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Role of H1 and DNA methylation in selective regulation of transposable elements during heat stress

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Summary

- Heat-stressed *Arabidopsis* plants release heterochromatin-associated transposable element (TE) silencing, yet it is not accompanied by major reductions of epigenetic repressive modifications. In this study, we explored the functional role of histone H1 in repressing heterochromatic TEs in response to heat stress.
- We generated and analyzed RNA and bisulfite-sequencing data of wild-type and *h1* mutant seedlings before and after heat stress.
- Loss of H1 caused activation of pericentromeric *Gypsy* elements upon heat treatment, despite these elements remaining highly methylated. By contrast, nonpericentromeric *Copia* elements became activated concomitantly with loss of DNA methylation. The same *Copia* elements became activated in heat-treated *chromomethylase 2* (*cmt2*) mutants, indicating that H1 represses *Copia* elements through maintaining DNA methylation under heat.
- We discovered that H1 is required for TE repression in response to heat stress, but its functional role differs depending on TE location. Strikingly, H1-deficient plants treated with the DNA methyltransferase inhibitor zebularine were highly tolerant to heat stress, suggesting that both H1 and DNA methylation redundantly suppress the plant response to heat stress.

Introduction

Transposable elements (TEs) constitute a large proportion of many eukaryotic genomes, including plants (McClintock, 1984; SanMiguel *et al.*, 1996; Kumar & Bennetzen, 1999; Havecker *et al.*, 2004). TEs lead to genetic variability and are therefore considered a major driving force for genome evolution (Mirouze & Paszkowski, 2011; Lisch, 2013; Belyayev, 2014; Grandbastien, 2015). Nevertheless, their ability to transpose can induce mutations and overall genetic instability, which has enforced the evolution of a complex epigenetic regulatory network to repress TE activation and transposition (Slotkin & Martienssen, 2007; Lisch, 2009; Zemach *et al.*, 2010; Gutzat & Mittelsten Scheid, 2012; Marí-Ordóñez *et al.*, 2013). As a consequence, in plants, most TEs are quiescent and both transcriptionally and transpositionally inactive (Ito *et al.*, 2011; Baubec *et al.*, 2014). However, under stress conditions, such as seasonal and daily temperature changes, TEs can be activated and increase genetic instability (Pecinka *et al.*, 2010; Tittel-Elmer *et al.*, 2010; Ito *et al.*, 2011; Bucher *et al.*, 2012; Cavrak *et al.*, 2014), yet only a small proportion of TEs become active under stress (Dubin *et al.*, 2018), revealing that there are so far largely unexplored mechanisms maintaining TE repression under stress conditions.

DNA methylation in the regulatory region of genes and TEs is crucial for silencing (Zemach *et al.*, 2010; Jones, 2012; Schübeler, 2015; Zhang *et al.*, 2018). Plants have DNA methylation in all three cytosine contexts (CG, CHG and CHH; H = A, T, C) (Feng *et al.*, 2010; Law & Jacobsen, 2010; Zemach *et al.*, 2013; Bewick & Schmitz, 2017), which are established and maintained by different mechanisms. The establishment and maintenance of CHH methylation are mediated by the DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) that is targeted by 24-nt small interfering RNAs in the RNA-directed DNA methylation (RdDM) pathway, which is mainly active on euchromatic TEs (Law & Jacobsen, 2010; Matzke & Mosher, 2014). In heterochromatic histone H1-containing regions, CHH methylation is maintained by CHROMOMETHYLASE 2 (CMT2), which can be recruited by lysine 9 dimethylation on histone H3 (H3K9me2) (Stroud *et al.*, 2014). Access of CMT2 to heterochromatic regions is mediated by the chromatin remodeller DECREASE IN DNA METHYLATION 1 (DDM1) (Zemach *et al.*, 2013; Lyons & Zilberman, 2017). Thus, DDM1 together with CMT2 mediate CHH methylation in pericentromeric regions, whereas DRM2 targets mainly euchromatic regions (Zemach *et al.*, 2013). CG and CHG methylation are established by the RdDM pathway, but the maintenance of CG methylation occurs by METHYLTRANSFERASE 1 (MET1) and CHG methylation by CMT3 (Law & Jacobsen, 2010; Du *et al.*, 2015; Bewick & Schmitz, 2017; Zhang *et al.*, 2018). CMT3 can be recruited by H3K9me2 (Law & Jacobsen, 2010; Du *et al.*, 2012, 2015).

†Deceased.

H3K9me2 and CHG methylation facilitate each other through regulatory feedback loops (Du *et al.*, 2012, 2014).

Heterochromatic regions are generally compact chromatin structures that are enriched for DNA methylation, H3K9me2 (Grewal & Jia, 2007; Vaillant & Paszkowski, 2007; Roudier *et al.*, 2009) and histone H1 (Zemach *et al.*, 2013; Rutowicz *et al.*, 2019; Choi *et al.*, 2020). H1 impedes the access of CMT2, causing increased DNA methylation on heterochromatic TEs upon H1 depletion (Zemach *et al.*, 2013; Lyons & Zilberman, 2017). Loss of H1 causes only a few TEs to be upregulated, but the dual loss of H1 and DNA methylation causes strong TE activation, revealing that DNA methylation and H1 cooperatively maintain heterochromatic TEs in an inaccessible and silent state (Choi *et al.*, 2020). In the vegetative cell of pollen, natural depletion of H1 allows access of the DNA glycosylase DEMETER to heterochromatic TEs, causing their activation by DNA demethylation (He *et al.*, 2019).

Temperature shifts from cold to high temperature cause transcriptional activation of genes and heterochromatic TEs in *Arabidopsis* (Grandbastien, 1998; Lämke & Bäurle, 2017). H2A.Z eviction leads to gene expression by affecting DNA accessibility upon high temperature, whereas the loss of DNA methylation on TEs enhances TE activation in response to heat (Kumar & Wigge, 2010; Tittel-Elmer *et al.*, 2010; Cavrak *et al.*, 2014). Resetting of this stress-induced epigenetic state on TEs requires the redundant action of DDM1 and MORPHEUS' MOLECULE 1 (MOM1) and CHROMATIN ASSEMBLY FACTOR 1 (CAF1) (Pecinka *et al.*, 2010; Iwasaki & Paszkowski, 2014). Given the connection between DDM1 and H1 and the enrichment of H1 on heterochromatic TEs, we hypothesized that H1 has a functional role in repressing TEs during temperature stress.

We explored this question using the *h1.1 h1.2* double mutants and found that H1 is required in particular for TE repression in response to heat stress. We discovered that the response to H1 loss differs depending on TE location; although pericentromeric *GYPSY* TEs became activated in heat-treated *h1* mutants despite maintaining high levels of DNA methylation, nonpericentromeric *COPIA* elements became activated concomitantly with loss of DNA methylation. Many of those activated *COPIA* elements also became activated in heat-treated *cmt2* mutants, suggesting that H1 repressed *COPIA* elements through maintaining DNA methylation. Heat-induced *COPIA* elements previously were shown to be strongly activated in DNA methylation impaired mutants upon heat treatment (Ito *et al.*, 2011; Thieme *et al.*, 2017). Most strikingly, H1-deficient plants treated with the DNA methyltransferase inhibitor zebularine were highly tolerant to heat stress, suggesting that both H1 and DNA methylation redundantly suppress the plant response to heat stress.

Materials and Methods

Plant material, growth conditions, heat stress and chemical treatments

All wild-type and mutant plants were in the *Arabidopsis thaliana* accession Columbia (Col-0) background. The *h1.1-1* (SALK_128430C) and *h1.2-2* (GK-116E08) plant lines were described previously (Rutowicz *et al.*, 2015); *h1.1-1 h1.2-2* double mutants were generated by crossing (H1, histone 1). The *cmt2-5* (SAIL_906_G03) and *cmt3-11* (SALK_148381) mutants were described previously (Chan *et al.*, 2006; Shen *et al.*, 2014) (CMT, CHROMOMETHYLASE 2).

Plants were grown on plates with 1/2 Murashige & Skoog medium including vitamins (Duchefa, Haarlem, The Netherlands) and 1% sucrose. Zebularine-treated plants were grown on ½MS medium supplemented with 40 µM zebularine (Abcam, Cambridge, UK). Plates were sealed with micropore tape and stratified for 2 d at 4°C in the dark. The plates were then transferred to a growth chamber with a 16 h : 8 h, light (110 µmol m^{-2} s^{-1}, 22°C) : dark (20°C) photoperiod. Ten-day-old seedlings were used for RNA extraction. For heat stress treatments, we incubated 10-d-old seedlings at 4°C for 1 h followed by 37°C for 24 h in the dark, as published previously (Shen *et al.*, 2014). The survival rate of heat-treated plants was determined by incubating 10-d-old seedlings in the dark at 4°C for 1 h followed by 37°C for 36 h and then removing to normal conditions for 2 d. Seedlings were defined as lethal if they showed bleaching of shoot apices and leaves.

RNA Isolation and quantitative reverse transcription (qRT)-PCR

RNA extraction was performed using the MagMAX™ Plant RNA Isolation Kit (Thermo Fisher Scientific, Göteborg, Sweden) followed by cDNA synthesis using a RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific). Experiments were performed in biological triplicates. Quantitative PCR with gene-specific primers (Supporting Information Table S1) was performed using a HOT FIREPol EvaGreen qPCR Supermix (Solis biodyne, Tartu, Estonia) according to the manufacturer's instructions. The reactions were performed using the 'CFX connect' Real-time PCR cycler detection system (Bio-Rad). For qPCR analysis of *ONSEN* copy numbers, *ACTIN2* was used to normalize DNA levels.

RNA sequencing

Except for the *h1* samples from where only duplicates were generated, RNA of biological triplicates was extracted (50 mg seedlings per replicate) using a RNeasy Plant Mini Kit (Qiagen). Libraries were generated using DNA-free RNA with the TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Sequencing was performed on an Illumina HiSeq2000 in 50-bp single-end mode. The correlation between replicates is shown Fig. S1 and details on the data in Table S2. A comparison between our RNA-seq data and previously published data (Choi *et al.*, 2020) of *h1* mutants under nonheat conditions is shown in Fig. S2.

DNA Isolation and bisulfite sequencing

For a single replicate, four seedlings of wild-type or the *h1* mutants were collected before and after heat stress. Around

500 ng genomic DNA was extracted using a MagJET Plant Genomic DNA Kit (Thermo Fisher Scientific). Libraries for two biological replicates were prepared by Novogene (Hongkong, China) and sequenced on an Illumina HiSeq2000 in 150-bp paired-end mode.

Bioinformatic analysis

For RNA analysis, untrimmed reads were mapped to the TAIR10 *Arabidopsis* reference genome using STAR (v2.5.3.a, (Dobin *et al.*, 2013)). Expression counts were generated using the R function *summarizeOverlaps* from the package HTSEQ in union mode on exons from the reference transcriptome AtRTD (Zhang *et al.*, 2017). Differential expression analysis was performed using the R/DESEQ2 (v1.20.0, (Love *et al.*, 2014)). Genes with a $\log_2$ fold change ≥ 1 or ≤ -1 and Bonferroni-adjusted *P*-value (padj) ≤ 0.05 were considered to be differentially expressed. Likewise, expression counts along transposable elements (TEs) were generated using *summarizeOverlaps* from R/HTSEQ in union mode along the coordinates of the 31 189 TAIR10 annotated TEs. Differential expression analysis was performed using DESeq2, using the same threshold values as for gene transcripts.

For DNA methylation analysis, the 150-bp-long pair-end reads were first quality trimmed by removing the first five bases from the 5′ end and the last 20 bases from the 3′ end. Reads were mapped to the reference genome TAIR10 in PE mode (–score_min L, 0, −0.6) using BISMARK v0.16.3. Mapped reads were deduplicated and cytosine methylation values calculated using the Bismark Methylation Extractor. Methylation reports were pooled for both replicates for further analyses.

Differentially methylated regions (DMRs) in each context were calculated only for demethylation in 50-bp bins considering as the fractional methylation threshold the bins with differences below the 1st decile. Those were parsed if they passed a fisher test ($P < 0.01$).

Published datasets were processed as follows: H1 chromatin affinity purification (ChAP) reads from GSE122394 (Choi *et al.*, 2020) control libraries were mapped to the TAIR10 genome using BOWTIE2. Coverage was calculated using the *coverage* function from R/NUCLER (Flores & Orozco, 2011) and normalized by the total number of mapped reads. The normalized input signal was subtracted from the H1.1 and H1.2 ChAP signal. Small RNA reads from GSE116067 (Tan *et al.*, 2018) were mapped to the TAIR10 genome using BOWTIE (-v 0-best). Pooled mapped reads were separated into two categories (21/22- and 24-nucleotides-long) and remapped using SHORTSTACK (Johnson *et al.*, 2016). The alignments were normalized by converting coverage values to reads per million values (Wang *et al.*, 2020).

Pericentrometic heterochromatin was considered to span the regions between the following coordinates: Chr1: 11 500 020–17 696 331; Chr2: 1100 003–7192 918; Chr3: 10 298 763–17 289 015; Chr4: 1500 001–2300 002; Chr4: 2800 003–6300 004 and Chr5: 8999 997–5982 772 (Copenhaver *et al.*, 1999).

Results

Absence of H1 affects gene and TE activation after heat stress

Histone H1 is abundant throughout the whole genome and mediates global nucleosome positioning in plants and animals, but only few genes and TEs become deregulated when H1 is deficient (Geeven *et al.*, 2015; Rutowicz *et al.*, 2019; Choi *et al.*, 2020). In plants, heat stress causes dispersal of heterochromatin, similar to H1-deficient mutants (Pecinka *et al.*, 2010; Rutowicz *et al.*, 2019). We therefore addressed the question of whether H1 has a role in antagonizing heat stress. Because H1.1 and H1.2 are constitutively expressed and highly redundant (Rutowicz *et al.*, 2019; Choi *et al.*, 2020), we used *h1.1 h1.2* double mutants, henceforth referred to as *h1* mutants. Expression levels of *H1.3* are very low under normal conditions (Ascenzi & Gantt, 1997; Rutowicz *et al.*, 2015) and *H1.3* was not induced by heat stress (Fig. S3), justifying the use of the *h1* mutants to explore the role of H1 in the heat response. We generated RNA-seq profiling data of wild-type and *h1* mutants before and after heat stress (4°C for 1 h followed by 37°C for 24 h in dark conditions). It was previously shown that loss of H1 activates gene expression but only weakly derepresses TEs (Rutowicz *et al.*, 2019; Choi *et al.*, 2020), we therefore focused initially on upregulated genes in *h1* mutants. We found that 301 genes ($\log_2$ fold change ≥ 1 and Bonferroni-adjusted *P*-value (padj) ≤ 0.05) were upregulated in *h1* mutants compared to wild-type (Fig. 1a; Table S3). Surprisingly, only 116 genes ($\log_2$ fold change ≥ 1 and Bonferroni-adjusted *P*-value (padj) ≤ 0.05) were activated in *h1* after heat stress compared to heat-treated wild-type (Fig. 1a; Table S3). Many genes (265), including 68 heat-responsive genes in wild-type that were activated in *h1,* were not changed or became downregulated in *h1* upon heat (Figs 1a,c, S4a,b), suggesting that heat attenuates the effect of loss of H1 on genes. In order to test whether H1 functions as a repressor after heat stress, we analyzed previously published H1 enrichment data (Choi *et al.*, 2020) on upregulated genes and downregulated genes in *h1* mutants upon heat stress. We found that upregulated genes in heat-treated *h1* mutants were significantly more enriched for H1 than downregulated genes (Fig. S5), indicating that H1 has mainly a repressive role after heat stress. We identified three over-represented gene ontology (GO) functional categories among the upregulated genes in *h1* mutants upon heat stress, corresponding to carbohydrate binding, hydrolase activity and nucleoside-triphosphatase (Fig. S6). Consistent with previous findings (Choi *et al.*, 2020), we found only few (13) TEs upregulated in *h1* mutants, but this number substantially increased (86 upregulated TEs) in *h1* upon heat compared to heat-treated wild-type (Fig. 1b,d; Table S4). Most of these TEs also were heat responsive in wild-type (Figs 1d, S4c). This reveals a repressive role of H1 on TEs in response to heat stress. Previous work revealed that loss of H1 together with loss of DNA methylation causes TE de-repression (Choi *et al.*, 2020). To understand the H1 repressive mechanism in response to heat, we generated bisulfite-sequencing data of wild-type and *h1* mutants with and without heat treatment. We found that CG

methylation levels in the 2-kb upstream region of the 265 upregulated genes in *h1* mutants without heat treatment (Fig. 1a) did not significantly change compared to wild-type, irrespective of heat treatment (Fig. 1e). By contrast, CHG and CHH methylation decreased in *h1* mutants without heat treatment, and also decreased to similar levels in wild-type and *h1* mutants upon heat treatment (Fig. 1f,g). Loss of CHH methylation in *h1* was partly attenuated by heat stress, providing a possible explanation why many genes became activated in *h1*, but not in heat-treated *h1* mutants (Fig. 1a,c). We identified CHG and CHH differentially methylated regions (DMRs) in *h1* mutants with and without heat treatment. We focussed on the 2-kb upstream region of those 265 genes that were upregulated in untreated *h1* mutants but did not change expression upon heat treatment (Fig. 1a,h). Upon heat treatment, we found that 49 genes gained hypermethylated CHG or CHH regions (Hyper DMRs), whereas 81 genes lost hypomethylated regions (Hypo DMRs; Fig. 1h) in *h1* mutants, which restored CHG or CHH methylation to wild-type levels upon heat (Fig. 1f,g), consistent with fewer genes being activated in *h1* mutants after heat stress. Together, our data reveal that heat treatment and loss of H1 both caused a reduction of CHG and CHH methylation (Fig. 1f,g), likely a consequence of opened chromatin structure (Zemach *et al.*, 2013; Rutowicz *et al.*, 2019). However, heat treatment of *h1* mutants partly restored CHH methylation (Fig. 1g,h), indicating that heat ameliorates the effect of loss of H1 on genes.

Gain of DNA methylation is not sufficient to repress *GYPSY* TEs in *h1* mutants after heat stress

In contrast with genes that were less affected in heat-treated *h1* mutants compared to heat-treated wild-type plants, we found increased numbers of TEs upregulated in heat-stressed *h1* mutants compared to heat-stressed wild-type plants (Fig. 1b,d). In order to understand the role of H1 on TE repression in response to heat, we analyzed H1 enrichment data (Choi *et al.*, 2020) to test whether upregulated TEs differ in their H1 enrichment compared to nonupregulated TEs. Indeed, we found that in wild-type, upregulated TEs in heat-treated *h1* mutants were more strongly enriched for H1 compared to TEs not affected by heat (Fig. 2a). Those upregulated TEs also had higher wild-type methylation levels in all three sequence contexts compared to nonupregulated TEs (Fig. 2b), consistent with H1 being associated with heavily methylated heterochromatic TEs (Rutowicz *et al.*, 2015; Choi *et al.*, 2020). Upregulated TEs belonged to different TE families, among which LTR/*GYPSY* ($P = 2.81e-24$), LTR/*COPIA* ($P = 9.88e-05$) and DNA/En-Spm ($P = 0.0045$) families were significantly enriched (Fig. 2c) (LTR, long terminal repeat). Among the most enriched retrotransposons, LTR/*COPIA* TEs were mildly increased in *h1* mutants after heat stress, whereas LTR/*GYPSY* TEs were more strongly upregulated (Fig. 2d). We analyzed DNA methylation levels of all *GYPSY* TEs and found that they gained DNA methylation in all sequence contexts in *h1* compared to wild-type, independent of being subjected to heat stress or not (Fig. 2e–g). This is consistent with previous findings revealing that absence of H1 causes gain of methylation in heterochromatic regions, but loss of methylation in euchromatic regions (Zemach *et al.*, 2013; Lyons & Zilberman, 2017). Upregulated *GYPSY* TEs in *h1* upon heat followed this trend and likewise gained DNA methylation of all three sequence contexts in *h1* mutants vs WT both before and after heat stress (Fig. 2h–j). This suggests that in the absence of H1, high levels of DNA methylation are not sufficient to repress a subset of TEs in response to heat stress. Thus, H1 has an indispensable and DNA methylation-independent repressive function in response to heat stress.

H1 and DNA methylation cooperatively control a subset of *COPIA* elements in response to heat

Upregulated *COPIA* elements in *h1* mutants upon heat were mainly *COPIA78/ONSEN* elements, including the eight full-length heat responsive *ONSEN* TEs (Ito *et al.*, 2013; Cavrak *et al.*, 2014; Pietzenuk *et al.*, 2016). In contrast with *GYPSY* TEs that strongly gained DNA methylation in *h1* mutants after heat stress, there were only mild changes in DNA methylation on *COPIA* TEs (Fig. 3a–c). However, those heat-responsive *COPIA78/ONSEN* elements that became upregulated upon heat stress, had increased DNA methylation levels in *h1* mutants without heat treatment, but experienced a strong loss of CG and CHG methylation upon heat treatment, independent of the presence or absence of H1 (Fig. 3d–f). Stronger activation of *COPIA78/ONSEN* elements in *h1* mutants upon heat compared to heat-treated wild-type suggests that under heat stress, DNA methylation and H1 cooperatively repress a subset of *COPIA* TEs.

Among those upregulated *COPIA* TEs were three *COPIA78/ONSEN* elements (*ONSEN 1* (*AT1TE12295*), *ONSEN 2* (*AT3TE92522*) and *ONSEN 3* (*AT5TE15240*)) that had more strongly reduced DNA methylation in all three sequence contexts in heat-treated *h1* mutants compared to heat-treated wild-type (Fig. 3g, S7). Heat treatment in combination with impaired epigenetic repression previously was shown to cause *ONSEN* transposition (Ito *et al.*, 2011; Thieme *et al.*, 2017), however, the copy numbers of *ONSEN* remained the same in the second generation of heat-treated *h1* mutants as in wild-type, revealing that H1 does not control *ONSEN* transposition (Fig. S8).

CMT2 controls *COPIA78/ONSEN* element silencing during heat stress

Natural *CMT2* variation was shown to correlate with CHH methylation variation and temperature seasonality in *Arabidopsis*, and *cmt2* mutants are more heat tolerant (Shen *et al.*, 2014; Dubin *et al.*, 2015). Because CMT2 acts on H1 containing loci (Zemach *et al.*, 2013), we explored the connection between H1 and CMT2 under heat stress. We generated transcriptome data of *cmt2* mutants before and after heat stress. Only 26 TEs became activated in *cmt2* mutants upon heating (Fig. 4a; Table S4). Among those, six TEs belonged to the *GYPSY* family, whereas 13 TEs belonged to the *COPIA78/ONSEN* subfamily. Among the upregulated TEs, 15 overlapped with TEs upregulated in heat-treated *h1* mutants (Fig. 4a), including 12 *COPIA78/ONSEN* TEs. The *COPIA78/ONSEN* elements were mildly upregulated

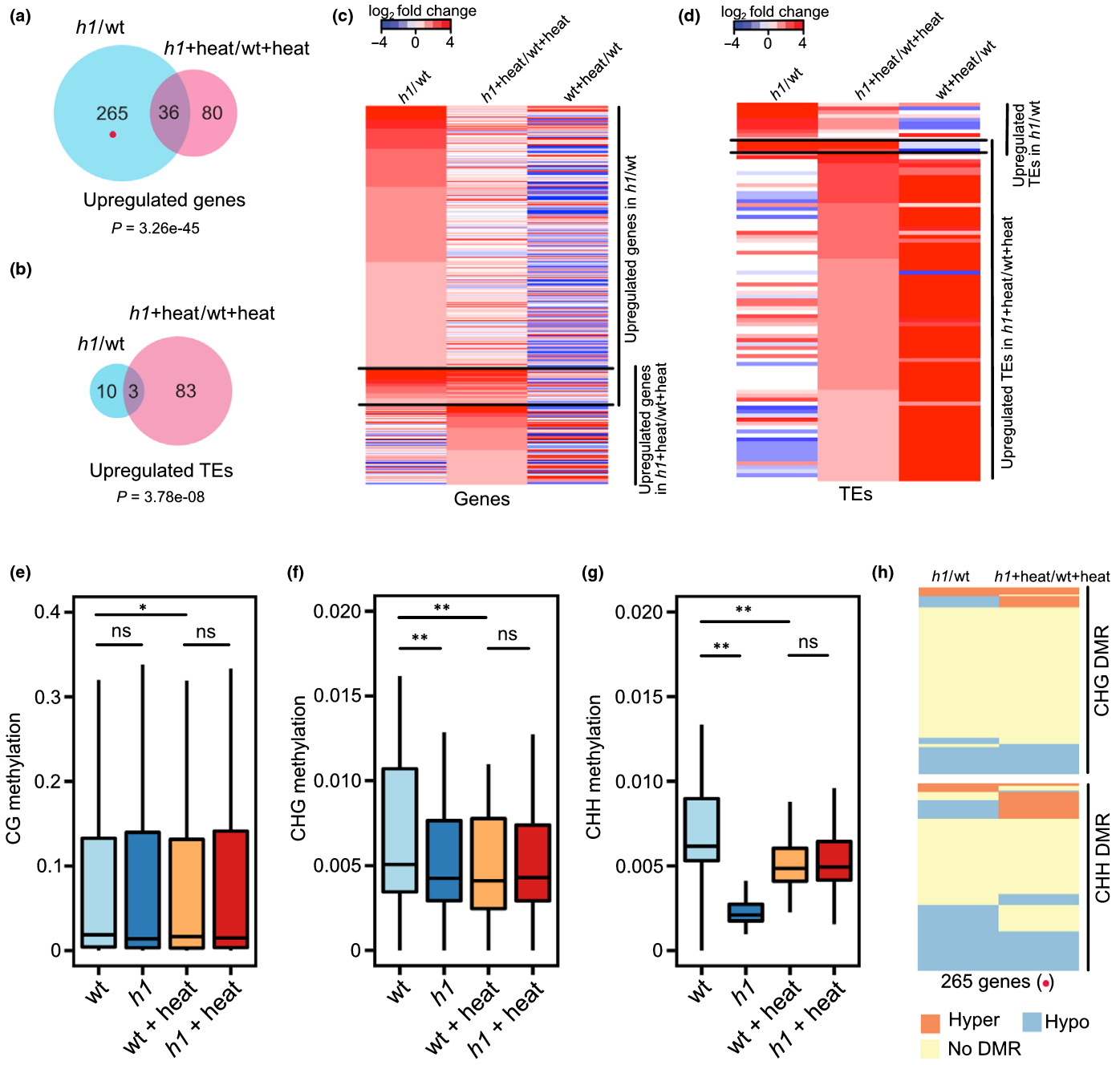

Fig. 1 Histone H1 is required for transposable element (TE) repression after heat stress in *Arabidopsis*. Venn diagrams show upregulated genes (log_2-fold change ≥ 1, adjusted *P*-value (padj) ≤ 0.05, (a)) and TEs (log_2-fold change ≥ 1, padj ≤ 0.05, (b)) in *h1* mutants vs wild-type (*h1*/WT) and *h1* plus heat vs WT plus heat (*h1* + heat/WT + heat). The red dot in (a) represents the same group of genes marked in panel (h). Heat maps show upregulated genes (c) and TEs (d) in *h1* mutants vs WT and *h1* plus heats WT plus heat. Averaged CG (e), CHG (f) and CHH (g) methylation in the −2 kb to transcription start site (TSS) region of 265 upregulated genes in *h1* mutants vs WT but not in *h1* plus heats WT plus heat are shown in four different conditions of WT, *h1*, WT plus heat (WT + heat), and *h1* plus heat (*h1* + heat). *, $P < 0.05$; **, $P < 0.01$; ns, not significant (Wilcoxon test). The lower and upper hinges of the boxplots correspond to the first and third quartiles of the data, the black lines within the boxes mark the median. CHG and CHH DMRs (h) in the −2 kb to TSS region of 265 upregulated genes in *h1* vs WT. Hyper and hypo refer to hyper- or hypomethylated regions, respectively, present in the upstream region of genes in *h1* vs WT or *h1* plus heat vs WT plus heat.

in heat-treated *h1* mutants but became strongly upregulated in heat-treated *cmt2* mutants (Fig. 4b), revealing that CMT2, like H1, plays a major repressive role on TEs of the *COPIA78/ONSEN* subfamily in response to heat stress. Heat stress-induced expression of *COPIA78/ONSEN* also was observed in RNA-directed DNA methylation (RdDM) mutants (Ito *et al.*, 2011), suggesting that *COPIA78/ONSEN* elements are common targets of the CMT2 and RdDM pathways. Consistently, upregulated *COPIA* elements strongly accumulated 24-nt siRNAs, differing from nonupregulated *COPIA* elements and *Gypsy* elements

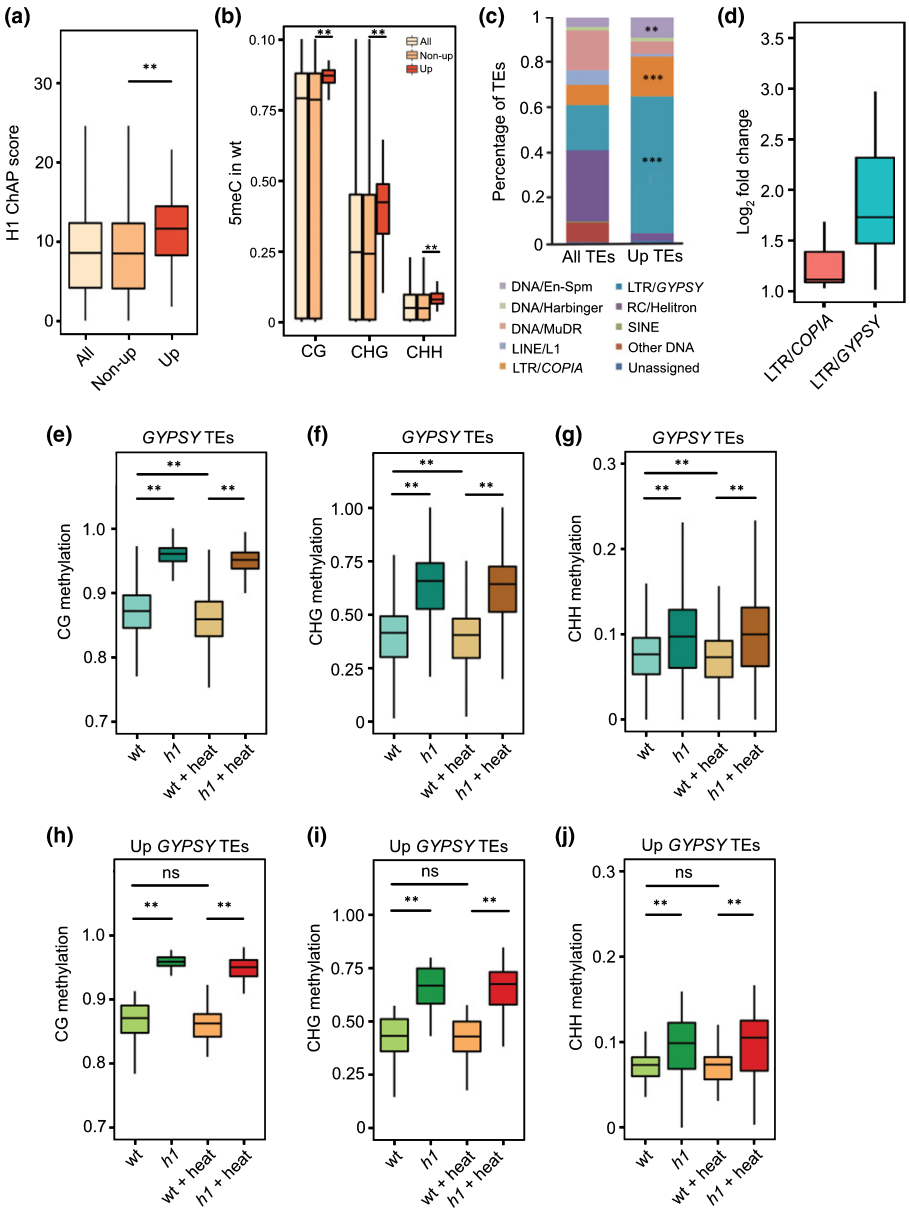

Fig. 2 Histone H1-mediated repression of *GYPSY* transposable elements (TEs) upon heat occurs independently of DNA methylation in *Arabidopsis*. Wild-type (WT) H1 enrichment (a) on all, nonupregulated and upregulated TEs in *h1* plus heat vs WT plus heat. **, $P < 0.01$ (Wilcoxon test). ChAP, chromatin affinity purification. H1 ChAP score was defined as the average ChAP coverage from start to end of the TE. Wild-type DNA methylation level (CG, CHG and CHH, (b)) on all, nonupregulated and upregulated TEs in *h1* plus heat vs WT plus heat. **, $P < 0.01$ (Wilcoxon test). Percentages of TEs are classified in different families (c). Up TEs refer to the upregulated TEs in *h1* plus heat vs WT plus heat. **, $P < 0.01$; ***, $P < 0.001$ (hypergeometric test). Expression level (log_2-fold change, (d)) of upregulated long terminal repeat (LTR)/*COPIA* and LTR/*GYPSY* elements in *h1* plus heat vs WT plus heat. Averaged CG (e), CHG (f) and CHH (g) methylation level on all *GYPSY* TEs in WT, *h1*, WT plus heat (WT + heat) and *h1* plus heat (*h1* + heat). Averaged CG (h), CHG (i) and CHH (j) methylation on upregulated *GYPSY* TEs in *h1* plus heat vs WT plus heat in four different conditions of WT, *h1*, WT plus heat and *h1* plus heat. **, $P < 0.01$; ns, not significant (Wilcoxon test). The lower and upper hinges of the boxplots correspond to the first and third quartiles of the data, the black lines within the boxes mark the median.

(Fig. 4c). The RdDM pathway targets the edges of long TEs, whereas CMT2 is required for TE body methylation on long heterochromatic TEs (Zemach *et al.*, 2013). In agreement with the dual regulation of *COPIA78* elements by both pathways, upregulated *COPIA* elements were significantly larger than upregulated *GYPSY* elements (Fig. 4d). Upregulated *COPIA* and *GYPSY* elements also differed in their chromosomal location: whereas upregulated *GYPSY* TEs were concentrated in the pericentromeric region, upregulated *COPIA* TEs were mainly dispersed along the chromosome arms (Fig. 4e), which may explain their different mode of regulation.

H1-deficient plants treated with zebularine have improved heat-stress tolerance

In order to further explore the interaction between H1-mediated TE repression and DNA methylation, we grew wild-type and *h1* plants on media supplemented with zebularine, a widely used inhibitor of DNA methyltransferases (Griffin *et al.*, 2016). Consistent with the transcriptome data, *COPIA78/ONSEN* expression increased upon heat treatment and was further increased in *h1* mutants upon heat as tested by qRT-PCR (Fig. 5a,b; note different axis scale). Zebularine treatment increased *COPIA78/ONSEN* expression upon heat treatment, but the effect was similar in wild-type and *h1* mutants (Fig. 5b), consistent with the strong effect of *cmt2* mutants on *COPIA78/ONSEN* expression upon heat treatment (Fig. 4b). By contrast, one *GYPSY* TE expression was increased in zebularine-treated wild-type and *h1* mutants, and strongly increased in heat-treated *h1* mutants compared to wild-type (Fig. 5c,d), indicating a synergistic repressive effect of H1 and DNA methylation on *GYPSY* repression upon heat treatment.

In order to functionally explore the impact of H1 on the temperature-stress response, we tested the sensitivity of wild-type and

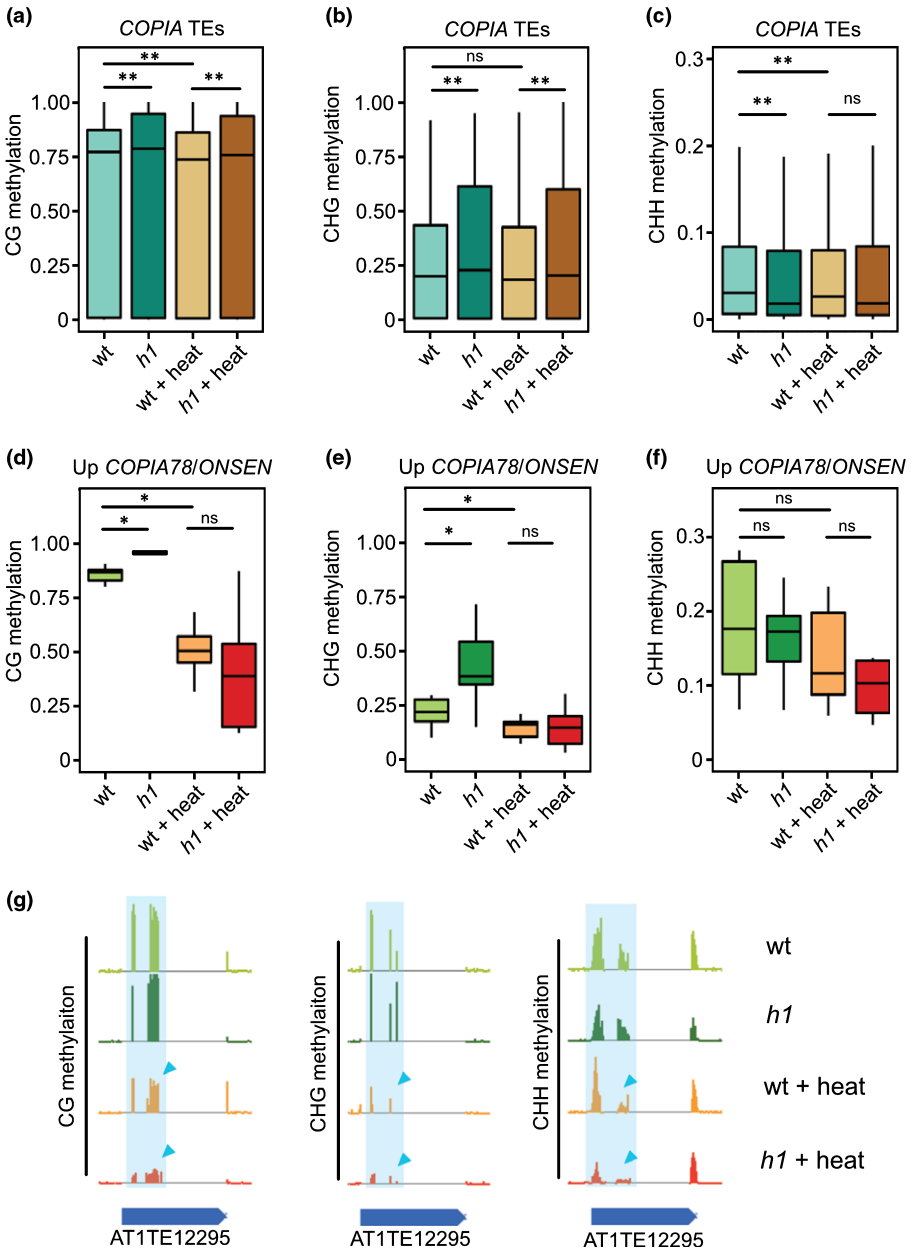

Fig. 3 Histone H1 represses *COPIA78/ONSEN* transposable elements (TEs) upon heat through maintaining DNA methylation in *Arabidopsis*. Averaged CG (a), CHG (b) and CHH (c) methylation on all *COPIA* TEs in wild-type (WT), *h1*, wt plus heat (WT + heat) and *h1* plus heat (*h1* + heat). Averaged CG (d), CHG (e) and CHH (f) methylation on upregulated *COPIA78/ONSEN* elements in *h1* plus heat vs WT plus heat in four different conditions of WT, *h1*, WT plus heat, and *h1* plus heat. *, $P < 0.05$; **, $P < 0.01$; ns, not significant (Wilcoxon test). The lower and upper hinges of the boxplots correspond to the first and third quartiles of the data, the black lines within the boxes mark the median. Example of CG, CHG and CHH methylation levels (g) on *ONSEN 1* (*AT1TE12295*). Triangle points to the decreased DNA methylation region.

h1 mutants to severe heat stress (1 h at 4°C followed by 36 h at 37°C in dark conditions) by measuring the survival rate of heat-treated plants after returning to normal conditions for 2 d. The leaves of most plants gradually became yellow and bleached and the plants finally died during these two days, resulting in ≤ 20% survival rate of wild-type plants. The *h1* mutants had a significantly higher survival rate (1.6-fold greater; $P = 0.016$, Wilcoxon signed-rank test) than wild-type (Fig. 5e,f). Strikingly, however, when the zebularine was present, the survival rate of wild-type and *h1* mutants strongly increased after heat stress, reaching *c.* 50% in wild-type and *c.* 86% in *h1* mutants (Fig. 5e,f), revealing a so far unknown potential of hypomethylation in combination with H1 deficiency to antagonize heat stress. We assessed whether the increased heat tolerance was a consequence of increased transposition rates of *COPIA78/ONSEN* TEs in *h1* mutants treated with zebularine and heat; however, we did not observe increased *ONSEN* copy numbers in the second generation of heat- and zebularine-treated *h1* mutants compared to wild-type (Fig. S9).

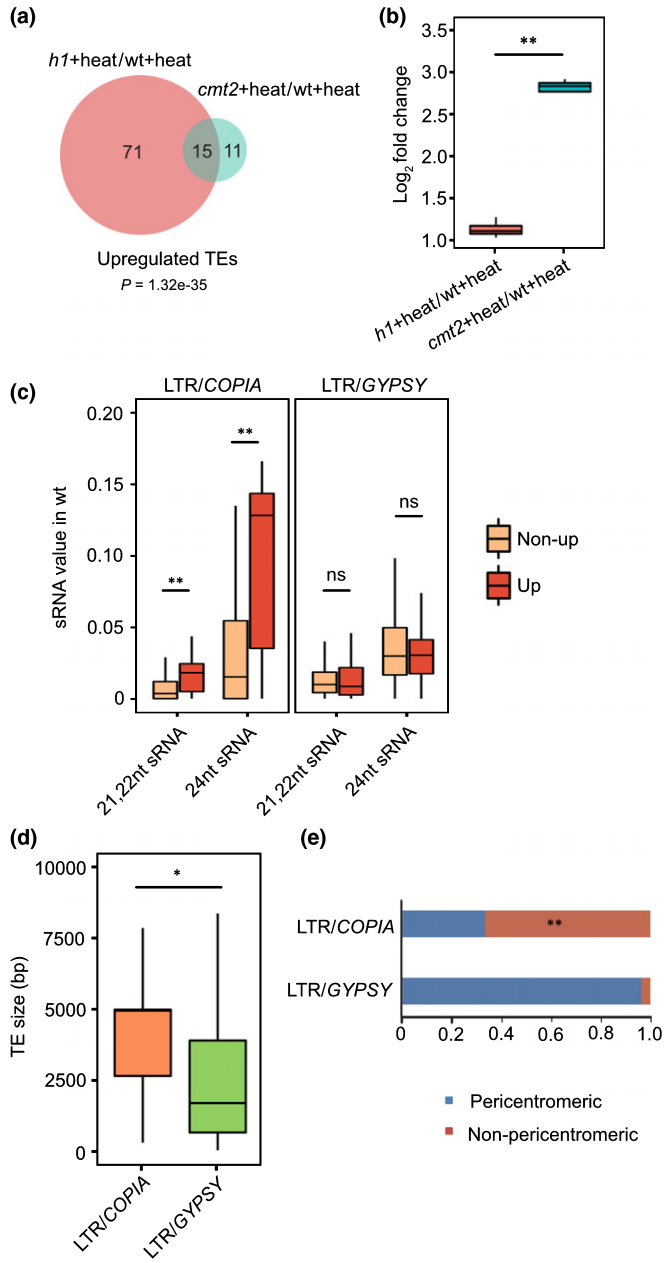

Fig. 4 CHROMOMETHYLASE 2 (CMT2) is required for *COPIA78/ONSEN* transposable element (TE) repression under heat conditions in *Arabidopsis*. Venn diagram (a) shows upregulated TEs in *h1* plus heat vs wild-type (WT) plus heat (*h1* + heat/WT + heat) and *cmt2* plus heats wt plus heat (*cmt2* + heat/WT + heat). Boxplot (b) shows the expression level of commonly upregulated *COPIA78/ONSEN* elements in *h1* plus heat vs WT plus heat and *cmt2* plus heat vs WT plus heat. **, $P < 0.01$ (Wilcoxon test). Boxplot (c) shows WT sRNA value on upregulated and nonupregulated long terminal repeat (LTR)/*COPIA* and LTR/*GYPSY* elements in *h1* plus heat vs WT plus heat. **, $P < 0.01$; ns, not significant (Wilcoxon test). Boxplot (d) shows the size of upregulated LTR/*COPIA* TEs and LTR/*GYPSY* TEs in *h1* plus heat vs WT plus heat. *, $P < 0.05$ (Wilcoxon test). The lower and upper hinges of the boxplots correspond to the first and third quartiles of the data, the black lines within the boxes mark the median. The percentages of upregulated LTR/*COPIA* and LTR/*GYPSY*-type TEs in *h1* plus heat vs WT plus heat are classified by location (e). Upregulated *COPIA* elements are significantly enriched in nonpericentromeric regions compared to all LTR/*COPIA* TEs. **, $P < 0.01$ (hypergeometric test).

Discussion

In this work, we explored the function of the linker histone H1 in response to heat stress in plants and discovered a novel role of H1 in modulating the plant response to heat. Although loss of H1 causes only a mild effect under normal conditions (Rutowicz *et al.*, 2015, 2019), we found that under heat stress, loss of H1 strongly activated transposable elements (TEs), mainly *GYPSY* and *COPIA* elements. Stress treatment of the *h1.3* mutant previously was reported to cause increase of CHH methylation at defined loci and reduced gene expression (Rutowicz *et al.*, 2015). Likewise, we observed that decreased CHH methylation in the 2-kb upstream region of upregulated genes in *h1* mutants was partly restored in heat-treated *h1*, suggesting that increased CHH methylation accounts for the dampened gene expressed in stressed *h1* mutants. Histone H1 together with DNA methylation were shown to jointly suppress aberrant intragenic transcription (Choi *et al.*, 2020). Thus, it isalso possible that the formation of aberrant transcripts in *h1* at least in part account for reduced transcript levels.

In heterochromatic regions of *Arabidopsis*, H1 impedes DNA methyltransferase accessibility to chromatin, leading to increased DNA methylation upon H1 depletion (Zemach *et al.*, 2013; Lyons & Zilberman, 2017). Although we found this effect very pronounced on *GYPSY* TEs, this effect was much weaker on *COPIA* TEs, indicating distinct regulation of both types of TEs by H1 (Fig. 6). In heat-treated *h1* mutants, gain of DNA methylation was not sufficient to repress *GYPSY* TEs, demonstrating a key repressive role for H1 on *GYPSY* TEs. Depletion of DNA methylation by zebularine treatment caused strongly increased expression of *GYPSY* TEs in heat-treated *h1* mutants, exceeding the upregulation in heat-treated *h1* without zebularine, revealing that H1 and DNA methylation synergistically repress *GYPSY* TEs under heat, consistent with previous work (Choi *et al.*, 2020).

Like *GYPSY* TEs that gained DNA methylation upon loss of H1, heat-induced *COPIA* TEs also gained DNA methylation in *h1* mutants. However, In contrast with *GYPSY* TEs, which maintained high levels of DNA methylation in *h1* mutants upon heat treatment, *COPIA* elements lost DNA methylation upon heat treatment in wild-type and *h1* mutants. Because *COPIA* elements became more strongly activated in heat-treated *h1* mutants than heat-treated wild-type, we conclude that the combination of reduced DNA methylation and loss of H1 caused transcriptional activation of *COPIA* elements upon heat. Nevertheless, most of the heat induced *COPIA* elements also were activated in heat-treated *chromomethylase 2* (*cmt2*) mutants, indicating that a complete loss of CHH methylation has the activating effect on the same *COPIA* elements as loss of H1 under heat.

The difference in chromosomal location may explain the different regulatory impact of DNA methylation on *GYPSY* and *COPIA* element expression in response to heat. *GYPSY*elements are located in compacted heterochromatic regions and possibly rely on Microrchidia (MORC) proteins for repression. MORC proteins act in heterochromatic regions and enforce silencing of TEs independently of DNA methylation, likely by affecting

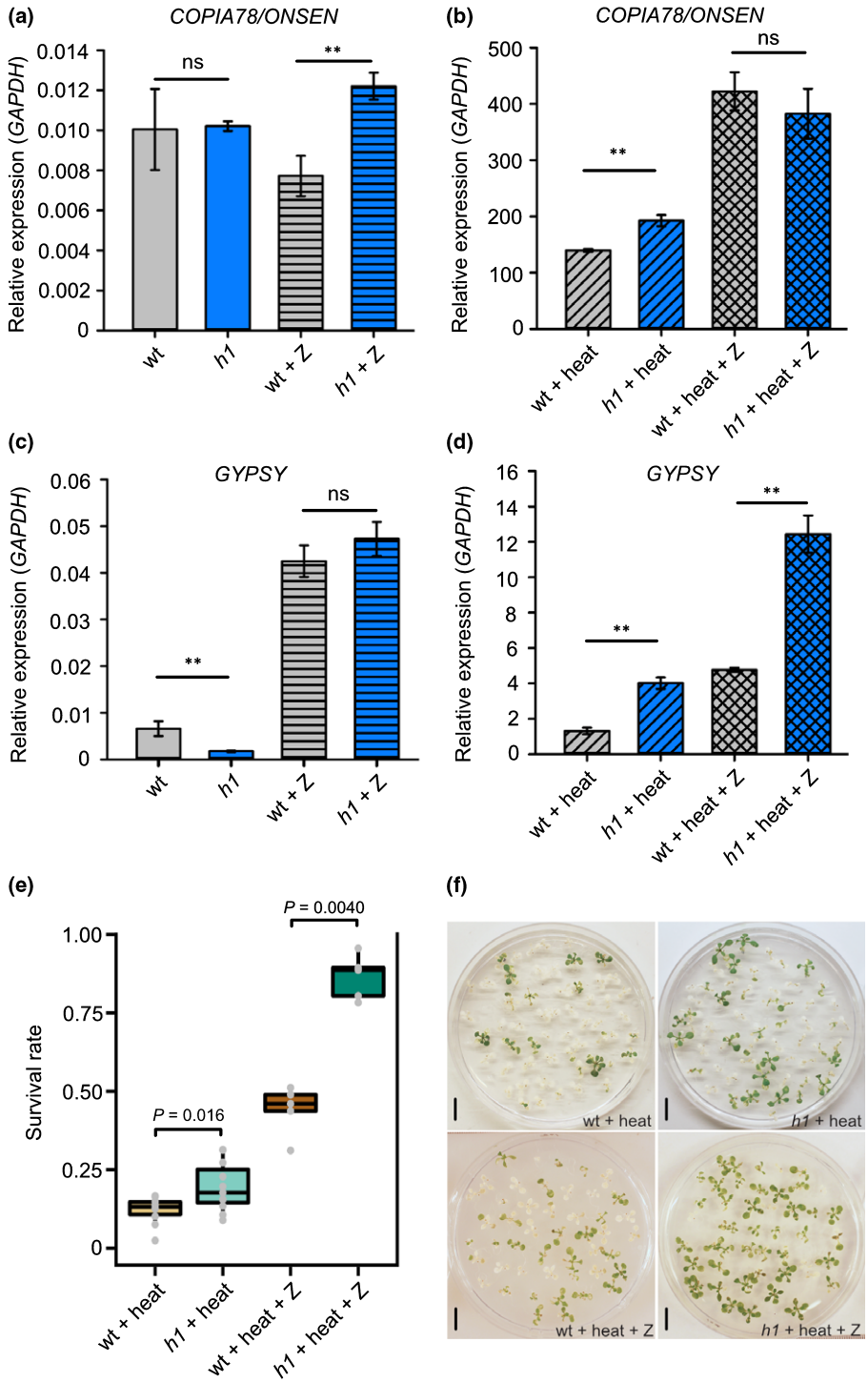

Fig. 5 Loss of histone H1 and DNA hypomethylation increase *Arabidopsis* heat tolerance. *COPIA78/ONSEN* (a) and *GYPSY* (c) transcripts in wild-type (WT), *h1*, WT plus 40 μm zebularine (WT + Z) and *h1* plus 40 μm zebularine (*h1* + Z). *COPIA78/ONSEN* (b) and *GYPSY* (d) transcripts in wt plus heat (WT + heat), *h1* plus heat (*h1* + heat), WT plus heat and 40 μm zebularine (WT + heat + Z) and *h1* plus heat and 40 μm zebularine (*h1* + heat + Z). Error bars represent 1 SD. **, $P < 0.01$; ns, not significant (Student's *t*-test). Survival rate (e) of seedlings of WT, *h1* mutants, WT plus 10 μm zebularine and *h1* plus 10 μm zebularine after 36 h heat stress and 48 h recovery under long day conditions. Each of three biological replicates corresponds to ≥ 90 seedlings. The lower and upper hinges of the boxplots correspond to the first and third quartiles of the data, the black lines within the boxes mark the median. Representative pictures (f) of plates with WT, *h1* mutants, WT plus 10 μm zebularine and *h1* plus 10 μm zebularine after 36 h heat stress and 48 h recovery under long day conditions. Bars, 1 cm.

chromatin structure (Moissiard *et al.*, 2012). Heat causes loss of chromocenter organization, similar to the effect caused by loss of H1 (Pecinka *et al.*, 2010; Rutowicz *et al.*, 2019; Choi *et al.*, 2020). Thus, the combination of H1 loss together with heat may cause a synergistic effect, opening heterochromatic pericentromeric regions and activating *GYPSY* TE expression. By

contrast, nonpericentromeric *COPIA* elements affected by heat, likely rely on the combination of CMT2 and H1 for stable repression (Choi *et al.*, 2020).

Most strikingly, we found that depletion of DNA methylation by zebularine enhanced heat tolerance and that this effect was strongly enhanced in *h1* mutants. Previous work revealed that loci controlling adaptive responses to the environment are frequent targets of TE insertions (Ito *et al.*, 2016; Quadrana *et al.*, 2016). *COPIA* TEs contain heat responsive factor binding elements (HREs) that confer heat response to the nearby genes (Ito *et al.*, 2011; Cavrak *et al.*, 2014; Pietzenuk *et al.*, 2016; Thieme *et al.*, 2017), and transcriptional activation of *COPIA78/ONSEN* elements correlates with environmental heat stress in most species of the Brassicaceae (Ito *et al.*, 2013). CHH methylation-deficient *cmt2* mutants are more heat-tolerant than wild-type (Shen *et al.*, 2014), consistent with the upregulated *COPIA* type TEs in both

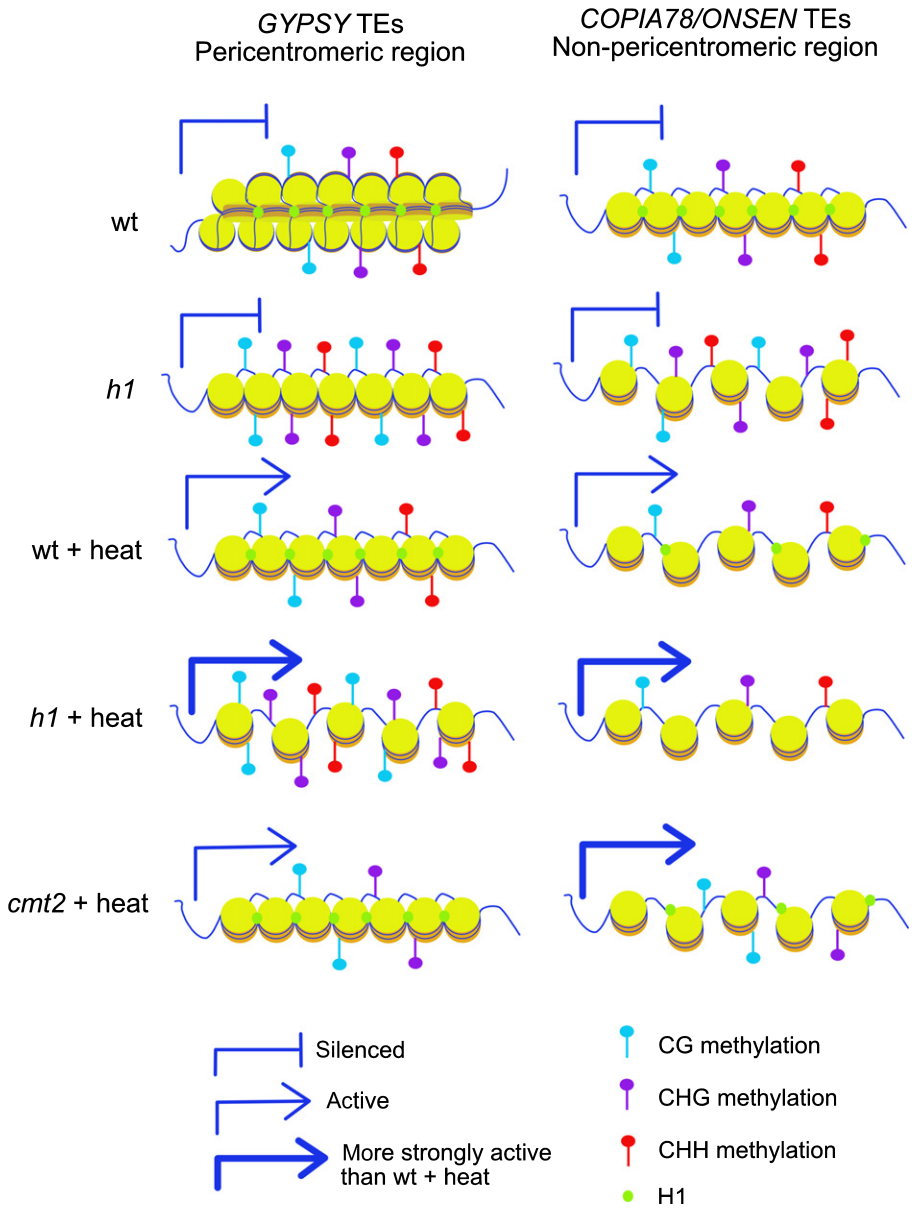

Fig. 6 Model depicting the role of histone H1 and DNA methylation in selective repression of *GYPSY* and *COPIA78/ONSEN* retrotransposons in *Arabidopsis*. Upregulated *GYPSY* transposable elements (TEs) in heat-treated *h1* mutants are located in pericentromeric regions, whereas upregulated *COPIA78/ONSEN* elements are dispersed in the chromosome arms. In *h1* mutants, chromatin structure is opened and the two groups of TEs gain DNA methylation and remain silenced. After heat stress of wild-type plants (WT + heat), DNA methylation level remains unchanged on upregulated *GYPSY* TEs, but decrease on upregulated *COPIA78/ONSEN* elements, thus likely contributing to their activation. Opened chromatin structure is likely sufficient to induce expression of *GYPSY* TEs. In heat-treated *h1* mutants (*h1* + heat), *GYPSY* TEs gain DNA methylation, which, however, is not sufficient for repression and *GYPSY* TEs become more strongly expressed compared to heat-treated WT. By contrast, *COPIA78/ONSEN* TEs lose DNA methylation in heat-treated *h1* mutants, which contributes to their upregulation, because *COPIA78/ONSEN* TEs, but not *GYPSY* TEs, are strongly activated in heat-treated *chromomethylase 2 (cmt2)* mutans (*cmt2* + heat).

cmt2 and *h1* mutants upon heat stress. We speculate that reduced DNA methylation and loss of H1 exposes heat-responsive elements that are epigenetically silenced under normal conditions. The combination of loss of H1 and reduced DNA methylation acts cooperatively on *COPIA* and *GYPSY* TE activation, possibly explaining the strongly enhanced heat tolerance of zebularine-treated *h1* seedlings. Whether or not this effect is heritable remains to be explored.

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Author contributions

LH conceived the study; SL and JdJ designed and performed the experiments; MST-A and JS-G analyzed high-throughput sequencing data; and SL and CK interpreted the data and wrote the manuscript. All authors approved the final version of the manuscript.

Data availability

The RNA and bisulfite-sequencing data in this study have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE152402.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Correlation analysis of RNA-seq samples.

Fig. S2 Comparison of data in this study with previously published data (Choi *et al.*, 2020).

Fig. S3 *H1.3* expression does not change in heat-treated *h1* mutants compared to heat-treated WT.

Fig. S4 A substantial proportion of upregulated genes in *h1* mutants and TEs in *h1* upon heat are heat-responsive in WT.

Fig. S5 H1 represses gene expression after heat stress.

Fig. S6 Gene ontology terms of upregulated genes in *h1* plus heat vs WT plus heat.

Fig. S7 Decreased CG, CHG and CHH methylation on upregulated *Copia78/ONSEN* elements.

Fig. S8 H1 does not affect *ONSEN* remobilization upon heat.

Fig. S9 *ONSEN* does not amplify in the progeny of heat and zebularine-treated WT and *h1* mutants.

Table S1 Primers used in the manuscript.

Table S2 Mapping statistics of RNA sequencing data.

Table S3 Lists of deregulated genes in *h1* mutants before and after heat stress.

Table S4 Lists of upregulated TEs in *h1* mutants and *cmt2* mutants before and after heat stress.

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