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Identification of genes preventing transgenerational transmission of stress-induced epigenetic states

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Abstract

Examples of transgenerational transmission of environmentally induced epigenetic traits remain rare and disputed. Abiotic stress can release the transcription of epigenetically suppressed transposons and, noticeably, this activation is only transient. Therefore, it is likely that mechanisms countering the mitotic and meiotic inheritance of stress-triggered chromatin changes must exist but are undefined. To reveal these mechanisms, we screened for *Arabidopsis* mutants impaired in the resetting of stress-induced loss of epigenetic silencing and found that two chromatin regulators, DDM1 and MOM1, act redundantly to restore pre-stress state and thus erase "epigenetic stress memory". In *ddm1* mutants, stress hyperactivates heterochromatic transcription and transcription persists longer than in the wild type. However, this newly acquired state is not transmitted to the progeny. Strikingly, although stress-induced transcription in *mom1* mutants is as rapidly silenced as in wild type, in *ddm1 mom1* double mutants, transcriptional signatures of stress are able to persist and are found in the progeny of plants stressed as small seedlings. Our results reveal an important novel function of DDM1 and MOM1 in rapid resetting of stress induced epigenetic states, and therefore also in preventing their mitotic propagation and transgenerational inheritance.

Significance Statement

Much attention has been drawn to research, which suggests that environmental factors, including stress and maternal care, alter the way the genetic code is executed. These epigenetic changes in gene regulation are thought to be stable enough to be heritable and thus may influence subsequent generations. Such prospects are as intriguing as they are troubling, since it is possible to imagine that accumulation of stress memories over several generations could make life difficult. Therefore, we have questioned whether mechanisms exist that could prevent the inheritance of stress-induced epigenetic changes and have discovered such mechanisms using forward genetics in *Arabidopsis*. Interestingly, one of the critical activities erasing stress memories is conserved between plants and mammals.

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Although environmentally induced traits and their transgenerational transmission in plants have been described repeatedly, trait stability and the involvement of epigenetic mechanisms in their generation remain controversial (1-3). In contrast, it has been well documented that environmental challenges such as elevated temperature can transcriptionally activate chromosomal loci normally silenced by repressive chromatin (4-7). However, this release of epigenetic silencing, unaccompanied by changes in DNA methylation or histone modifications, is only transient (4, 5). Such non-canonical release of transcriptional gene silencing (TGS) is similar to alterations in epigenetic regulation observed in the *mom1* mutant, where release of TGS occurs without major changes in epigenetic marks (8-13). Although, molecular mechanisms used by MOM1 in TGS regulation are not well understood, genetic studies have linked MOM1 activity to small interfering RNAs (14) and RNA processing (15). In addition, structure/function studies have suggested that a conserved domain of MOM1 forms a homodimer, which is possibly required as a binding platform for additional silencing factors (13, 16).

The rapid re-silencing of heterochromatic transcription induced by heat stress seems to involve changes in nucleosome occupancy and re-silencing is delayed in mutants with impaired chromatin assembly (5). These observations suggest that suppressive chromatin has certain plasticity in response to stress, but also a robust buffering system that resets its pre-stress state. This counters the persistence of stress-induced epigenetic alterations during subsequent development and thus their transmission to the progeny.

Results and Discussion

To identify factors involved in the erasure of "epigenetic stress memory", we performed a genetic screen using *Arabidopsis* line *LUC25* carrying a transcriptionally silenced transgene encoding firefly luciferase (*LUC*) (14), which as an endogenous chromosomal TGS target loci can be transiently activated after heat stress. First, we introduced the *mom1* mutation into *LUC25* (*mom1 LUC25*). The *mom1* mutation partially releases silencing of *LUC25*, producing weak luciferase signals in roots but not aerial parts, where the *LUC* transgene remains silenced. Importantly, the *LUC* transgene in *mom1 LUC25* is strongly activated by heat stress, similar to *LUC25* (Fig. S1A). We presumed that the introduction of the *mom1* mutation would enhance

stress-induced luciferase signals, increasing clarity and thus the efficiency of the mutant screen. Moreover, although the *mom1* mutation does not directly influence the kinetics of stress-induced TGS release, MOM1 is involved in buffering epigenetic states of chromatin (11). We hypothesized, therefore, that any deficiency in such buffering would facilitate phenotypic detection of additional epigenetic regulators involved in the rapid restoration of TGS after stress and, thus, in the erasure of epigenetic stress memory.

M2 seedlings of mutagenized *mom1 LUC25* were germinated for 5 days and individuals showing enhanced luciferase signals prior to stress treatment were removed, since these plants release TGS constitutively. The remaining seedlings were subjected to heat stress and plants showing significantly stronger and/or longer-lasting luciferase signals were selected and grown to maturity (Fig. S1B). We examined their M3 progeny to determine whether selected phenotypes were heritable. Interestingly, several plants selected in the M2 produced progeny uniformly showing high luciferase signals *prior* to heat stress in the M3. Since such "constitutive" phenotypes had been discarded in the previous M2 generation, we concluded that their appearance in the M3 reflects transgenerational transmission of heat stress-induced TGS release that occurred in the previous plant generation (Fig. 1A). In other words, we may have recovered mutant plants severely impaired in the erasure of epigenetic stress memory. Focusing on four independent lines with these characteristics, we identified causal mutations by a combination of genetic mapping and whole genome sequencing. Two independent mutations resided in a gene encoding nucleosome remodeler DDM1 (17-20) (Fig. 1B). The DDM1 protein, conserved between plants and mammals, is required for maintenance of DNA methylation, thus TGS (21, 22). It has been suggested that DDM1 alters accessibility of H1-containing heterochromatin to DNA methyltransferases (23).

Recovery of *ddm1* mutants in our screen was both surprising and disturbing. Surprising, since *ddm1* mutants are known to release epigenetic silencing independently of stress and, therefore, should have been eliminated from the screen in the M2. Disturbing, since *ddm1* mutants have a transgenerationally progressive effect on the loss of DNA methylation and silencing release (18, 19). Thus, luciferase activity observed in the M3 could simply reflect this *ddm1* property rather than transgenerational memory of heat stress. To address these reservations, we first analyzed DNA methylation of the *LUC* promoter. Cytosine methylation patterns were only slightly altered, however, surprisingly high levels of methylation remained in the M3 of both mutant lines (Fig. 1

C and D), which is unusual for *ddm1* mutants strictly associated with hypomethylation-mediated TGS release (17, 20, 24, 25). This supported the possibility that the heat stress-activated state of the *LUC* transgene, which is independent of DNA methylation (4, 5), can in fact be maintained and transmitted to the next generation in the *ddm1* background. This would define a novel and potentially crucial activity of DDM1 in reversing TGS after it has been destabilized by environmental changes/stress.

The only way to test this hypothesis was by the re-creation and analysis of the *ddm1* mutant line with a naïve and, thus, still-silenced *LUC* transgene (Fig. 2A). To obtain such a line, we crossed the commonly used allele of *ddm1* (*ddm1-2*) (18) into *LUC25* and subjected the F2-segregating progeny to temperature stress. Importantly, under control growth conditions, luciferase signals remained at the *LUC25* level and no differences between segregating F2 individuals were recorded (Fig. 2B). This suggested that the *LUC* transgene remained silent in the first generation of *ddm1* mutants and explains the initially unexpected presence of *ddm1* among plants subjected to heat stress during the mutant screen. Notably, heat stress applied to segregating F2 seedlings revealed individuals with very strong luciferase signals (Fig. 2B) in proportion close to expected segregation ratio for plants homozygous *ddm1* mutation containing *LUC* plants (18.75%). The genotyping of the segregating population at *DDM1* and *LUC* loci confirmed that, all of these plants were found to be homozygous for the *ddm1-2* mutation and contained *LUC* transgene in homozygous or hemizygous state, which had no influence on the intensity of LUC signals. Furthermore, we performed an analogous genetic experiment introducing the *ddm1-2* allele into another line (L5) (26) carrying a silent transgenic locus for the glucuronidase marker gene (*GUS*). As with the *LUC* transgene, we observed heat stress-dependent hyperactivation of *GUS* expression in *ddm1-2* mutants (Fig. S2). These results demonstrated that DDM1 down-regulates stress-induced heterochromatic transcription. Moreover, this novel *DDM1* activity appears to be independent of changes in DNA methylation, with which so far DDM1 was very tightly associated.

However, we found that in *ddm1-2* mutants the stress-hyperactivated LUC transgene was re-silenced within a few days (Fig. 2B) and there was no difference in LUC signals in the progeny (F3 generation) between stressed and non-stressed *ddm1-2 LUC25* plants (Fig. 2D).

Considering the genetic screen was performed in the *mom1 LUC25* background, we repeated the genetic reconstruction experiment including the *mom1* mutation. *ddm1-2* was crossed with *mom1 LUC25* and stress-induced LUC activation in the segregating F2 populations was examined as before, as well as its inheritance in the F3 (Fig. S3). LUC phenotyping and subsequent genotyping of F2 plants showed that the *mom1* mutation alone did not affect stress-induced expression of *LUC*, confirming the previous observations with the *mom1 LUC25* line. Although, the stress-induced LUC activation levels in the *ddm1-2 mom1* double mutants were similar to those in the *ddm1-2* single mutants (Fig. S4A), however, the LUC signals remained high only in *ddm1-2 mom1* double mutants (Fig. 2C). Furthermore, in the next (F3) generation, progeny from stressed *ddm1-2 mom1 LUC25* plants showed significantly higher LUC signals than non-stressed *ddm1-2 mom1 LUC25* controls (Fig. 2D and Fig. S4B), indicating that the stress-induced active state of the *LUC* transgene initiated at the small seedling stage could persist throughout plant development and be transgenerationally transmitted, however only in *ddm1 mom1* double mutants.

To further investigate stability and possible transgenerational inheritance in *ddm1 mom1* double mutants of stress-triggered TGS release, we examined transcriptional changes at endogenous chromosomal loci. For this we first performed RNA-seq analyses on four independent populations of stressed and control-treated *ddm1-2 mom1* double mutant plants. This genome-wide approach should uncover chromosomal loci stably activated following the stress subjected to the parental plants. The MDS plot analysis of the RNA-seq data revealed well-clustered biological repetitions for each individual line (Fig. 3A), indicating the robustness of the RNA-seq data. Noticeably, the largest difference between biological repetitions was seen for *ddm1 mom1* double mutant seedlings derived from ancestors not subjected to stress. This may simply reflect an intrinsic property of *ddm1 mom1* double mutants, which exhibit variation in heterochromatin silencing among individual plants (27). Most importantly, the samples were clearly clustered according to whether seedlings of the previous generation were stressed or not (Fig. 3A). Therefore, the genome-wide transcriptional profiles supported and extended our previous conclusion based on transgenic loci that temperature stress-activated transcription occurs genome wide, and that newly acquired transcriptional signatures can be transgenerationally inherited in *ddm1 mom1* double mutants.

Of the loci affected in the progeny of stressed *ddm1-2 mom1*, compared with progeny of control-treated *ddm1-2 mom1*, 340 loci were up-regulated more than twofold ($P<0.01$) and 484 down-regulated less than twofold ($P<0.01$) (Fig. 3B). Approximately 60% and 20% of the up-regulated and down-regulated transcripts, respectively, are derived from transposable elements (TEs) (Fig. 3B). These results are consistent with our previous demonstration that heterochromatic regions enriched in TEs are predominantly transcriptionally activated by temperature stress, and that euchromatic regions are either activated or repressed by this treatment (4). Such a transcriptional signature of the genome-wide stress-induced alteration of transcription appears to be inherited by the progeny of *ddm1 mom1* double mutant plants.

Due to economic constraints, we refrained from genome-wide analyses of plant populations constituting various experimental controls. Using the transcriptional profiling data described above we selected several genomic loci displaying heritable stress-induced alteration of transcription in *ddm1 mom1* double mutants, and examined by quantitative RT-PCR persistence of their transcriptional activation in wild type and single *mom1* or *ddm1* mutant plants relative to stress treatments in the preceding generation. No significant differences in transcript levels were found between the progenies of stressed and control wild-type plants or single mutants, which is in contrast to *ddm1 mom1* double mutants (Fig. 3C). This supports our previous conclusion, derived from the properties of transgenic loci, that *DDM1* and *MOM1* both act redundantly in resetting chromatin status destabilized by heat stress, which prevents transgenerational propagation of transcriptional stress memory (Fig. 4).

A closer look at the results of the genome-wide transcriptional analyses reveals that DDM1 and MOM1 are not the only factors reverting the properties of chromatin affected by stress. We found previously that approximately 3,000 loci are activated under stress conditions analogous to those used here (4) and, thus, only a small fraction (340) remain transgenerationally active in the *ddm1 mom1* double mutants. This suggests that the prevention of transgenerational transmission of stress memory extends far beyond the activities of DDM1 and MOM1 and, thus, that the unequivocal demonstration of transgenerational transmission of environmentally-induced epigenetic traits remains a significant challenge.

Materials and Methods

Plant materials

The *LUC25* line was described previously(14). *mom1 LUC25* was obtained by crossing *mom1-6* and *LUC25*. *mom1-6* seeds were obtained from INRA-Versailles, Genomic Resource Center (FLAG_340E12) and *ddm1-2* seeds were provided by Dr. E. Richards. *LUC25* and *mom1-6* are in the Wassilewskija (WS) background and *ddm1-2* in the Columbia (Col-0) background.

Mutagenesis and screening

mom1 LUC25 seeds (20,000) were mutagenized in 0.3% EMS for 15 h. After washing with water, seeds were germinated on soil to give 78 M2 pools, each derived from approximately 150 M1 plants. For each pool, 1,000 seeds were plated on 1/2 MS medium (0.8% agar, 1% sucrose) and screened for mutants by spraying with a luciferin (Biosynth) solution (31.5 mg per 100 ml water) and examining treated seedlings using an Aequoria dark box with a mounted ORCAII CCD camera (Hamamatsu). Luciferase luminescence and chlorophyll auto-fluorescence image overlays were created using the Wasabi software package (Hamamatsu). Isolated mutants were crossed with wildtype in the Col-0 background to generate mapping populations.

DNA methylation analysis

Bisulphite sequencing was performed as described previously (14). Sequencing data were analyzed with Kismeth (http://katahdin.mssm.edu/kismeth)(28). Primers used for bisulphite sequencing are listed in Table S1.

Histochemical GUS staining

Staining was performed on cotyledons of 12-day-old plants as described previously (13).

RAN-seq analyses

Total RNA samples were isolated from 7-day-old seedlings using TRI reagent (Sigma). The libraries were prepared using a TruSeq RNA Sample Prep Kit (Illumina) and sequenced using HiSeq 2500 (Illumina) with single-end 50 base reads. The trimmed reads were mapped with the TopHat v1.3.3 software to the TAIR10 annotations. The normalization and differential expression analysis were performed with R/Bioconductor package edgeR v.2.6.12 (29).

Real-time qPCR analysis

Total RNA was isolated from 7-day-old seedlings using TRI reagent (Sigma). After RQ1 DNase treatment (Promega), cDNA was synthesized with the Superscript VILO cDNA synthesis kit (Life Technologies). Real-time qPCR analyses were performed using Power SYBR Green PCR Master Mix (Life technologies) in ABI7900FT (Life technologies). PCR conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Primers are listed in Table S1.

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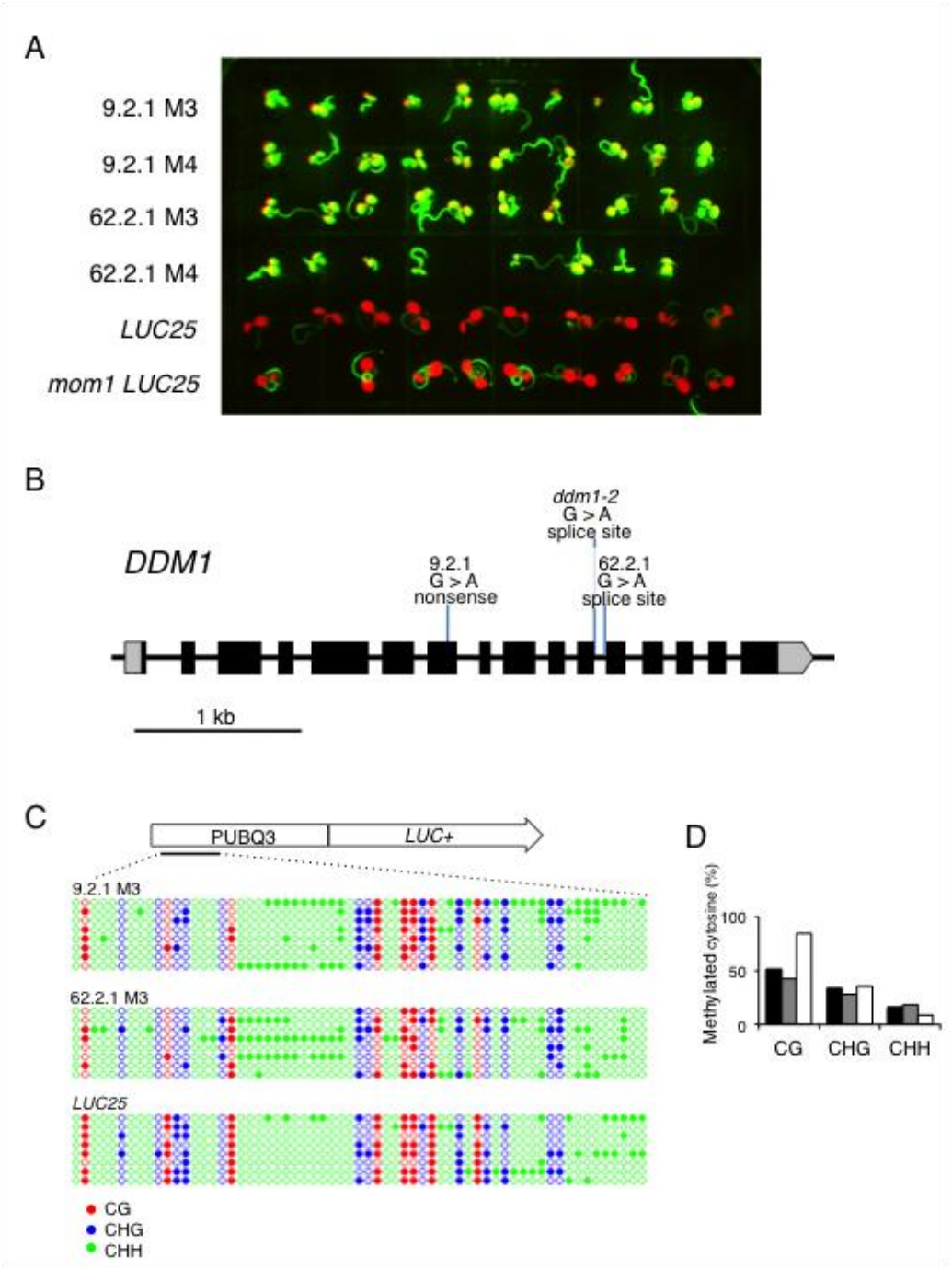
Figure Legends

Fig. 1. Identification and characterization of mutants showing transgenerational transmission of the heat-stress release of TGS. (A) Bioluminescence images of the progeny (M3 and M4) of two mutant candidates (9.2.1 and 62.2.1) and of the controls (*LUC25*, and *mom1 LUC25*), all grown under control conditions at 21°C. The two mutant lines are M3 progeny of heat stressed M2 parents recovered from the mutant screen. The green and red signals are luciferase luminescence and auto-fluorescence of chlorophyll, respectively. **(B)** Two new mutant alleles of the *DDM1* gene (9.2.1 and 62.2.1) were identified in the screen. The *ddm1-2* allele was used for the experiments presented in Fig. 2. **(C)** DNA methylation distribution at the ubiqutin3 promoter of the *LUC* transgene in 9.2.1 (M3), 62.2.1 (M3), and *LUC 25* (WT). Colored and open circles represent methylated and unmethylated cytosines respectively, with red representing CG sites, blue CHG and green CHH (H can be A,T or C). **(D)** Percentage of cytosine methylation in the ubiqutin3 promoter. Black bars, 9.2.1; grey bars, 62.2.1; white bars, *LUC25*.

Fig. 2. Inheritance of stress-induced transcriptional activation of *LUC*. (A) Crossing scheme for the re-creation of the *ddm1-2* mutant line with the naïve *LUC* transgene. P0: *ddm1-2* was crossed with WT *LUC25*, F1: heterozygous for *ddm1* and carrying the hemizygous naïve *LUC* transgene, F2: *ddm1* homozygous mutants are segregated in the progeny.F2 seedlings were separated into two subpopulations, one of which was subjected to heat stress. Bioluminescence images were captured (panel B) and each plant was genotyped at the *DDM1* and *LUC* loci. **(B)** Bioluminescence images of segregating progeny of a hybrid between *ddm1-2* and *LUC25* (Fig. 2A). The F2 seedlings were expected to include 18.75 % of individuals homozygous for the *ddm1-2* mutation and carrying the *LUC* transgene, these were predicted to display enhanced luminescence. Rows of *LUC25* and *mom1 LUC25* plants are shown as a control. Note, LUC signals in *mom1* after heat stress are restricted to roots. **(C)** Bioluminescence images of segregating progeny of a cross between *ddm1-2 mom1 LUC25* (for details Fig. S3). The F2 progeny is expected to include 4.69 % of individuals homozygous for both *ddm1-2 and mom1* and carrying the *LUC* transgene. White arrowheads point towards *ddm1-2 mom1* double mutant plants, as revealed by the genotyping of the population at *DDM1* and *MOM1* loci. Quantification of LUC signals is displayed in Fig. S4. **(D)** Bioluminescence images of *ddm1 LUC25* and *ddm1 mom1 LUC25* F3 progenies (as depicted on panel A) of F2 heat stressed parents at the seedlings stage (+stress) or non-stressed controls (-stress). Two independent F3 populations (upper or lower row) derived from two *ddm1 LUC25* or *ddm1 mom1 LUC25* F2 plants (panel B and C).

Fig. 3. Genome-wide analysis of transcriptional changes in the progeny of heat-stressed *ddm1 mom1*. (A) Multidimensional scaling (MDS) plot (R/Bioconductor) showing the overall similarity of RNA expression patterns between samples using RNA-seq data of two biological repetitions. These were performed with two independent populations (circled) of *ddm1 mom1* progeny plants obtained from stressed or non-stressed parents at the seedling stage (*mom1* transcriptome was used as an additional control). **(B)** Pie charts showing the difference in functional distribution of up- and down-regulated loci between heat-stressed or non-stressed double mutants. **(C)** Relative levels of mRNA of selected targets as determined by qRT-PCR. Values were normalized to 18s ribosomal RNA. The mean of one ~~sample~~ of stressed *ddm1 mom1* was set to 1. White and gray bars indicate the progeny of control plants and heat-stressed plants, respectively. Pooled F3 plants (approximately 20 individuals), progeny of two independent F2 plants for each category were used for these analyses. Gene annotations of each target: AT1G43880 - ATLANTYS1 (TE), AT2G05564 - VANDAL2 (TE), AT5G29560 - caleosin-related family protein, AT5G34790 - VANDAL20 (TE), AT2G12345 - ATHILA3 (TE), AT5G48850 - ATSDI1, SULPHUR DEFICIENCY-INDUCED 1. Error bars indicate standard deviation of results from three repeated experiments. $*P < 0.01$, student's *t* test.

Fig. 4. Model summarizing a putative epigenetic mechanism preventing the transmission of "stress memory" to progeny. Schematic illustration on a possible chromatin states upon heat stress. Although heterochromatic loci are transcriptionally activated by temperature stress in WT and *mom1* mutants, they are rapidly re-silenced after stress is removed. Stress-induced transcription is hyper-activated and persists longer in *ddm1* mutants than in WT and *mom1*; however, the altered transcriptional status is not transmitted to the progeny. In contrast, stress-induced transcriptional activation in *ddm1 mom1* double mutants is transgenerationally inherited. The transcriptional activation in *ddm1 mom1* could due to altered positioning of nucleosome or other modifications of chromatin properties.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9.2.1 M4
62.2.1 M3
62.2.1 M4
LUC25
mom1 LUC25
B
DDM1
ddm1-2
G > A
splice site
9.2.1
G > A
nonsense
62.2.1
G > A
splice site
1 kb
C
PUBQ3
LUC+
9.2.1 M3
62.2.1 M3
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D
Methylated cytosine (%)
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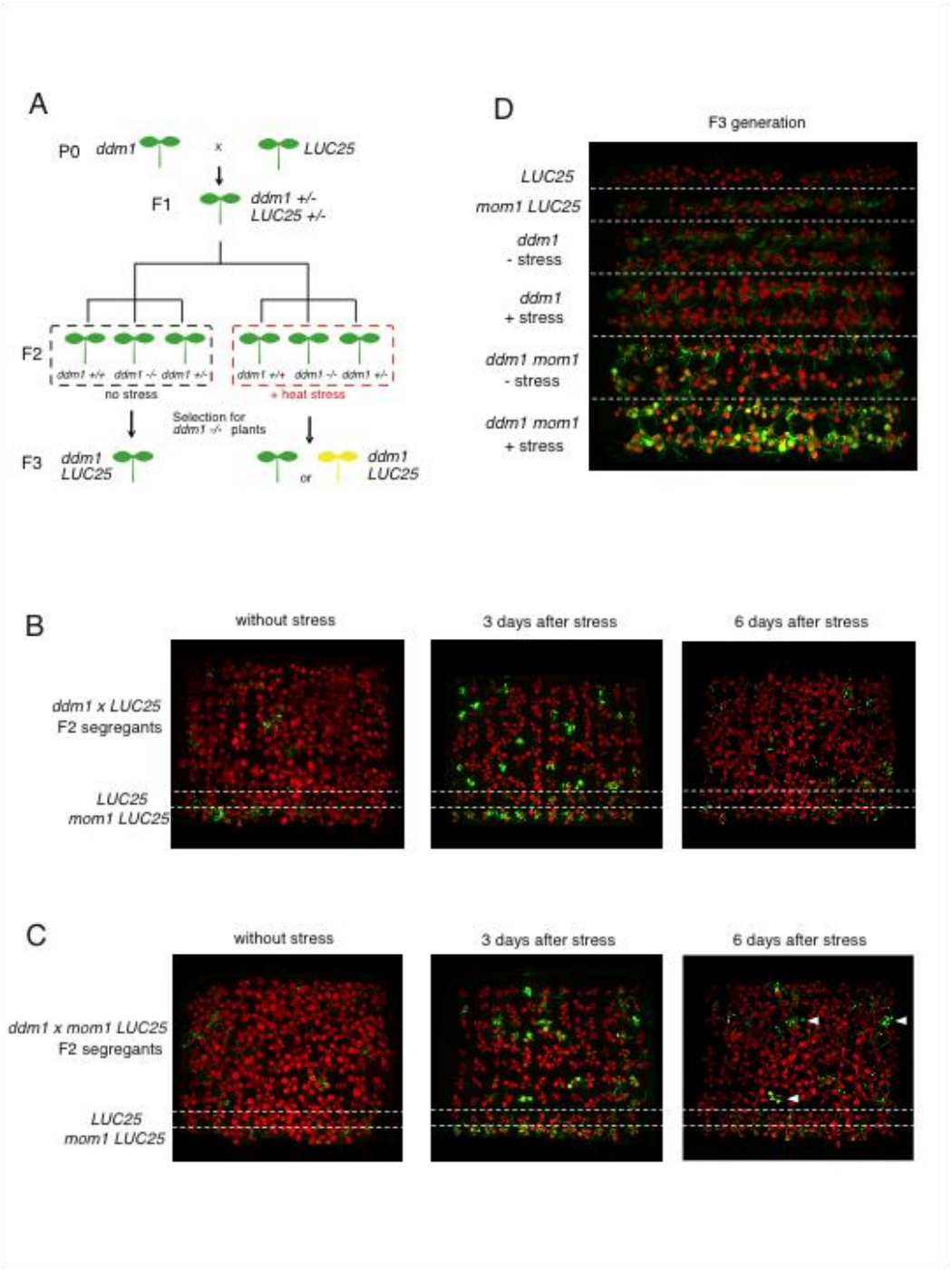
50
0
CG
CHG
CHH

Fig. 1


A
P0 ddm1 x LUC25
F1 ddm1 +/- LUC25 +/-
F2
ddm1 +/+ ddm1 -/- ddm1 +/-
no stress
ddm1 +/+ ddm1 -/- ddm1 +/-
+ heat stress
Selection for ddm1 -/- plants
F3 ddm1 LUC25
or
ddm1 LUC25
D
F3 generation
LUC25
mom1 LUC25
ddm1 - stress
ddm1 + stress
ddm1 mom1 - stress
ddm1 mom1 + stress
B
without stress
3 days after stress
6 days after stress
ddm1 x LUC25 F2 segregants
LUC25 mom1 LUC25
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without stress
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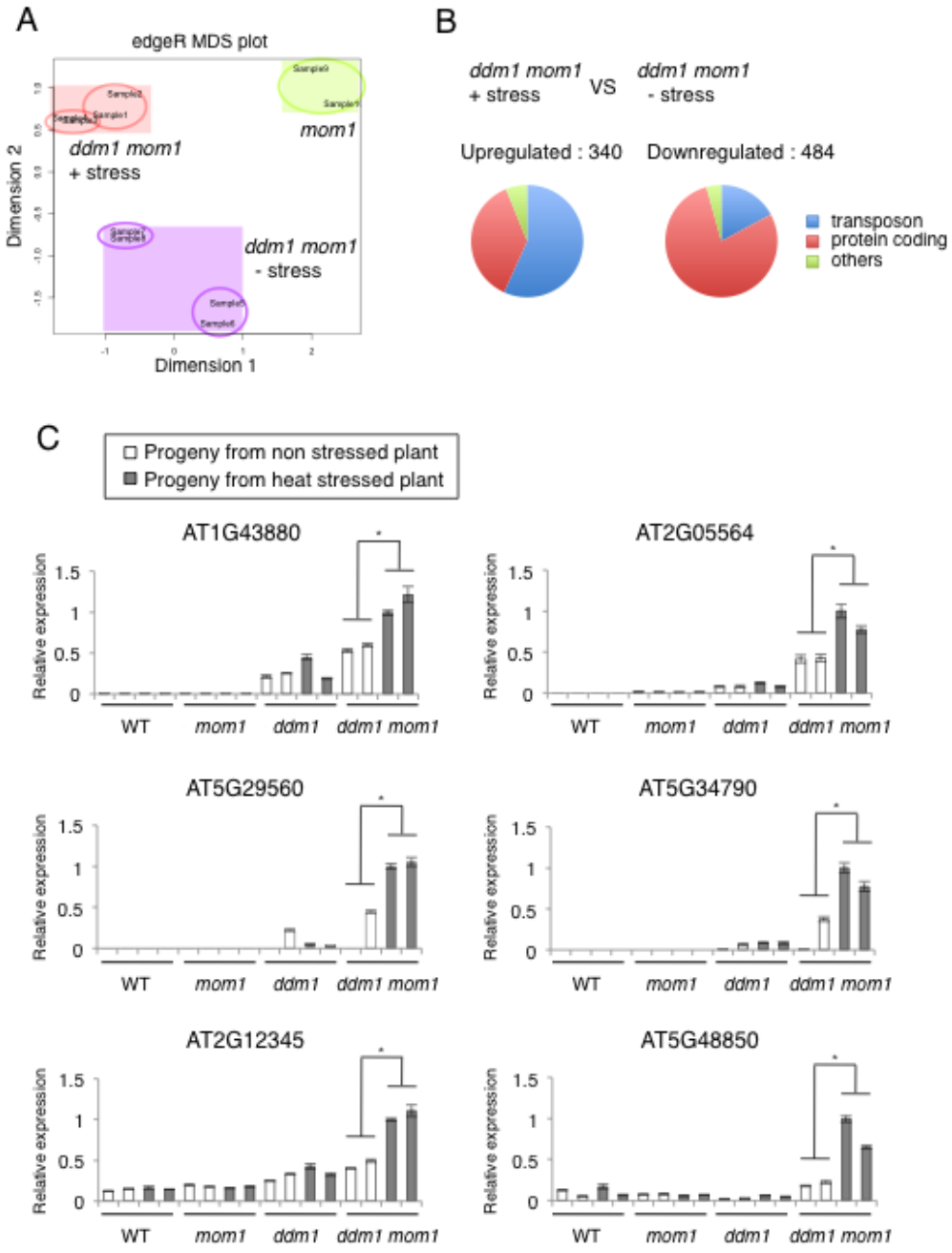
ddm1 x mom1 LUC25 F2 segregants
LUC25 mom1 LUC25

Fig. 2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edgeR MDS plot
mom1
ddm1 mom1
+ stress
ddm1 mom1
- stress
Dimension 2
Dimension 1
B
ddm1 mom1
+ stress
VS
ddm1 mom1
- stress
Upregulated : 340
Downregulated : 484
transposon
protein coding
others
C
Progeny from non stressed plant
Progeny from heat stressed plant
AT1G43880
AT2G05564
AT5G29560
AT5G34790
AT2G12345
AT5G48850
Relative expression
WT
mom1
ddm1
ddm1 mom1

Fig. 3

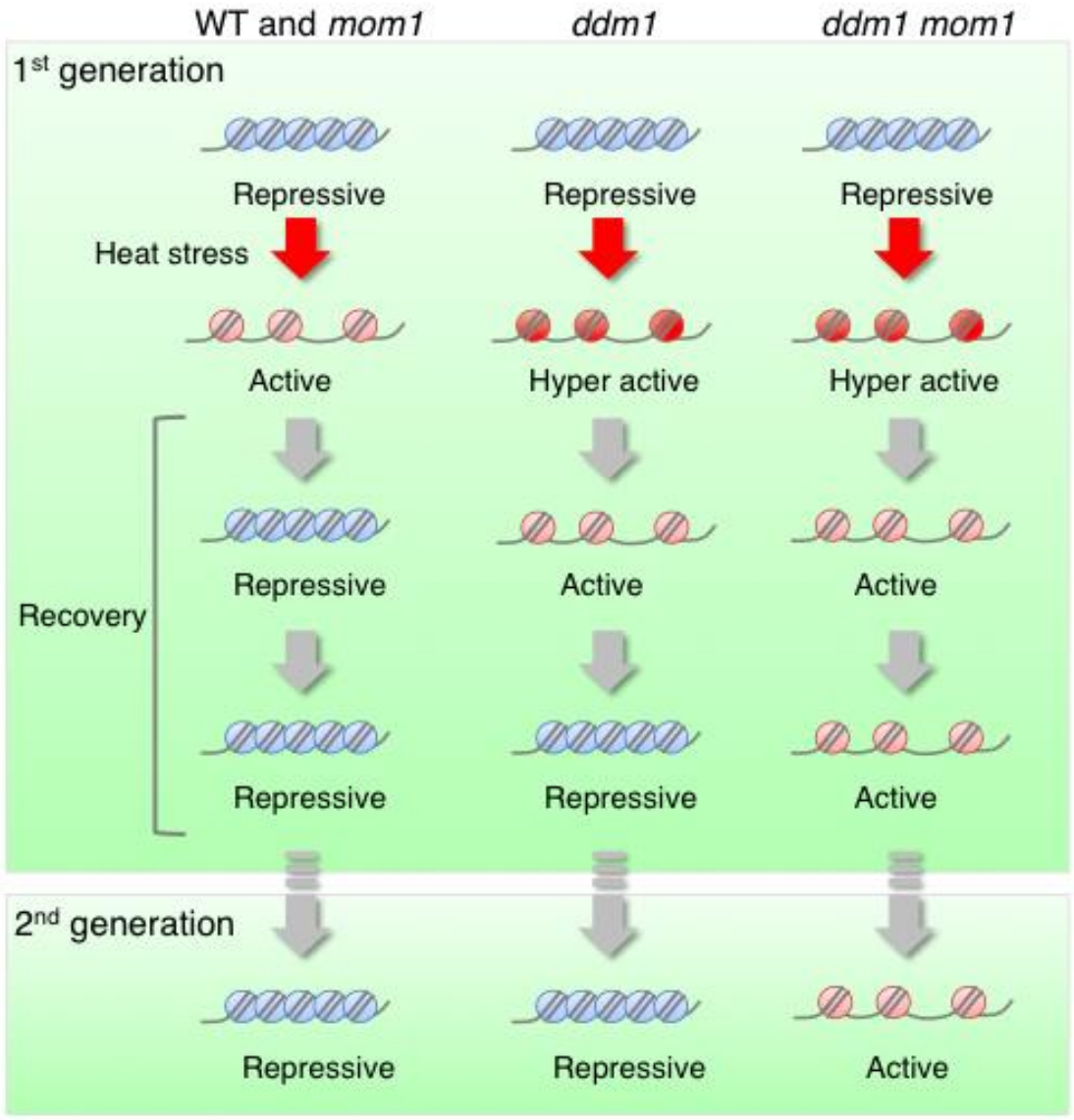
WT and mom1
ddm1
ddm1 mom1
1st generation
Repressive
Repressive
Repressive
Heat stress
Active
Hyper active
Hyper active
Recovery
Repressive
Active
Active
Repressive
Repressive
Active
2nd generation
Repressive
Repressive
Active

Fig. 4