *et al*, 2003). In mammals, H3K4me3 marks genes that are poised for expression (Guenther *et al*, 2007). Transcriptional stress memory in plants has been described for recurring drought stress (Ding *et al*, 2012a), hyper-osmotic stress (Sani *et al*, 2013), and defense priming (Conrath, 2011; Jaskiewicz *et al*, 2011). Priming refers to the ability for quicker and more effective activation of specific cellular defenses upon a previous exposure to stress (Hilker *et al*, 2015). Originally used mainly for biotic stress responses, it is now also used to describe abiotic stress responses. In the above-mentioned cases of priming, molecular responses were associated with lasting changes in chromatin modifications. Transcriptional memory following drought stress was correlated with elevated H3K4me3 and stalled RNA polymerase II that is phosphorylated at Ser5 (Ding *et al*, 2012a). Whether chromatin modifications are involved in priming-like phenomena in response to other abiotic stresses such as HS is unknown. What triggers and maintains the deposition of these plant memory marks remains unclear in all reported cases. In particular, the interaction of such chromatin modifications with the transcription factors that govern the induction or sustained activation of the corresponding loci remains unclear.

Moderate heat stress (HS) allows a plant to acquire thermotolerance and subsequently withstand high temperatures that are lethal to a plant in the naïve state (Mittler *et al*, 2012). After returning to non-stress temperatures, acquired thermotolerance is maintained over several days, and this maintenance is genetically separable from the acquisition itself (Charng *et al*, 2006, 2007; Meiri & Breiman, 2009; Yeh *et al*, 2012; Stief *et al*, 2014a). We refer to this maintenance of acquired thermotolerance as HS memory. While the molecular events that lead to the acquisition of thermotolerance are relatively well understood, little is known about the mechanism of HS memory. The acquisition of thermotolerance involves the activation of heat shock transcription factors (HSFs) that induce the expression of heat shock proteins (HSPs), which protect cellular proteins from denaturation (Scharf *et al*, 2012). This HS response is conserved in plants, animals, and fungi (Richter *et al*, 2010). Beyond its function in the HS response, mammalian HSF1 has important roles in aging and pathologies (Vihervaara & Sistonen, 2014). Yeast and animals have only one or a few copies of HSF genes, yet many plants contain more than 20 copies with specialized functions. Among the 21 HSFs in *Arabidopsis thaliana*, so far eight have been shown to act in the responses to HS (Charng *et al*, 2007; Schramm *et al*, 2008; Ikeda *et al*, 2011; Liu *et al*, 2011; Scharf *et al*, 2012). Of these, HSFA2 has received special attention, as its expression is highly induced by heat (Nishizawa *et al*, 2006; Schramm *et al*, 2006). Interestingly, *HSFA2* is not required for the acquisition of thermotolerance, but specifically for its maintenance (Charng *et al*, 2007). Similar effects have been described for *HSA32* (Charng *et al*, 2006), and *miR156* (Stief *et al*, 2014a). Microarray analyses have identified a number of HS memory-related genes that were classified based on their sustained induction after HS, which lasts for at least 3 days (Stief *et al*, 2014a). They comprise many small HSPs (such as *HSP21*, *HSP22.0*, and *HSP18.2*), but also *ASCORBATE PEROXIDASE 2* (*APX2*). Their expression pattern is in strong contrast to that of HS-inducible non-memory genes such as *HSP70* and *HSP101*, whose expression peaks soon after HS and declines relatively quickly. *HSFA2* was reported to be required for the maintenance of high expression levels of several HS memory-related genes, but not for their induction, suggesting they could be direct targets of HSFA2 (Nishizawa *et al*, 2006; Charng *et al*, 2007). This idea was confirmed by *in vitro* binding studies (Schramm *et al*, 2006), but so far not *in planta*. How HS induction is maintained on some HSPs for several days but not on others remains an open question. Also, it is unknown how the molecular responses to a recurring HS during the memory period differ from the responses to the initial HS, that is, whether there is a transcriptional memory in the classical sense.

In general, the term "transcriptional memory" has been used to describe at least two different phenomena (D'Urso & Brickner, 2014). The first phenomenon is where transcriptional response upon a stimulus (such as induction) is modified if the gene was recently active compared to a copy of the gene that was not active before (type 1). An example is transcriptional memory of INO1 in yeast (Light *et al*, 2010). The second type of transcriptional memory refers to a phenomenon during which gene expression levels are lastingly modified after a transient signal (type 2). A classic example here is the epigenetic silencing of *FLC* in response to cold by vernalization (Berry & Dean, 2015). During HS memory, a modified re-induction after a second HS would indicate type 1, whereas the sustained induction of HS memory-related genes indicates type 2. For the sake of distinction and clarity, we here refer to type 1 transcriptional memory as such, whereas type 2 transcriptional memory will be referred to as sustained induction.

This study investigates a possible involvement of histone modifications during HS memory and their interaction with HSFA2. We show that HSFA2 is required for the maintenance of induction of HS memory-associated genes and directly binds to these loci *in planta*. Interestingly, this binding occurs transiently during the first few hours after HS. In addition, we identify sustained H3K4me3 and H3K4me2 as chromatin marks that discriminate HS memory-related genes from other HS-inducible genes. Elevated H3K4me3 and H3K4me2 levels persist after HSFA2 association with the locus has declined. Loci that maintain very high levels of H3K4 methylation show an increased response after a recurring HS 2 days after the primary HS, consistent with the definition of transcriptional memory. Thus, HS memory consists of two components, first, the sustained induction of HS memory-associated genes, and second, differential response upon recurring HS. Both components are associated with elevated levels of H3K4me3 and H3K4me2 and depend on functional HSFA2. In summary, our study identifies H3K4 methylation as a HS memory mark and reveals its dependency on a transiently binding transcription factor.

Results

Prolonged expression of HS memory-related genes is associated with changes in chromatin modifications

We hypothesized that sustained activation of HS memory-related genes may be associated with changes in chromatin modifications

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and that such modifications may prime the plant for recurring HS. To test this hypothesis, we investigated H3K4me3, H3K4me2, and H3K9 acetylation (H3K9ac) at the *HSP22.0* and *HSP70* loci as representatives of the HS memory-related and non-memory HS-inducible genes, respectively.

We analyzed histone modifications in the *A. thaliana* accession Col-0 by chromatin immunoprecipitation followed by quantitative real-time PCR (ChIP-qPCR) 4, 28 and 52 h after an acclimatizing HS (ACC), consisting of 60 min at 37°C, 90 min recovery at 23°C, 45 min at 44°C (Stief *et al*, 2014a). A temperature of 44°C is commonly used in *A. thaliana* HS studies (Yeh *et al*, 2012). In nature, full insolation causes leaf temperatures to rise much higher than air temperatures (Salisbury & Spomer, 1964). During the 2 days following ACC, *HSP22.0* transcript levels remain elevated while *HSP70* transcript levels are elevated only during the first 28 h (Stief *et al*, 2014a). For both genes, we analyzed one genic region at the 5′-end of the gene and one flanking region about three kb away in an intergenic region. For H3K9ac, we observed strong enrichment at 4 h that was specific for the genic region at both loci (Fig 1). This enrichment declined rapidly in *HSP70*, where it was no longer significantly enriched at 28 h and undetectable at 52 h relative to no-HS (NHS) control conditions (Fig 1B). In contrast, H3K9ac at *HSP22.0* declined more slowly and was still significantly enriched at 52 h (Fig 1A). We next investigated H3K4me3 levels at *HSP70*. A moderate enrichment (threefold) was observed 4 h after ACC that declined over the next 2 days and returned to baseline levels by 52 h. In contrast, *HSP22.0* showed strongly elevated H3K4me3 levels relative to NHS (up to 75-fold enrichment). These remained very highly elevated over the course of the experiment. For H3K4me2, no HS-dependent changes were observed at the *HSP70* locus (Fig 1B). However, at *HSP22.0*, enrichment of H3K4me2 was observed following HS. Interestingly, this modification accumulated only at the later time points (28, 52 h), while at 4 h no significant enrichment compared to the NHS control was found. Together, these results indicate that H3K4me3 and H3K4me2 accumulated on *HSP22.0* especially toward the later phases during HS memory, when expression and acetylation levels have declined (Figs 1A and 2A). Thus, given their sustained enrichment in comparison with H3K9ac, H3K4me3 and H3K4me2 at *HSP22.0* may store information of recent transcriptional activity. Interestingly, the genic regions of *HSP22.0* and *HSP70* displayed a slight enrichment of H3K4me2 and H3K4me3 relative to their respective intergenic control regions also in the absence of any HS (compare regions 1 and 2 in Fig 1, see Fig EV1 for no antibody control). This suggests that *HSP22.0* and *HSP70* may both be constitutively poised for rapid activation in response to elevated temperatures. Notably, our results for *HSP70* confirm the notion that there is no close correlation between high expression levels and H3K4me2 accumulation. At the physiological level, HS memory is detectable for at least 3 days after ACC (Stief *et al*, 2014a). Thus, the duration of the physiological memory phase is in good accordance with elevated H3K4me3 and H3K4me2 at *HSP22.0*.

Sustained activation of HS memory-related genes in response to ACC depends on *HSFA2*

HSFA2 was previously shown to be required for the maintenance of acquired thermotolerance (HS memory), but not acquisition *per se* (Charng *et al*, 2007; see also Stief *et al*, 2014a). We next investigated whether the expression profiles of HS memory-related and non-memory HS-inducible genes depended on HSFA2 during a time course of 3 days (76 h) following ACC (Fig 2A). To put further analyses on a broader basis, we investigated four memory genes and two HS-inducible non-memory genes. For the memory-related genes *HSP18.2*, *HSP22.0,* and *HSP21,* we observed unchanged induction 4 h after ACC in *hsfa2* mutants compared to wild type. This induction declined faster in *hsfa2* mutants over the next 3 days. The dependency on *HSFA2* was most pronounced in *HSP18.2* and somewhat weaker in *HSP21* and *HSP22.0*. For the memory gene *APX2*, transcript levels in *hsfa2* mutants were already lower at 4 h and declined strongly thereafter. These results indicate that for *HSP18.2*, *HSP21,* and *HSP22.0*, *HSFA2* is not required for the initial induction, but rather for the maintenance of high expression levels. For *APX2*, *HSFA2* was required for all time points analyzed. However, it is important to keep in mind that the first time point analyzed was already 4 h after ACC. In contrast, transcript levels of the non-memory genes *HSP70* and *HSP101* declined much faster. Moreover, *HSP70* and *HSP101* transcript levels were not dependent on *hsfa2*. These findings confirm and extend previous work (Charng *et al*, 2007).

To estimate whether transcript levels represented ongoing transcriptional activity, we sought to analyze unspliced transcript levels as a proxy for ongoing transcriptional activity (Bäurle *et al*, 2007; Kabelitz *et al*, 2014). Unspliced *APX2* transcripts were significantly induced in wild type between 4 and 52 h, indicating that active transcription continues for at least 2 days after ACC (Fig EV2A). In *hsfa2,* we observed a significantly lower induction of unspliced *APX2* transcripts at 4 h that was undetectable at later points. The same was true for the HS memory gene *HSP21* (Fig EV2B). In contrast, unspliced *HSP70* transcripts in wild type and *hsfa2* were strongly induced at 4 h, but quickly declined thereafter (Fig EV2C). Neither *HSP18.2* nor *HSP22.0* contains an intron, thus precluding corresponding analyses for these genes. In summary, the overall dynamics of unspliced transcripts closely mimicked that of the spliced transcripts, indicating that the sustained accumulation of *APX2* and *HSP21* transcripts reflects ongoing transcriptional activity for at least 52 h after ACC. In contrast, unspliced *HSP70* transcripts were not induced at 28 h and thereafter, corroborating the distinction between the two classes. Thus, our results indicate that HSFA2 regulates *APX2* and *HSP21* at the level of transcription.

We next sought to confirm our findings at the protein level. Unfortunately, antibodies were only available for HSP21 and HSP101. We analyzed protein levels of HSP21 and HSP101 in wild type and *hsfa2* after ACC using commercially available antibodies. HSP21 protein levels peaked only at 28–52 h after ACC and remained highly elevated until 76 h (Fig 2B). In contrast, HSP21 levels in *hsfa2* were much reduced between 28 and 76 h. HSP101 was induced by ACC and peaked at 4 h. Elevated protein levels were still observed at 76 h. There was no difference in *hsfa2,* indicating that *HSP101* expression did not depend on HSFA2. Thus, HSP101 protein levels are still elevated at 76 h despite the earlier decline in transcript levels, suggesting that the HSP101 protein may have a longer half life than the HSP21 protein. In summary, our protein analysis correlates well with the corresponding transcript analysis and indicates that proteins may be more stable than transcripts.

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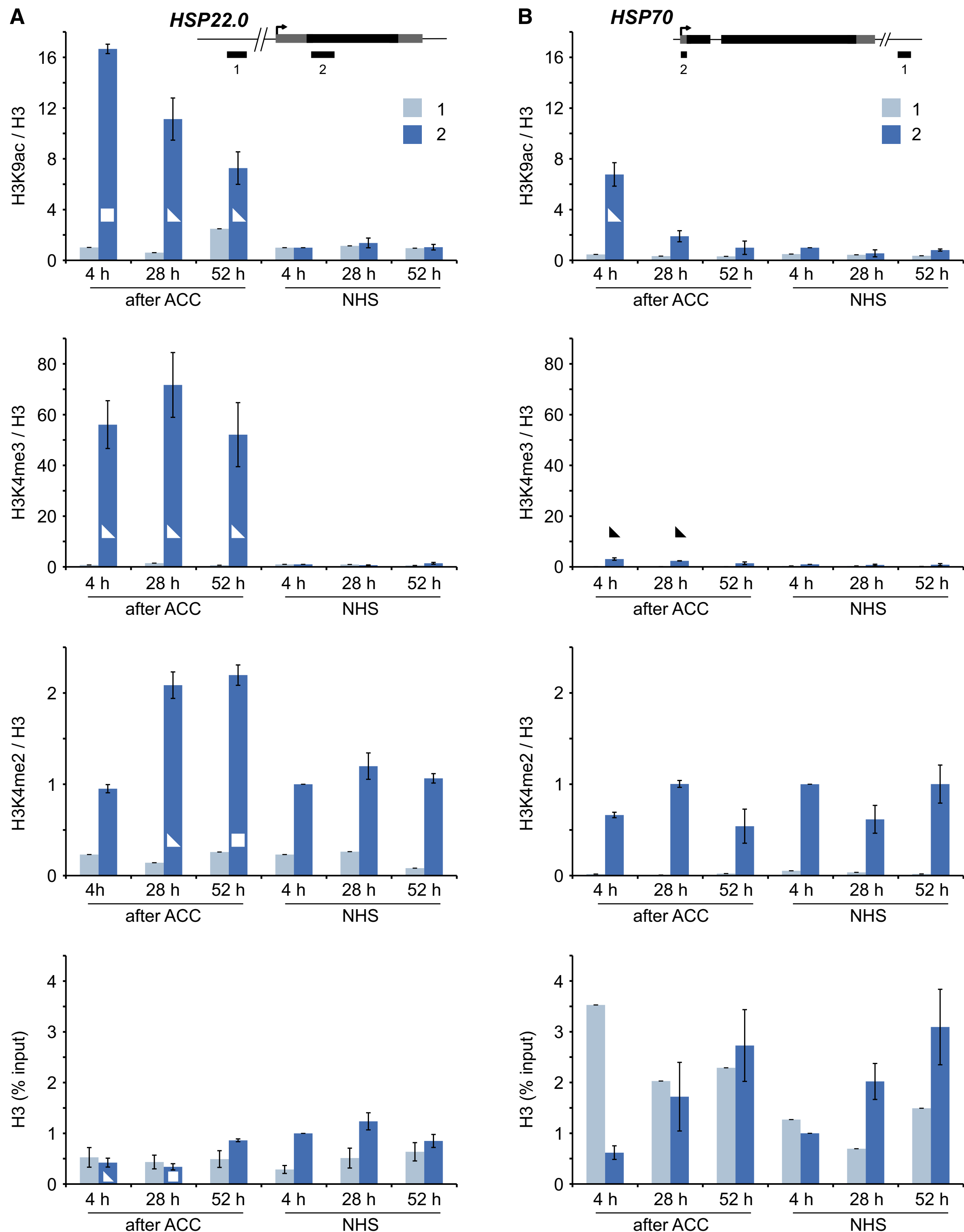

Figure 1. HS induces sustained H3K4me3 and H3K4me2 methylation at *HSP22.0*, but not at *HSP70*.

A, B Dynamics of histone modifications after an acclimatizing HS (ACC) or no HS (NHS) in Col-0 wild type at *HSP22.0* (A) and *HSP70* (B). Seedlings were subjected to ACC or a control treatment (NHS) 4 days after germination. At the indicated time points after the treatments, ChIP-qPCR was performed with antibodies against H3K9ac, H3K4me3, H3K4me2, and H3. Schematics show positions of regions analyzed. Amplicon positions relative to TSS are *HSP22.0*: 1, −2,570 bp; 2, +235 bp. HSP70: 1, 4,192 bp downstream of the 3′UTR; 2, +47 bp. Data shown are averages over three biological replicates. Amplification values were normalized to input, H3 and 4 h NHS region 2. The bottom panel shows the H3 signal normalized to input and 4 h NHS region 2. Squares and triangles within bars mark significant differences ($P < 0.01$ and $P < 0.05$, respectively, Student's *t*-test) between ACC and NHS samples of the same time point. Error bars indicate SE.

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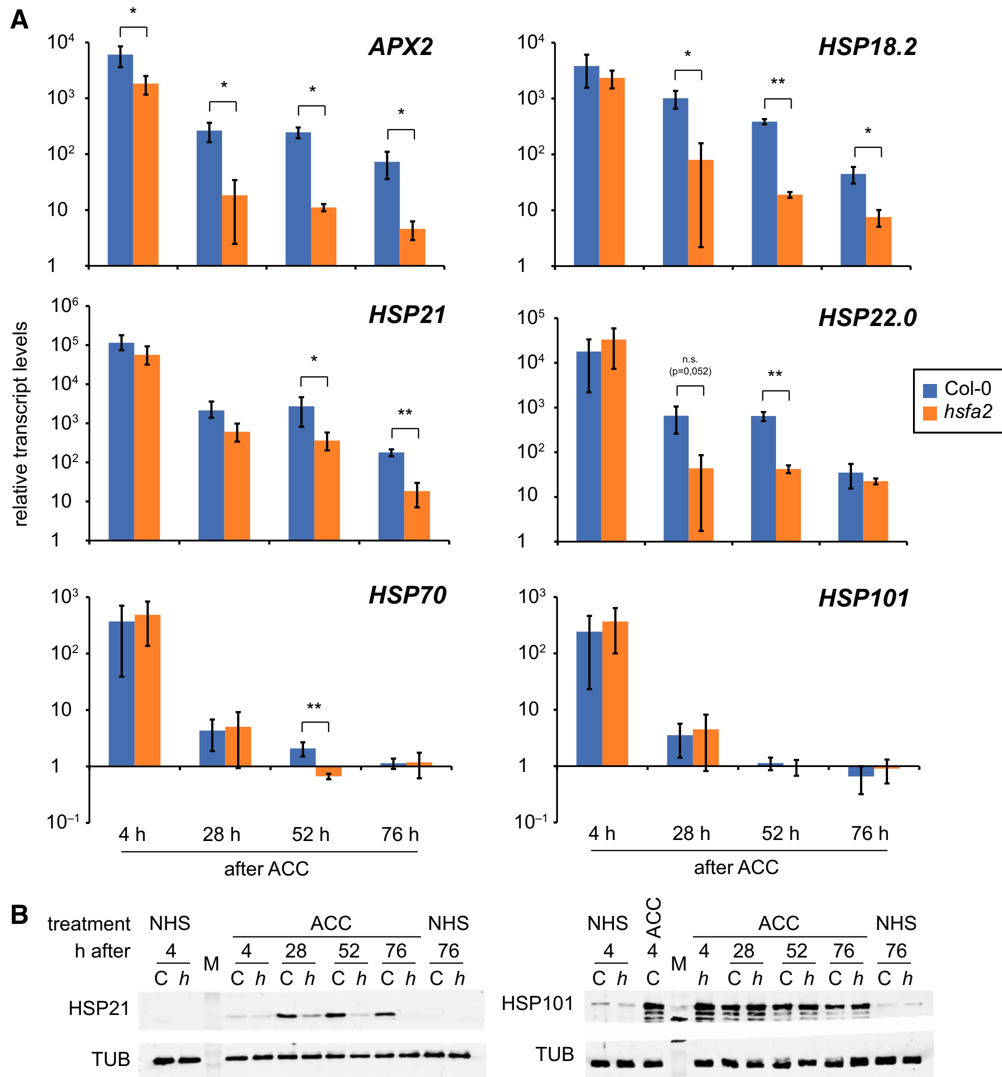

Figure 2. Sustained induction of HS memory-related gene expression after an acclimatizing HS depends on HSFA2.

A Transcript levels of the indicated HS-inducible genes after ACC in Col-0 (blue bars) and *hsfa2* (orange bars) as determined by quantitative reverse transcription PCR (qRT–PCR). Transcript levels were normalized to *TUB6* and the respective NHS harvested at the same time point ([GENE OF INTEREST$_{ACC \times h}$/TUB6$_{ACC \times h}$]/[GENE OF INTEREST$_{NHS \times h}$/TUB6$_{NHS \times h}$]). Note that the *y*-axis is on a $\log_{10}$ scale. Error bars show SD of at least three biological replicates. Asterisks show significant differences between the genotypes at the same time point based on Student's *t*-test (**P* < 0.05; ***P* < 0.01).

B Immunoblotting of HSP21 and HSP101 in Col-0 (C) and *hsfa2* (*h*) at the indicated time points after ACC or NHS. α-tubulin was used as a loading control. M, marker.

HSFA2 is required for sustained accumulation of H3K4me3 and H3K4me2

We next asked whether HSFA2 is also required for the accumulation of H3K4me3 and H3K4me2 at the memory-associated loci. To this end, we compared the accumulation of H3K4me2, H3K4me3, and H3K9ac in response to ACC in wild-type and *hsfa2* mutants by ChIP-qPCR. We analyzed *HSP18.2*, *HSP22.0,* and *APX2* as HS memory-related loci, and *HSP70* as a HS-inducible non-memory locus whose expression is independent of *HSFA2* (see Fig 2A). In wild type, H3K4me3 was strongly induced at the 5′-region of *HSP18.2*, *HSP22.0,* and *APX2* throughout the analyzed time course (4–52 h after ACC, compared to NHS controls, Figs 1A and 3A). There was no enrichment in intergenic regions flanking the loci. For *HSP22.0* and *APX2*, H3K4me3 remained very high at 52 h after ACC, and for *HSP18.2,* the levels were still elevated, but lower than at 4 h (Fig 3A). In contrast, at the *HSP70* locus, we observed only a moderate increase in H3K4me3 after ACC (Figs 1B and 3A). H3K4me3 accumulation at *HSP18.2*, *HSP22.0,* and *APX2* was dependent on *HSFA2* as *HSP18.2* and *APX2* had significantly lower H3K4me3 in *hsfa2* at all time points, and *HSP22.0* had a trend for lower H3K4me3 at 4 and 28 h and significantly lower levels at 52 h (Fig 3A). The slight increase in H3K4me3 at *HSP70* at 4 and 28 h was largely independent of *HSFA2*, in line with the expression analysis, which suggested that HSP70 is not a target. In summary, we observed sustained H3K4me3 accumulation at HS memory-related loci (*HSP18.2*, *HSP22.0*, *APX2*) that lasted for several days after ACC; this sustained H3K4me3 accumulation required functional HSFA2.

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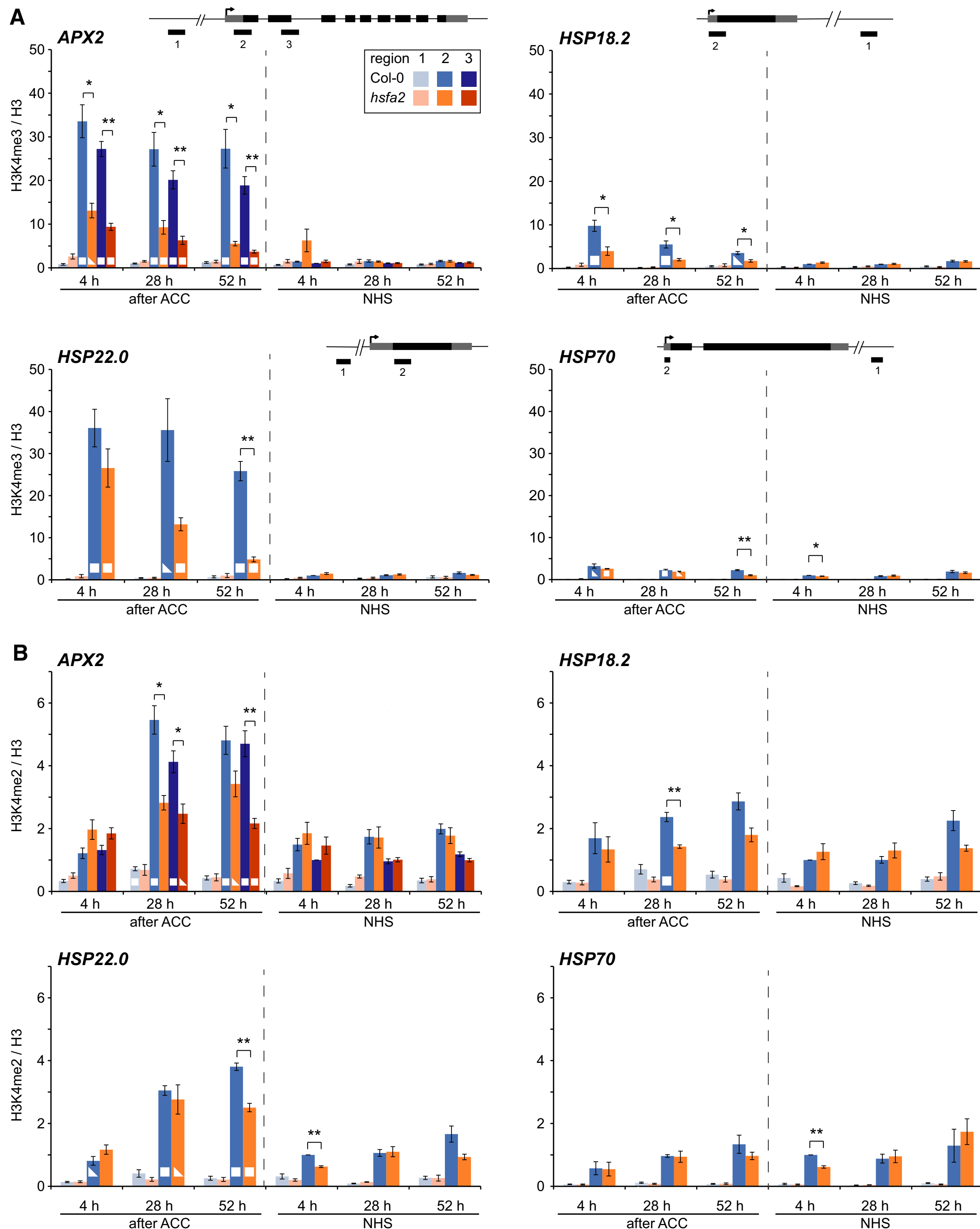

Figure 3. Sustained H3K4me3 and H3K4me2 at HS memory-related loci depends on HSFA2.

A, B H3K4me3 (A) or H3K4me2 (B) levels after an acclimatizing HS (ACC) in Col-0 and *hsfa2* at *HSP18.2*, *HSP22.0*, *HSP70*, and *APX2* as detected by ChIP-qPCR. Col-0 (blue bars) and *hsfa2* (orange bars) seedlings were subjected to ACC or no treatment (NHS) 4 d after germination. At the indicated time points after the treatment, ChIP-qPCR was performed with antibodies against H3K4me3 (A), H3K4me2 (B) and H3 (for normalization). Schematics show positions of regions analyzed (gray bars, UTR; black bar, exons). Intergenic control region 1 is 3,123 bp (*APX2*) or 2,570 bp (*HSP22.0*) upstream of the TSS, or 5,311 bp (*HSP18.2*) or 6,725 bp (*HSP70*) downstream of the TSS, respectively. Data are averages over four biological replicates. Amplification values were normalized to input and H3 and the Col-0 4 h NHS region 2 (*HSP18.2, HSP22.0,* and *HSP70*) or region 3 (for *APX2*). *$P < 0.05$; **$P < 0.01$ for differences between genotypes at the same time point and treatment; squares and triangles within bars mark significant differences ($P < 0.01$ and $P < 0.05$, respectively) between ACC and NHS samples of the same time point and genotype, Student's *t*-test. Error bars indicate SE.

We next analyzed H3K4me2 accumulation (Fig 3B). Similar to what we observed previously for *HSP22.0* (Fig 1A), H3K4me2 at the 5′-region of *HSP18.2* and *APX2* was clearly enriched after ACC in wild type (Fig 3B). Remarkably, this enrichment tended to increase during the course of the experiment and reached highest levels only at 28 or 52 h after ACC. For *HSP70*, H3K4me2 after ACC was not increased. At the *APX2* locus, H3K4me2 enrichment was high and depended on *HSFA2* at 28 h and 52 h. Similar results were obtained for *HSP18.2* and *HSP22.0*. In contrast, H3K4me2 levels at *HSP70* were not induced after ACC and this did not change in *hsfa2*. For all genes analyzed, no accumulation of H3K4me2 was observed at the intergenic region. In summary, H3K4me2 does not directly correlate with transcriptional activity. Instead, our findings support the notion that H3K4me2 acts as a mark of recent transcriptional activity in HS memory and that *HSFA2* is required for the sustained accumulation of this mark.

Lastly, we analyzed H3K9ac levels after ACC (Fig 4). At *HSP70*, we observed enrichment at 4 h but not thereafter. This enrichment was not affected in *hsfa2*. At *HSP18.2* and *APX2*, H3K9ac was enriched strongly at 4 h and weaker thereafter and the enrichment depended on *HSFA2*. At *HSP22.0*, sustained accumulation of H3K9ac depended on HSFA2 during the later time points (Figs 1A and 4). Thus, H3K9ac levels during HS memory largely follow transcriptional activity, however, at most HS memory-related genes investigated, acetylation levels appear to decline earlier than transcription. In summary, especially for *APX2* and *HSP18.2*, H3K4me3 and H3K4me2 persist longer than H3K9ac after ACC.

HSFA2 expression is induced by HS and peaks during the first day after HS

Following ACC, *HSFA2* transcript levels peaked at 4 h and declined rapidly thereafter (Fig EV3A). This is in contrast to the expression, H3K4me3 and H3K4me2 dynamics of the suspected target genes *HSP18.2*, *HSP21*, and *HSP22.0*, which all show more sustained changes (Fig 2A). One possibility is that HSFA2 protein is much more stable than its transcripts. To investigate this, we created transgenic *A. thaliana* plants expressing a fusion of the yellow fluorescent protein (*YFP*) to the endogenous *HSFA2* gene (*pHSFA2::HSFA2-YFP*) in the *hsfa2* background. The construct complemented

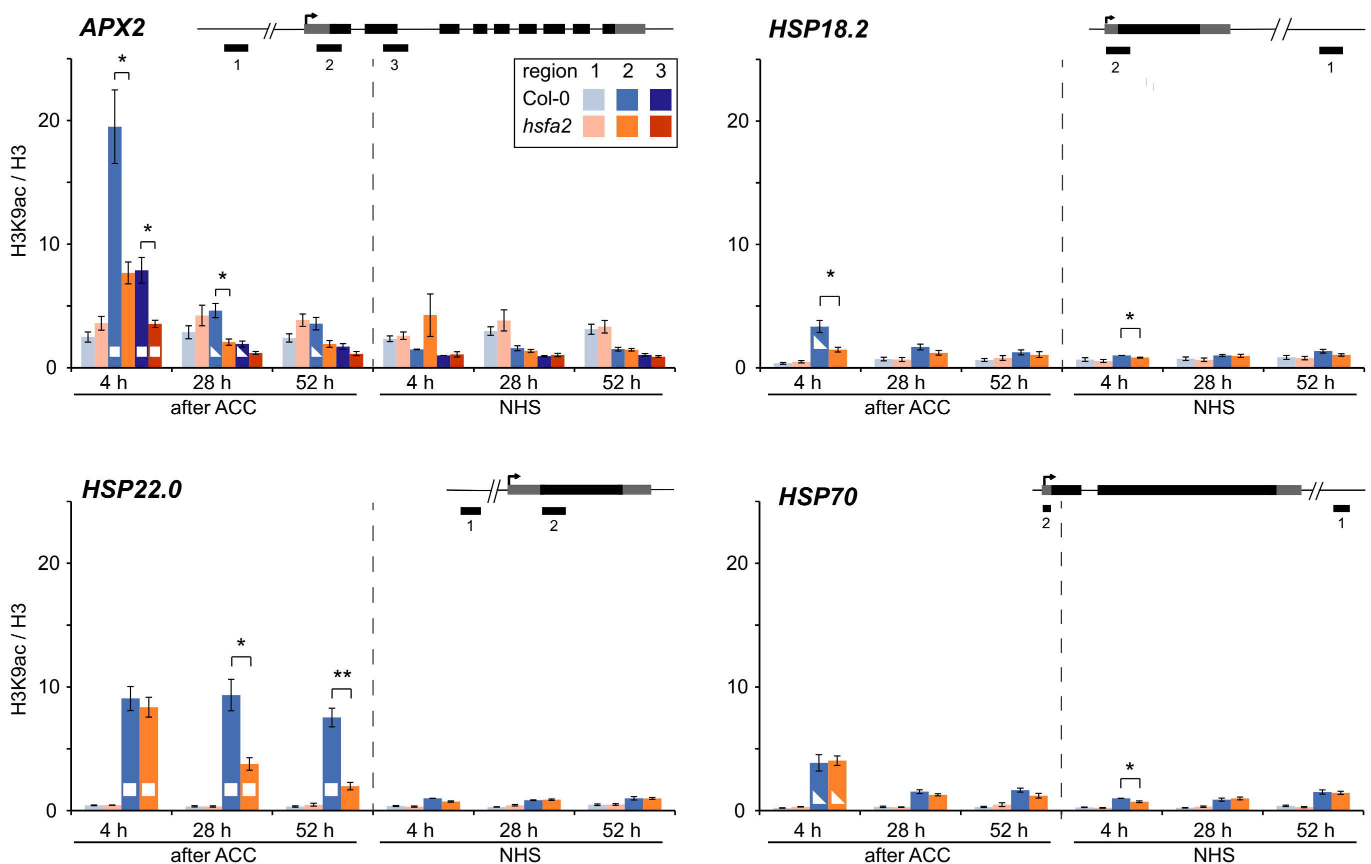

Figure 4. Histone H3K9ac profiles after ACC in Col-0 and *hsfa2* at *HSP18.2*, *HSP22.0*, *HSP70*, and *APX2* as detected by ChIP-qPCR.
Col-0 (blue bars) and *hsfa2* (orange bars) seedlings were subjected to ACC or control treatment (NHS) 4 days after germination. At the indicated time points after the treatments, ChIP-qPCR was performed with antibodies against H3K9ac or H3. Schematics (gray bars, UTR; black bar, exons) show positions of regions analyzed. Intergenic control region 1 is 3,123 bp (*APX2*) or 2,570 bp (*HSP22.0*) upstream of the TSS, or 5,311 bp (*HSP18.2*) or 6,725 bp (*HSP70*) downstream of the TSS, respectively. Data are averages over four biological replicates. Amplification values were normalized to input and H3 and the Col-0 4 h NHS region 2 (*HSP18.2*, *HSP22.0*, and *HSP70*) or 3 (for *APX2*). **P* < 0.05; ***P* < 0.01 for differences between genotypes at the same time point and treatment; squares and triangles within bars mark significant differences (*P* < 0.01 and *P* < 0.05, respectively) between ACC and NHS samples of the same time point and genotype, Student's *t*-test. Error bars indicate SE.

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the *hsfa2* mutant as evidenced by complementation of the loss of HS memory phenotype (Fig EV3B) and by restoration of HSFA2-dependent protein levels after HS (Fig EV3C). Immunoblotting with an anti-GFP antibody indicated that HSFA2-YFP levels peaked at 2–4 h after ACC (Fig EV3D), thereby closely resembling the endogenous *HSFA2* transcript levels (Fig EV3A). In addition, we investigated the expression and localization of HSFA2-YFP in the root tips of seedlings that were treated with ACC 3 days after germination (Fig EV4). We observed a strong induction of HSFA2-YFP after 1 h of 37°C that was maintained until 8 h after ACC. The HSFA2-YFP signal was mostly nuclear. The next day, fluorescence levels were markedly decreased, similar to the signal in NHS seedlings (Fig EV4H and I). The signal also appeared more diffuse with potentially a cytoplasmic component. These results corroborate the immunoblotting results and the endogenous transcript analysis and indicate that HSFA2 is a strongly heat-inducible protein that peaks within hours after heat exposure and then rapidly decreases in abundance. Interestingly, these results suggest that by the time the effect of a loss of HSFA2 is apparent at the transcript and physiological levels, its protein levels are already much reduced.

HSFA2 associates directly with the promoter of HS memory-related genes

To further investigate the interaction with HS memory-related loci, we next studied their association with HSFA2-YFP *in planta* using ChIP-qPCR. HSF transcription factors are known to bind heat shock elements (HSE, 5′- nnGnAnnTnCtn-3′) (Nover *et al*, 2001). We identified putative HSEs in the promoters of *APX2*, *HSP18.2*, *HSP22.0*, and *HSP70* (Fig 5, insets) (Schramm *et al*, 2006; Jung *et al*, 2013). We then performed ChIP at 0.5, 4, or 28 h after ACC or NHS. Besides regions containing HSEs, we assayed intergenic regions flanking each gene to estimate technical background (about three kb away, see legend of Fig 5 for details). HSFA2-YFP was enriched around the promoter-proximal HSEs of *HSP18.2*, *HSP22.0,* and *APX2*, compared to NHS control samples and to the intergenic regions not spanning an HSE (Fig 5). This is consistent with the notion that HSFA2 directly regulates these HS memory-related genes through binding of promoter-proximal HSEs. We also found a slight enrichment of HSFA2-YFP at HSE-containing regions of *HSP70* and *ACT7* after ACC compared to NHS controls and regions not containing an HSE (Fig 5D and E). Interestingly, at *HSP18.2*, *HSP22.0,* and *APX2,* the strongest enrichment was observed 30 min after the end of the ACC treatment (Fig 5A–C). For *HSP18.2*, HSFA2 binding was already strongly decreased at 4 h after ACC, while the decrease was slower at *HSP22.0* and *APX2*. At 28 h, HSFA2 binding was hardly detectable. Thus, HSFA2 binds to *HSP18.2*, *HSP22.0,* and *APX2* directly and predominantly during the first few hours after ACC.

Transcriptional memory after recurring HS depends on HSFA2

We next asked whether *HSP18.2*, *HSP22.0*, *APX2,* and *HSP70* activation is modified upon a second HS 2 days after a primary HS. To this end, we revised the HS treatment so that each HS was for 1 h at 37°C. This shortened the duration of the HS treatment and allowed us to investigate (relatively) early time points after HS. In addition, this HS is not lethal even for non-acclimatized seedlings, which facilitated interpretation of the results. HS was applied either 4 days after germination, 6 days after germination, or both 4 and 6 days after germination. Expression was monitored 1, 2, and 4 h after the end of the HS. For the treatment on day 4, transcript levels were analyzed also 28 and 52 h after the HS to allow estimation of transcript levels at the time of the second HS. Generally, we observed a similar HS-dependent induction of expression compared to the ACC treatment, however with a more rapid decline of transcript levels (Fig 6). For *APX2*, we observed a very strong hyper-induction after repeated HS compared to the single HS on day 6 or on day 4 (Fig 6A). For *HSP22.0*, we observed a trend for increased induction at 1 h after the repeated HS (day 4 + 6), compared to seedlings that had received only the second (day 6) HS (Fig 6C). For *HSP18.2* and *HSP70,* the induction profiles were similar for all three treatments (HS on day 4, day 6, day 4 + 6; Fig 6B and D). Thus, *APX2* and possibly *HSP22.0* show transcriptional memory as the induction upon repeated HS is increased compared with the induction after either single HS. This transcriptional memory is active for at least 2 days, consistent with the maintenance of elevated H3K4me3 and H3K4me2 at these loci. It was abolished in *hsfa2* mutants, which induced expression of *APX2* and *HSP22.0* to the same level regardless if a single HS or a repeated HS was applied (Fig 6A and C).

To facilitate the comparison between transcriptional memory following HS and the chromatin states investigated above, we subsequently modified the experiment using the ACC treatment as primary HS and a HS of 1 h at 37°C as second HS after a lag phase of 2 d or 3 d. We observed hyper-induction in plants that had received the ACC treatment 2 days before for *APX2*, *HSP22.0,* and *HSP21*, but not for *HSP18.2* and *HSP70* (Fig EV5), thus confirming that *APX2* and *HSP22.0* show transcriptional memory after HS. The effect was reduced, but not fully abolished in HSFA2, suggesting that the stronger primary heat treatment (ACC) may trigger the activation of an additional factor acting redundantly with HSFA2. Taken together, we demonstrated transcriptional memory in response to HS for *APX2* and *HSP22.0* that correlated well with sustained H3K4me3 and H3K4me2. In addition, both processes required HSFA2.

Discussion

Transcriptional memory was described in yeast, mammals, and plants as the phenomenon where gene induction is faster or stronger upon a repeated signal following an intervening lag phase or repressive phase (D'Urso & Brickner, 2014; Stief *et al*, 2014b; Avramova, 2015). In yeast, the mechanistic understanding of transcriptional memory is most advanced, and several non-exclusive mechanisms have been proposed (Brickner *et al*, 2007; Laine *et al*, 2009; Tan-Wong *et al*, 2009; Light *et al*, 2010, 2013). They involve a lasting modification of chromatin, either through histone modifications, histone variants, gene looping, or altered subnuclear localization of the memory loci. In plants, transcriptional memory is less well understood. It is increasingly recognized that plants can remember a past exposure to environmental stress and this may serve them to be better prepared for a future stress incident. In the case of HS, such a memory has been thoroughly characterized at the whole plant and molecular level (Charng *et al*, 2006, 2007; Meiri & Breiman, 2009; Stief *et al*, 2014a,b). While a number of reports have implicated epigenetic regulators in plant HS responses (Liu *et al*,

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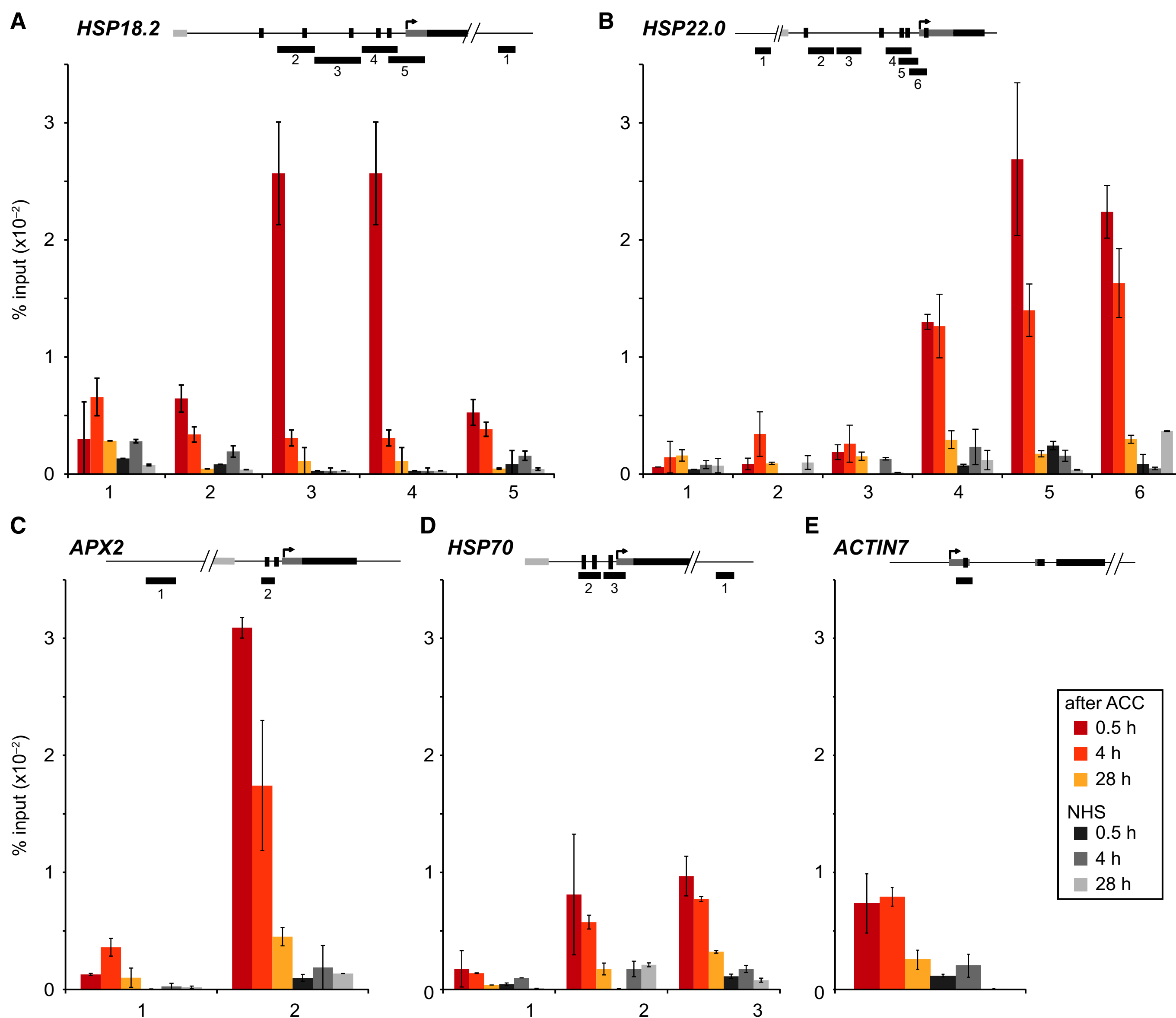

Figure 5. HSFA2 associates with HS memory-related genes transiently after an acclimatizing HS.

A–E Binding of HSFA2-YFP to the promoter regions of *HSP18.2* (A), *HSP22.0* (B), *APX2* (C), HSP70 (D), and *ACTIN7* (E) was determined by ChIP-qPCR. Quantifications represent the enrichment relative to input in %. Red–orange–yellow bars represent time points (0.5 h–4 h–28 h) after a ACC and gray bars represent the respective NHS control samples. Schematics show the promoter region of the respective gene with regions analyzed (arrow, TSS; gray bar, 5′UTR; black bar, exon; black boxes, heat shock elements (5′-nnGnAnnTnCtn-3′)). Regions are drawn to scale. Intergenic control region 1 is 4,610 bp (*HSP18.2*) and 4,192 bp (*HSP70*) downstream of the 3′UTR and 2,570 bp (*HSP22.0*) or 3,123 bp (*APX2*) upstream of the TSS, respectively. One representative experiment out of at least four biological replicates is shown. Error bars are SD of three replicates.

2015), the involvement of chromatin modifications in HS memory was hitherto unknown. Here, we have shown that HS induces sustained accumulation of H3K4me3 and H3K4me2 at specific loci. In particular, H3K4me2 peaks later than the corresponding transcript levels, hence marking these loci as recently transcriptionally active. We further demonstrated that increased H3K4me3 and H3K4me2 at some of these loci were associated with increased activation upon a recurring HS. Thus, the physiologically defined phenomenon of HS memory has a molecular correlate in the transcriptional memory at *APX2* and *HSP22.0*. Moreover, both the transcriptional memory at these loci and the sustained H3K4me3 and H3K4me2 depend on an HSF transcription factor that transiently associates with these loci.

Previously, transcriptional memory in response to drought stress was reported and this was associated with elevated H3K4me3 and Ser5P RNA polymerase II levels, suggesting stalling of RNA polymerase II (Ding *et al*, 2012a). H3K4me2 was not investigated in this context. Interestingly, neither of the tested transcriptional regulators (ABA-dependent transcription factors and the histone methyltransferase ATX1) was required for drought transcriptional memory (Ding *et al*, 2012a). Here, we uncovered that HS memory is

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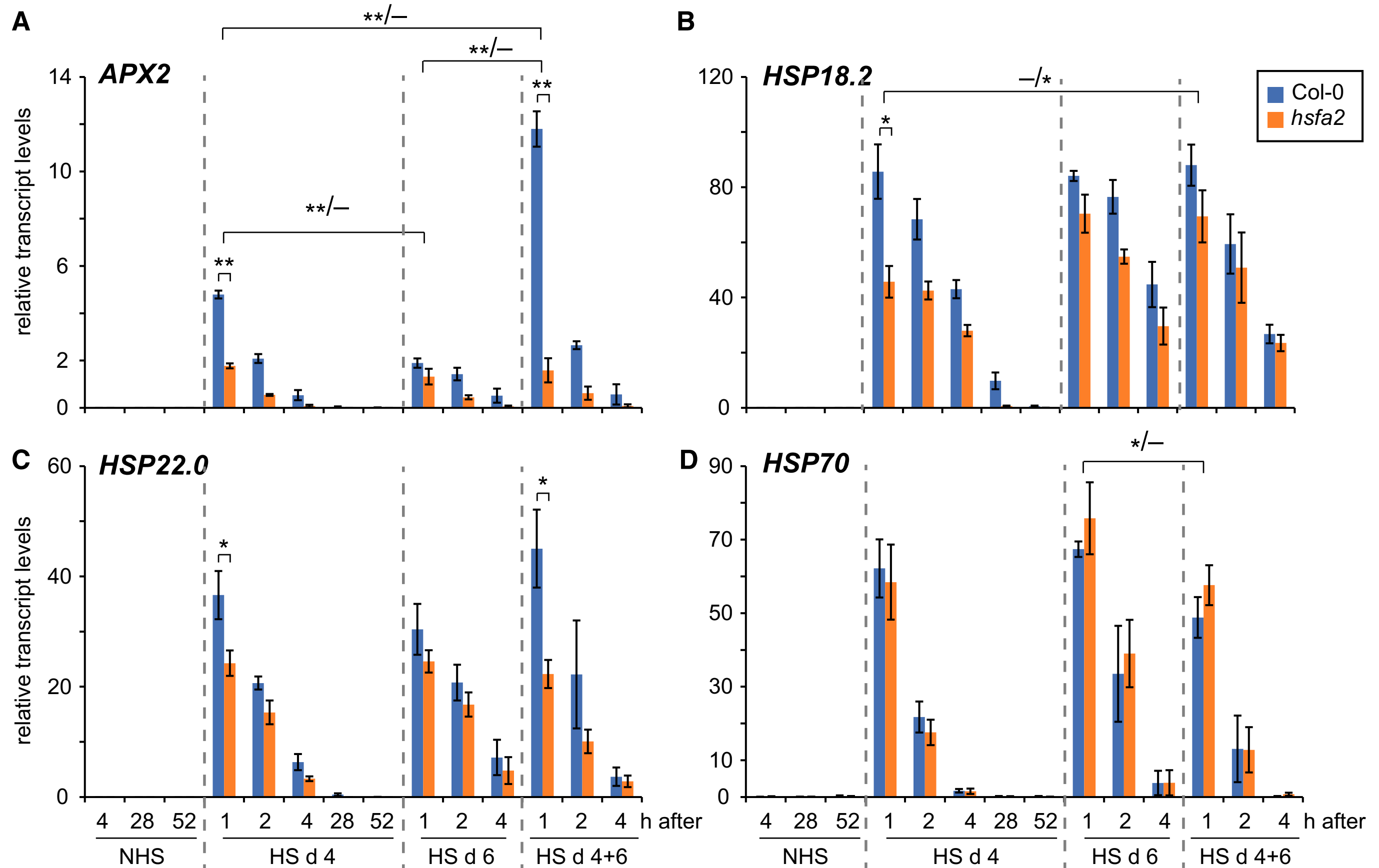

Figure 6. Expression profiles of HS memory-related genes after a recurring HS show HSFA2-dependent transcriptional memory.

A–D Response of selected HS-inducible loci upon recurring HS as determined by qRT–PCR. Transcript levels of *APX2* (A), *HSP18.2* (B), *HSP22.0* (C), and *HSP70* (D) in Col-0 (blue bars) and *hsfa2* (orange bars) at the indicated time points after a single HS or two HS separated by 2 days. Plants were subjected to 37°C for 60 min on either day 4, day 6, or on day 4 + day 6 after germination. NHS samples were harvested at the same time points as HS day 4 samples. Transcript levels of four biological replicates normalized to *TUB6* are shown. Small parentheses compare the two genotypes at the same condition, and large parentheses compare the same genotype across two treatments (at 1 h after treatment) with Col-0 indicated first (Col-0/*hsfa2*). Error bars show SE over four biological replicates. *$P < 0.05$; **$P < 0.01$; Student's *t*-test.

associated with elevated H3K4me3 and H3K4me2. Moreover, we identified a transcription factor that is required for both, hypermethylation of H3K4 and transcriptional memory at *APX2* and *HSP22.0*, providing a molecular framework for HS memory in plants. During HS memory, H3K4me3 and H3K4me2 both accumulate strongly and especially during the later stages (28, 52 h) at HS memory-related genes. In contrast, the HS-inducible non-memory locus *HSP70* accumulates at 4 and 28 h moderate levels of H3K4me3 but no H3K4me2, despite strong transcriptional activation. The accumulation of H3K4me3 and H3K4me2 is particularly high and long-lasting at *APX2* and *HSP22.0*. It is somewhat lower at *HSP18.2*. Interestingly, this appears to correlate with the strength of transcriptional memory upon recurring HS, being most pronounced for *APX2*, somewhat weaker for *HSP22.0* and undetectable for *HSP18.2* and *HSP70*. These findings are in line with the notion that H3K4me3 and/or H3K4me2 act as a mark of recent transcriptional activity (Ng *et al*, 2003; Guenther *et al*, 2007; Light *et al*, 2013). Interestingly, H3K4me2 appeared to peak even later than H3K4me3, suggesting that both marks may have slightly different functions. This is in line with genome-wide ChIP-chip experiments in *A. thaliana* that found a good correlation of active transcription with H3K4me3, but not H3K4me2 (Zhang *et al*, 2009). Thus, H3K4me2 may convey a more sustained mark of recent transcriptional activity than H3K4me3. However, experiments that specifically modify one but not the other H3K4 methylation mark will be required to disentangle the functions of H3K4me3 and H3K4me2. Also, it remains to be investigated whether the sustained accumulation of H3K4 methylation is caused by sustained methylation, reduced demethylation, or reduced nucleosome replacement.

The responses of plants to chronic or recurring stress are not well understood, despite their economic and ecological relevance. HSFA2 is a master regulator of the HSF signaling network involved in cellular responses to various types of environmental stress. HSFA2 is induced not only upon HS, but also upon drought stress, osmotic stress, salt stress, high light, and hypoxia (Nishizawa *et al*, 2006; Ogawa *et al*, 2007; Banti *et al*, 2010; Liu *et al*, 2011; Jung *et al*, 2013). *HSFA2* expression is activated by HSFA1 isoforms (Liu *et al*, 2011; Nishizawa-Yokoi *et al*, 2011). Constitutive overexpression of HSFA2 enhances heat, salt, and osmotic stress tolerance (Nishizawa *et al*, 2006; Ogawa *et al*, 2007). Several putative target genes of

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HSFA2 were identified based on differential gene expression, *in vitro* binding studies and transient protoplast reporter assays (Nishizawa *et al*, 2006; Schramm *et al*, 2006). Here, we showed direct binding of HSFA2 to putative target genes using ChIP in *A. thaliana*, thereby confirming earlier predictions. Unexpectedly, HSFA2 was associated with its target genes most highly as early as 30 min after ACC. In contrast, gene expression studies and physiological analyses indicate that HSFA2 is specifically required for the maintenance of acquired thermotolerance and not its induction (this study and (Charng *et al*, 2007)). Accordingly, for *HSP18.2* and *HSP22.0* expression, the strongest effect in *hsfa2* was observed only 1–2 days after ACC, but was weak or undetectable 4 h after ACC (Fig 2). Notably, even at 4 h after ACC, binding of HSFA2 at *HSP18.2* and *APX2* was already reduced compared to 0.5 h after ACC. For *APX2*, the analysis of unspliced transcript levels indicated that transcription was elevated until at least 52 h after ACC. Also, transcriptional activity was lower in *hsfa2* throughout the experiment (at 4, 28, and 52 h after ACC). The initial induction of the HS memory-related genes is mediated by isoforms of HSFA1 (HSFA1a, HSFA1b, HSF1d, HSFA1e) that also induce *HSFA2* expression (Liu *et al*, 2011). In contrast to *HSFA2*, *HSFA1* isoforms are constitutively expressed and rapidly activated upon HS. As overexpression of *HSFA2* constitutively activates its target genes (Nishizawa *et al*, 2006), it can be assumed that this sequence of events (HSFA1 acting before HSFA2) is primarily caused by the absence of HSFA2 protein upon the first response to HS rather than the inability of HSFA2 to induce inactive target genes.

The binding kinetics of HSFA2 and the expression kinetics of its target genes suggest that HSFA2 may act according to the "hit-and-run" model for transcription factors. This model was first proposed several decades ago (Schaffner, 1988) and has recently received experimental support with the development of better spatial and temporal resolution of binding studies (Para *et al*, 2014; Charoensawan *et al*, 2015). According to this model, transcription factors bind transiently to their targets, even when activating lasting gene expression. This may be achieved by recruiting sequence-specific or general transcriptional activators that induce lasting gene expression. Thus, our findings suggest that HSFA2 recruits (directly or indirectly) chromatin-modifying factors such as H3K4 histone methyltransferases to memory loci to mediate their sustained induction and hyper-inducibility upon a recurring HS (Fig 7). The model is supported by our finding that HSFA2 is required for sustained accumulation of H3K4me3 and H3K4me2. Direct interaction of sequence-specific transcription factors with the H3K4 methyltransferase Set1-containing COMPASS complex has been described in several organisms including plants (Ruthenburg *et al*, 2007; Bertero *et al*, 2015; Song *et al*, 2015). *A. thaliana* contains up to ten histone H3K4 methyltransferases that are related to yeast Set1. Further studies will be required to identify the interacting proteins recruited by HSFA2. The lack of sustained activation and H3K4 hyper-methylation in *hsfa2* mutants and at HSFA2-independent loci such as *HSP70* suggests that such interacting proteins may be specifically recruited by HSFA2, but not by HSFA1 isoforms.

In summary, we have identified transcriptional memory in response to HS and show that this is associated with elevated H3K4 methylation levels and dependent on a HSF transcription factor that transiently binds these loci. Similarly, sustained activation of HS memory loci requires *HSFA2* and is associated with high levels of

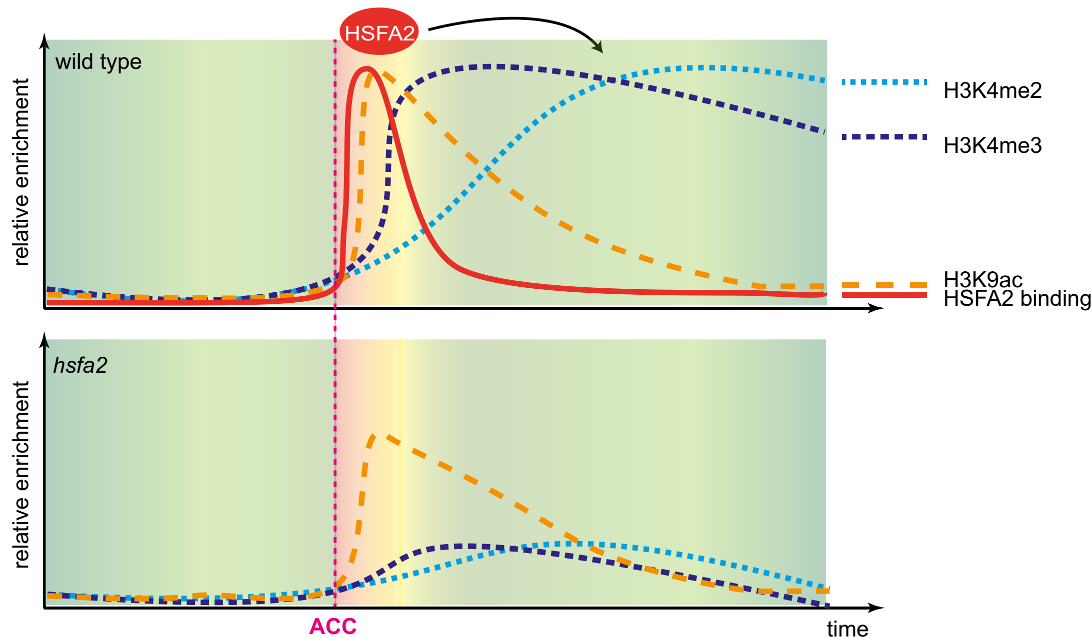

Figure 7. Transient binding of HSFA2 governs sustained H3K4 methylation after HS.
Top panel: Transient binding of HSFA2 after HS induces sustained induction of H3K4me2 (light blue) and H3K4me3 (dark blue) through direct or indirect recruitment of chromatin-modifying factors. H3K4me3 and H3K4me2 levels remain elevated after H3K9ac (orange) and HSFA2 binding (red) have decreased and hence mark recent transcriptional activity of the locus, leading to sustained induction and transcriptional memory upon recurring stress. Bottom panel: In *hsfa2*, H3K4me2 and H3K4me3 remain low, while the H3K9ac levels are only slightly changed compared to wild type. Profiles were drawn based on the data obtained for the *APX2* locus.

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H3K4me3 and H3K4me2. Our findings have implications for the engineering of heat-resistant plants and for the basic research question how stress memory and transcriptional memory are realized mechanistically.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (ecotype Col-0) seedlings were grown on GM medium (1% [w/v] glucose) under a 16-h/8-h light/dark cycle at 23°C/21°C. The *hsfa2-1* allele (Charng *et al*, 2007) was obtained from the Nottingham Stock Centre (SALK_008978). Seeds were stratified for 5 days at 4°C in darkness. Heat treatments were performed on 4-day-old seedlings unless stated otherwise. For expression analyses, ChIP experiments, and protein extraction, seedlings were treated with an acclimatizing HS (ACC) of 37°C for 60 min, 23°C for 90 min, and 44°C for 45 min starting 8 h after light onset. After HS, plants were returned to normal growth conditions. For the repeated HS experiment, each HS was 60 min at 37°C on the indicated days unless otherwise stated. Maintenance of acquired thermotolerance assays were performed as described (Stief *et al*, 2014a). Accession numbers of the genes studied were *HSP18.2* (*At5g59720*), *HSP21* (*At4g27670*), *HSP22.0* (*At4g10250*), *HSP70* (*At3g12580*), *HSP101* (*At1g74310*), *APX2* (*At3g09640*), *HSFA2* (*At2g26150*), *ACT7* (*At5g09810*), and *TUB6* (*At5g12250*).

Construction of *pHSFA2::HSFA2-YFP*

To generate *pHSFA2::HSFA2-venusYFP*, a 2.8-kb fragment encompassing the promoter and coding sequence of *HSFA2* was amplified from genomic DNA mutating the stop codon (Hsfa2 5-FSalI GTCGACGGACTCTGCGAGCAAAGCTAC, Hsfa2 5-RnostpSmaI CC CGG GGA AGG TTCCGAACCAAGAAAACCC) and fused to *venusYFP*. A 0.7-kb fragment containing the 3′UTR and further sequences (Hsfa2 3-FSacI GAGCTCTATACGGTATTAACACATCTACTTG, Hsfa2 3-RERI GAATTCCTTATAGTTACGTGTTGTGTTGT) was added. The resulting *pHSFA2::HSFA2-YFP* construct was transformed into *hsfa2-1*.

Chromatin immunoprecipitation

Seedlings were harvested and cross-linked under vacuum in ice-cold MC buffer/1% (v/v) formaldehyde (Kaufmann *et al*, 2010) for 2 × 5 min or 2 × 10 min for histone ChIP or HSFA2-YFPChIP, respectively. Chromatin was extracted as described (Kaufmann *et al*, 2010). Chromatin was sonified using a Diagenode Bioruptor (18 cycles/30 sec on/off) on low intensity settings. For histone ChIP, equal amounts of chromatin from the same preparation were immunoprecipitated at 4°C over night using antibodies against H3 (Abcam, ab1791), H3K4me2 (Millipore, 07-030), H3K4me3 (Abcam, ab8580), or H3K9ac (Abcam, ab10814). For HSFA2-YFP-ChIP, chromatin was incubated with α-GFP paramagnetic beads for 1 h at 4°C and chromatin was recovered using a GFP isolation kit (Miltenyi Biotec). Immunoprecipitated DNA was quantified by qPCR (LightCycler480, Roche). Primers used are listed in Appendix Table S1.

Protein extraction and immunoblotting

Total protein was extracted from seedlings using extraction buffer (50 mM Bis–Tris, pH 7.6, 10% (v/v) glycerol, 5 mM DTT, 1% (w/v) polyvinylpolypyrrolidone, complete Mini Protease inhibitor Cocktail (Roche)). One microgram of protein was loaded per lane and separated on either 12% (for HSP101 and HSFA2-YFP) or 14% (for HSP17.6, HSP17.7, and HSP21) SDS–PAGE gels. For immunodetection of proteins, the following antibodies were used: α-GFP (Abcam, ab290), α-tubulin (Sigma, T5168), α-HSP21, α-HSP101 (Agrisera, AS08285, AS07253). Secondary antibodies (goat-α-mouse-IRdye 800CW, #926-32210, goat-α-rabbit-IRdye 800CW, #926-32211, LICOR) were used for infrared detection.

RNA extraction, cDNA synthesis, and qRT–PCR

RNA extraction, cDNA synthesis, and qRT–PCR were done as described previously (Stief *et al*, 2014a). All data were normalized to *TUB6* using the comparative CT method. Data for Fig 2 was further normalized to NHS control ([GENE OF INTEREST$_{ACC\ x\ h}$/TUB6$_{ACC\ x\ h}$]/[GENE OF INTEREST$_{NHS\ x\ h}$/TUB6$_{NHS\ x\ h}$]) before averaging. For statistical analysis, unpaired Student's *t*-test was used as indicated. Primer sequences are listed in Appendix Table S1.

Microscopy

Transgenic or wild-type seedlings grown and treated as described above were imaged on a Zeiss LSM710 confocal microscope.

Expanded View for this article is available online.

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Author contributions

JL, KB, and IB conceived and designed the experiments. JL, KB, SA, and IB performed the experiments and analyzed the data. JL and IB wrote the manuscript with input from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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