New Phytologist

Research

Transgenerational phenotype aggravation in CAF-1 mutants reveals parent-of-origin specific epigenetic inheritance

Iva Mozgova[1,2]*, Thomas Wildhaber[3]*, Minerva S. Trejo-Arellano[1], Jiri Fajkus[4], Pawel Roszak[3], Claudia Köhler[1] and Lars Hennig[1]

[1]Department of Plant Biology, Uppsala BioCenter, Swedish University of Agricultural Sciences and Linnean Center for Plant Biology, SE-75007 Uppsala, Sweden; [2]Institute of Microbiology of the Czech Academy of Sciences, Centre Algatech, Opatovický mlýn, CZ-37981 Třeboň, Czech Republic; [3]Department of Biology and Zurich-Basel Plant Science Center, ETH Zurich, CH-8092 Zurich, Switzerland; [4]Laboratory of Functional Genomics and Proteomics, National Centre for Biomolecular Research, Faculty of Science, Masaryk University, CZ-61137 Brno, Czech Republic

Author for correspondence:
Lars Hennig
Tel: +46 18 673326
Email: Lars.Hennig@slu.se

Received: *20 December 2017*
Accepted: *5 February 2018*

New Phytologist (2018)
doi: 10.1111/nph.15082

Key words: *Arabidopsis thaliana*, CAF-1, Chromatin, Development, DNA methylation, epigenetics, histone.

Summary

- Chromatin is assembled by histone chaperones such as chromatin assembly factor CAF-1. We had noticed that vigor of *Arabidopsis thaliana* CAF-1 mutants decreased over several generations. Because changes in mutant phenotype severity over generations are unusual, we asked how repeated selfing of Arabidopsis CAF-1 mutants affects phenotype severity.
- CAF-1 mutant plants of various generations were grown, and developmental phenotypes, transcriptomes and DNA cytosine-methylation profiles were compared quantitatively.
- Shoot- and root-related growth phenotypes were progressively more affected in successive generations of CAF-1 mutants. Early and late generations of the *fasciata (fas)2-4* CAF-1 mutant displayed only limited changes in gene expression, of which increasing upregulation of plant defense-related genes reflects the transgenerational phenotype aggravation. Likewise, global DNA methylation in the sequence context CHG but not CG or CHH (where H = A, T or C) changed over generations in *fas2-4*. Crossing early and late generation *fas2-4* plants established that the maternal contribution to the phenotype severity exceeds the paternal contribution.
- Together, epigenetic rather than genetic mechanisms underlie the progressive developmental phenotype aggravation in the Arabidopsis CAF-1 mutants and preferred maternal transmission reveals a more efficient reprogramming of epigenetic information in the male than the female germline.

Introduction

Nuclear DNA is packaged into chromatin, which affects and regulates major cellular processes such as transcription, replication, DNA-repair and silencing of transposable elements (TEs). The basic unit of chromatin is the nucleosome, a hetero-octamer of histones H2A, H2B, H3 and H4, which organizes 147 bp of DNA. Chromatin assembly factor 1 (CAF-1) is a histone chaperone that initiates nucleosome formation by depositing H3–H4 dimers on free DNA after replication or DNA repair (Ramirez-Parra & Gutierrez, 2007b; Yu *et al.*, 2015). CAF-1 consists of three subunits, which are conserved in all eukaryotes (Verreault *et al.*, 1996; Kaufman *et al.*, 1997) and are called FASCIATA 1 (FAS1), FASCIATA 2 (FAS2) and MULTICOPY SUPRESSOR OF IRA 1 (MSI1) in Arabidopsis (Kaya *et al.*, 2001). Lack of CAF-1 is lethal in mammalian cells and causes developmental arrest in *Drosophila* (Nabatiyan & Krude, 2004; Song *et al.*, 2007).

In Arabidopsis, *fas1* and *fas2* mutants are viable (Kaya *et al.*, 2001), whereas *msi1* mutants are embryo-lethal due to the crucial function of MSI1 in Polycomb repressive complexes (PRC2) (Köhler *et al.*, 2003; Guitton *et al.*, 2004). The *fas1* and *fas2* CAF-1 mutants develop several phenotypic defects, including stem fasciation, abnormal leaf and flower morphology, and disorganization of the shoot and root apical meristems (Reinholz, 1966; Leyser & Furner, 1992; Kaya *et al.*, 2001). CAF-1 mutants have also defects in cell fate specification (Costa & Shaw, 2006; Exner *et al.*, 2006). Recently, it has been shown that some of the developmental phenotypes are a result of unrestricted activation of defense genes (Mozgova *et al.*, 2015). Additionally, CAF-1 is required for the organization of heterochromatin and maintenance of transcriptional gene silencing, including inactivation of certain TEs (Kaya *et al.*, 2001; Kirik *et al.*, 2006; Ono *et al.*, 2006; Schönrock *et al.*, 2006), regulation of endoreduplication (Exner *et al.*, 2006; Kirik *et al.*, 2006; Ramirez-Parra &

*These authors contributed equally to this work.

Gutierrez, 2007a,b), regulation of cell cycle duration (Ramirez-Parra & Gutierrez, 2007a,b; Abe *et al.*, 2008; Chen *et al.*, 2008) and homologous recombination (Endo *et al.*, 2006; Kirik *et al.*, 2006). More recently, a functional link was found between CAF-1 and maintenance of telomeres and ribosomal DNA (rDNA) (Mozgova *et al.*, 2010). In *fas1* and *fas2*, telomere shortening and loss of rDNA occurs in an increasingly severe fashion over several generations. It also had been noticed that vigor of *fas1* and *fas2* mutants decreased over several generations (Mozgova *et al.*, 2010) but this effect was not thoroughly documented. As most mutants in genetic model systems have stable phenotypes, a possible change in phenotype severity over generations of the *fas1* and *fas2* CAF-1 mutants is remarkable. Here, we asked how repeated selfing of *fas1* and *fas2* affects phenotype severity. We found that plant size, juvenile–adult phase transition and maternal reproduction are progressively more affected in successive generations of the *fas1* and *fas2* CAF-1 mutants. Fully expanded leaves of early and late *fas2* generations display only limited changes in gene expression, of which upregulation of plant defense-related genes reflects the transgenerational phenotype aggravation. The developmental phenotypes of the CAF-1 mutants but not the tandem repeat copy-number are readily complemented by the presence of a functional CAF-1 complex. By crossing early and late generation mutants, we find that the maternal contribution to the phenotype severity exceeds the paternal contribution. Together, we establish that epigenetic rather than genetic mechanisms underlie the progressive developmental phenotype aggravation in the Arabidopsis CAF-1 mutants.

Materials and Methods

Plant material

All experiments used *Arabidopsis thaliana* (L.) Heynh. accession Col-0 and the *fas1-4* (SALK_N828822) and *fas2-4* (SALK_N533228) mutants (Exner *et al.*, 2006). For further information and scheme of plant propagation see Supporting Information Methods and Fig. S1. For the *fas1-4 35S::FAS1* lines, the *FAS1* cDNA was amplified (for primers see Table S1) and recombined into pMDC32 (Curtis & Grossniklaus, 2003) followed by transformation into *fas1* generation 4 (G_4) plants. The relative quantification of 45S rDNA repeat number and telomere length analysis was performed as described (Mozgova *et al.*, 2010).

Plants were grown at 21°C with humidity of 60% under 16 h : 8 h, or 8 h : 16 h, light : dark cycles corresponding to long-day or short-day conditions, respectively, at 120 µmol m^{-2} s^{-1}. Plants under AGRONOMICS conditions (Baerenfaller *et al.*, 2012) were cultivated at 8 h 22°C : 16 h 21°C, light : dark short-day cycles, with humidity of 60% and a light intensity of 100 µmol m^{-2} s^{-1}. To expose plants to waterlogging stress, pots with 10-d-old plants were submerged in water up to soil level for the entire duration of the plant life. The submerging water was exchanged two times per week. Stressed plants were cultivated under long-day cycles as described above.

Rosette diameter, silique length, shoot length and length of silique number 6 were measured after plants were fully grown and carried only mature siliques. The juvenile–adult phase transition was determined by emerging of trichomes on the abaxial (lower) surface of rosette leaves. For details on characterization of ovule development see Methods S1.

Gene expression analysis

Reverse transcription polymerase chain reaction (RT-PCR) was performed as described previously (Mozgova *et al.*, 2015) (for primers see Table S1). For transcriptome analysis, wild-type (WT), *fas2-4* G_1 and G_6 plants were grown for 48 d according to AGRONOMICS standard conditions (Baerenfaller *et al.*, 2012). Leaf number 6 was harvested at ZT (zeitgeber time) = 7 and processed for transcriptome profiling on Affymetrix AGRONOMICS1 Arabidopsis tiling arrays (Rehrauer *et al.*, 2010) as described (Mozgova *et al.*, 2015). The experiment was performed in biological triplicates with each triplicate consisting of pooled leaves from five, seven and nine plants for WT Col, *fas2-4* G1 and *fas2-4* G6, respectively. Data were normalized and analyzed as described (Rehrauer *et al.*, 2010; Müller *et al.*, 2012). Differential expression was tested with LIMMA (Smyth, 2004) followed by multiple testing correction (Storey & Tibshirani, 2003). Genes were considered to be differentially expressed when $q < 0.05$ and fold-change > 1.5. Differentially expressed genes (DEGs) were assigned to one of eight principal classes of change by regression analysis. Briefly, gene expression vectors per gene were (0, 1) normalized before squared distances to each of the eight principal expression profiles were calculated. Each of the selected DEGs was assigned to the profile class for which the sum of squared distances was minimal.

Analysis of cytosine-DNA methylation by bisulfite sequencing

Bisulfite-converted DNA was sequenced in paired-end mode of 90 nucleotides long reads using an Illumina HiSeq2000 sequencer (Illumina Inc., San Diego, CA, USA). Quality control was performed with in-house scripts. Adapter trimming and removal of low complexity sequences was done using reaper (Davis *et al.*, 2013). Reads with total sequencing quality of < 30 Phred or shorter than 10 nucleotides were discarded. Clean reads were mapped to the reference genome TAIR10 using bismark (Krueger & Andrews, 2011) allowing at most one mismatch per 25-nucleotide seed. Forward and reverse reads were mapped independently (Table S2). Conversion rates and methylation status of cytosines were obtained using bismark_methylation_extractor. Cytosines in the plastid genome are not methylated, allowing estimates of bisulfite conversion efficiency. The mean conversion rate was 96% and the estimated mean false positive methylation rates were < 5% (Table S3). Cytosines that were covered by at least seven reads in WT, G1 and G6 were considered for the calculation of differentially methylated regions (DMRs). The Arabidopsis TAIR10 genome sequence was divided into windows of 100 bp; windows containing less than three covered

cytosines were discarded. To avoid calling DMRs with artificially small or large methylation differences due to low coverages, the 10th percentile of bins with lowest coverage was selected and bins with the 20% smallest and 20% largest average methylation values were discarded. Bins with a *P*-value ≤ 0.01 (Fisher's exact test) were considered as significant. Methylation changes are expressed as the relative difference between the given genotypes. DMRs were classified in eight nonoverlapping classes according to the transgenerational trends of change observed for DEGs.

Accession numbers

Data are available at GEO and ArrayExpress (accession numbers GSE104456 and E-MTAB-6136).

Results

Developmental phenotypes of CAF-1 mutants change over generations

In order to test for a potential change in phenotype severity in Arabidopsis Chromatin assembly factor 1 (CAF-1) mutants, *fas2-4* plants of the first to the sixth generation were grown in parallel (Fig. S1). Note that generation 1 (G_1) refers to the first homozygous mutant plants that segregate from a selfed heterozygous parental plant. There was a clear transgenerational aggravation of mutant plant vigor (Fig. 1a–c). The phenotype aggravation was visible as early as 13 d after induction of germination, as the plantlets of later generations appeared considerably smaller than those of earlier generations (Fig. 1a). This difference was even more pronounced at 17 (Fig. 1b) and 31 d (Fig. 1c). Reduced rosette and silique size and accelerated bolting were most obvious. Decreasing seed set led to hardly any seeds being produced by G_6 plants under our conditions.

Quantification of *fas2-4* developmental phenotypes showed a gradual transgenerational phenotype aggravation (Fig. 1d–h). Rosette diameter of fully grown plants decreased from *c.* 60% of WT rosette size in *fas2-4* G_1 to *c.* 25% in G_6 plants (Fig. 1d). The number of total rosette leaves decreased gradually from *c.* 90% of WT numbers in *fas2-4* G_1 to < 60% in G6 plants (Fig. 1e). Rosette leaf numbers in *fas2-4* were mainly affected by the number of adult leaves (Fig. 1f,g), which gradually decreased from *c.* 60% in *fas2-4* G_1 to < 10% in G_6 plants (Fig. 1g). Frequently, G_6 plants completely lacked adult leaves and formed only juvenile leaves before bolting (Fig. 1f,g). These results showed a gradual reduction of total leaf number as a consequence of the gradual reduction of adult leaf numbers in later generations of *fas2-4* mutant plants. Similar effects of generation on rosette diameter and bolting were observed for *fas1-4* (see below, Fig. 6a), demonstrating that the transgenerational aggravation is neither specific for a particular allele nor for loss of FAS2 but is characteristic for disrupted CAF-1 function in Arabidopsis.

The *fas1* and *fas2* mutants have defects not only in aboveground organs originating from shoot apical meristems (SAM), but also in root growth and development (Leyser & Furner, 1992; Kaya *et al.*, 2001; Costa & Shaw, 2006; Ramirez-Parra &

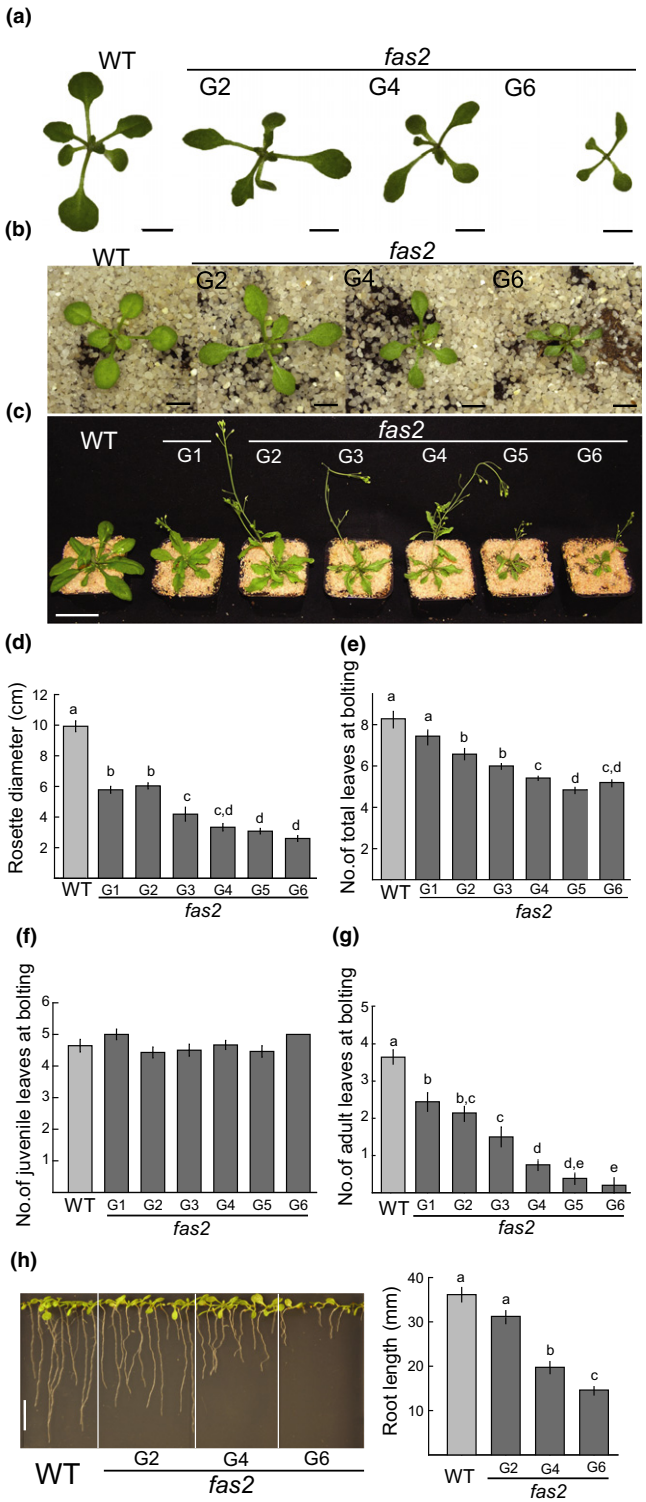

Fig. 1 Transgenerational phenotype aggravation in *Arabidopsis thaliana fas2* mutant plants. (a–c) Examples of phenotypes of plants (a) 13 d after germination (dag), (b) 17 dag and (c) 31 dag. (d) Quantification of rosette diameter of fully grown plants ($n = 14$ plants). (e–g) Quantification of the number of (e) total, (f) juvenile and (g) adult rosette leaves at bolting ($n = 14$ plants). (h) Example and quantification of root length in seedlings at 10 dag ($n = 25$–30 seedlings grown on four different plates). Bars represent means ± SE. Different letters above bars indicate significant difference ($P < 0.05$ in Student's *t*-test). Bars: (a, b) 5 mm; (c) 5 cm; (h) 1 cm. WT, wild-type; G, generation.

Gutierrez, 2007a,b; Pavlistova *et al.*, 2016). Therefore, we asked whether transgenerational aggravation also occurs for root phenotypes. Indeed, the impaired root growth of *fas2-4* became more severe in later generations (Fig. 1h). Together, both SAM-related and root apical meristem-related phenotypes show transgenerational aggravation in *fas2-4* plants, most likely due to disturbed CAF-1 function.

Defects in ovule development cause reduced seed set in *fas2-4*

As we had noticed reduced seed set in *fas2-4* plants, we next studied reproductive development in more detail. Because reduced seed set often causes reduced silique length, we measured silique length in several generations of *fas2-4* (Fig. 2a). Silique length greatly decreased from 70% in *fas2-4* G_1 to < 20% in G_6, demonstrating a transgenerational aggravation similar to that of other tested phenotypes. In order to identify reasons for reduced fruit length, seed set was quantified in naturally pollinated WT, *fas2-4* G_2 and G_6 plants (Fig. 2b). Under our experimental conditions, WT siliques had *c.* 45 normally developed seeds and five nonfertilized ovules. By contrast, siliques of *fas2-4* G_2 had *c.* 20 normally developed seeds and 10 unfertilized ovules. In *fas2-4* G_6, no normally developed seeds could be found and siliques contained 25 unfertilized ovules. Seed abortion was always below 1%. Thus, reduced silique length in *fas2-4* is caused by two effects. First, *fas2-4* forms *c.* 40% fewer ovules per flower than WT. This phenotype is almost completely penetrant in G_2 as it does not differ considerably between G_2 and G_6 (Fig. 2b). Second, lack of FAS2 caused reduced efficiency of fertilization. This phenotype is only partially visible in G_2 (30% of unfertilized ovules) but fully penetrant in G_6 (100% of unfertilized ovules). Together, reduced number of ovules formed and a large fraction of unfertilized ovules explain the short siliques and low seed set in *fas2-4* mutants.

Next, we investigated ovule development in more detail. Stages of WT ovule development include integument outgrowth, integument fusion and closure (Fig. 2c-i–ii), and finally formation of mature ovules (Fig. 2c-iii). In *fas2-4* G2 plants (Fig. 2c-iv–viii), integuments of most ovules started to grow (Fig. 2c-iv), fused and closed normally (Fig. 2c-v). In some ovules, however, integuments did not fuse properly (Fig. 2c-vi) and tissue was protruding out of the ovule with the mature ovule sometimes bearing incompletely fused integuments (Fig. 2c-vii, viii). In G_6 plants (Fig. 2c-ix–xi), integuments grew only very incipiently (Fig. 2c-ix) before arresting prematurely (Fig. 2c-x). The unfused integuments determine defective ovules, which cannot be fertilized (Fig. 2c-xi). Note that no normally developed ovules could be found in G_6, consistent with the complete lack of developing seeds in these plants.

In summary, these results demonstrate that ovule development is strongly impaired in subsequent *fas2-4* generations, most likely leading eventually to complete sterility.

Transgenerational aggravation of transcriptome changes in *fas2-4* plants

In order to test whether the transgenerational aggravation of developmental phenotypes of *fas2-4* is also reflected in the transcriptomes, we profiled gene expression in fully expanded rosette leaves of WT, *fas2-4* G_1 and G_6 mutant plants. Although there were only 62 significantly upregulated and 26 downregulated genes in *fas2-4* G1 compared to WT, 295 and 62 genes were up- and downregulated, respectively, in G_6 (Fig. 3a; Table S4). Of the 62 genes upregulated in G_1, about one third also were found upregulated in G6. Likewise, five of the 26 genes downregulated in G_1 also were downregulated in G_6. No genes were found to be dysregulated in an opposite manner between G_1 and G_6 (i.e. downregulated in G_1 and upregulated in G_6, or vice versa). Together, considerably more genes changed expression in the late than in the early generation of *fas2-4*. In total, 417 genes were affected in at least one mutant sample.

Although CAF-1 is needed for normal heterochromatin formation (Kirik *et al.*, 2006), previous studies did not find widespread activation of TE genes in Arabidopsis CAF-1 mutants (Schönrock *et al.*, 2006). The tiling array used here probes 2424 TE genes. Of these, only one (*AT4G04410*, a copia-like retrotransposon) was mildly upregulated in *fas2-4* G_1 and none in *fas2-4* G_6, consistent with maintained repression of heterochromatically silenced TE genes, even in late generations of *fas2-4*. Activation of *CACTA* TEs was observed in some cells in a fraction of mutant plants (Ono *et al.*, 2006). Because microarrays may lack the sensitivity to detect weak activation, we used RT-PCR to probe possible activation of *CACTA* TEs in seedlings of *fas2-4* G_2 and G_6. However, no transcripts could be detected for *CACTA* or the *TA2* TE in any mutant material (Fig. 3b). By contrast, transcripts of another silenced sequence (*TSI*) were readily detected in all *fas2-4* samples, consistent with earlier reports (Takeda *et al.*, 2004; Schönrock *et al.*, 2006). Note that *TSI* is not probed by the used microarray. The lack of strong signals for TEs *CACTA* and *TA2* is consistent with the reported rare stochastic activation (Ono *et al.*, 2006). Together, neither transcriptome nor RT-PCR data indicate an increasing loss of heterochromatic gene silencing in late generations of *fas2-4*.

Arabidopsis CAF-1 interacts with the repressive machinery of Polycomb group (PcG) proteins (Jiang & Berger, 2017). At a genome-wide scale, however, histone 3 lysine 27 trimethylation (H3K27me3)-positive PcG target genes were not preferentially enriched among genes upregulated in leaves of *fas2-4* G1 or G6 mutant plants (Fig. S2a). By contrast, H3K27me3-positive genes were considerably enriched among genes upregulated in *fas2* seedlings similar to the overlap between H3K27m3-positive genes and genes upregulated in H3.1-deficient plants. Because seedlings but not expanded leaves contain replicating cells, it is tempting to speculate that a CAF-1-PcG protein interaction enforces repression via H3K27me3 in dividing cells, but that eventually full repression by PcG proteins can be established even in the absence of CAF-1.

CAF-1 is considered to be the major histone chaperone for H3.1-H4, and both CAF-1 and H3.1 deficiency affect expression of similar numbers of genes (Jiang & Berger, 2017; Fig. 3a). There is, however, only limited overlap between genes upregulated in CAF-1 or H3.1-deficient plants (Fig. S2b). Although this overlap is statistically significant, the limited scale of overlap suggests that lack of CAF-1 affects Arabidopsis not only by reduced H3.1 incorporation but also by other means.

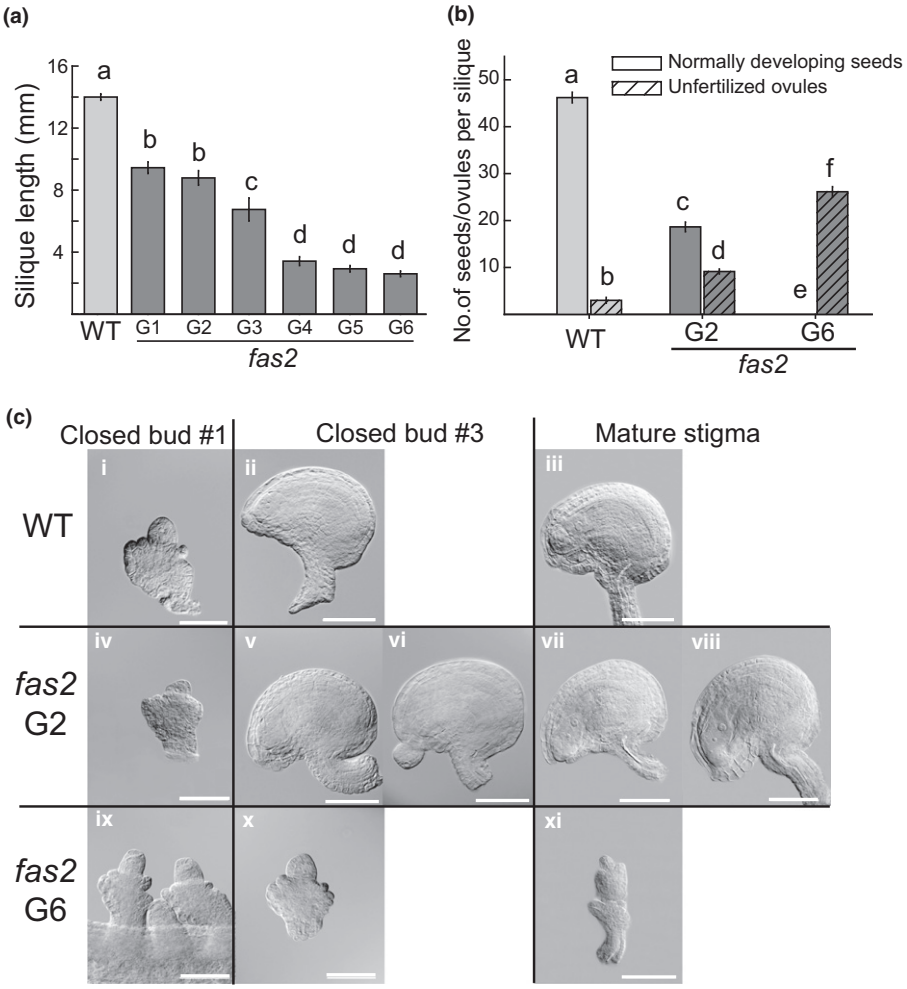

Fig. 2 Transgenerational loss of fertility in *Arabidopsis thaliana fas2* mutant plants. (a) Progressive reduction of the length of silique number 4 over six successive generations of *fas2*. Silique size was scored when all siliques on the plant were fully mature. (b) Reduced number of developing seeds and increasing number of unfertilized ovules in generations (G) G_2 and G_6 of *fas2*. Bars represent means ± SE. Different letters above bars indicate significant difference ($P < 0.05$ in Student's *t*-test). (c) Transgenerational aggravation of ovule developmental phenotypes in *fas2*. Ovules were taken from gynoecia from the first and third youngest closed buds (closed bud #1, closed bud #3, resp.) and from gynoecium with mature stigma. Bars, 50 μm. WT, wild-type.

As we were interested in the pattern of changes across generations for the 417 genes with altered transcription, we assigned each gene to one of eight principal classes of change (see the Materials and Methods section for details; Table 1; Fig. 3c,d). A majority of the 330 upregulated genes (Fig. 3c, classes 1–4), belonged to class 2 – that is, was not affected in G1 but mainly in G_6 (Tables 1, S5; Fig. 3c). Another considerable group of 84 upregulated genes was changed to some degree in G_1 but much more in G_6 (class 1). By contrast, only 46 genes were affected in a similar way in G_1 and G_6 (class 3). Finally, < 10% (26 genes) of the upregulated genes were affected mainly in G_1 but barely in G_6 (class 4). For the 87 downregulated genes (Fig. 3d, classes 5–8), the majority of 46 genes belonged to class 5 – that is, was affected in G_1 but even more in G_6. Only eight genes were affected in G_6 but not in G_1 (class 6). Eleven genes were affected in a similar way in G_1 and G_6 (class 7). Finally, 22 genes were downregulated in G_1 but not in G_6 (class 8). Together, most genes showed either a gradual increase in expression changes from G_1 to G_6 or were exclusively affected in G_6, consistent with the much more severe developmental phenotype of *fas2-4* G_6 than G_1 plants.

Gene ontology (GO) analysis revealed that upregulated genes of the dominating classes 1 ($Expr_{Col} < Expr_{G1} < Expr_{G6}$) and 2 ($Expr_{Col} \approx Expr_{G1} < Expr_{G6}$) were significantly enriched for terms related to plant defense (Table 2). The most common group of downregulated genes (class 5, $Expr_{Col} > Expr_{G1} > Expr_{G6}$) was enriched for the term 'chloroplast' and downregulated genes of class 8 ($Expr_{Col} > Expr_{G1} < Expr_{G6}$) were enriched for terms related to abiotic stress. Activation of plant defense genes in *fas2-4* has been reported before (Mozgova *et al.*, 2015) and the present analysis shows that this activation is considerably more pronounced in *fas2-4* G6 than in *fas2-4* G_1, revealing a transgenerational aggravation of distorted gene expression in the *fas2-4* CAF-1 mutant.

Together, activation of defense-related genes reflected the transgenerational aggravation of developmental phenotypes of *fas2-4*, whereas TE silencing appeared independent of the tested mutant generation.

Sequence context-specific transgenerational changes of DNA methylation in *fas2* mutant plants

Arabidopsis CAF-1 mutants interact synthetically with reduction in DNA cytosine methylation (Schönrock *et al.*, 2006) and DNA methylation was reported to be altered in CAF-1 mutants (Pontvianne *et al.*, 2013; Stroud *et al.*, 2013). Therefore, we asked

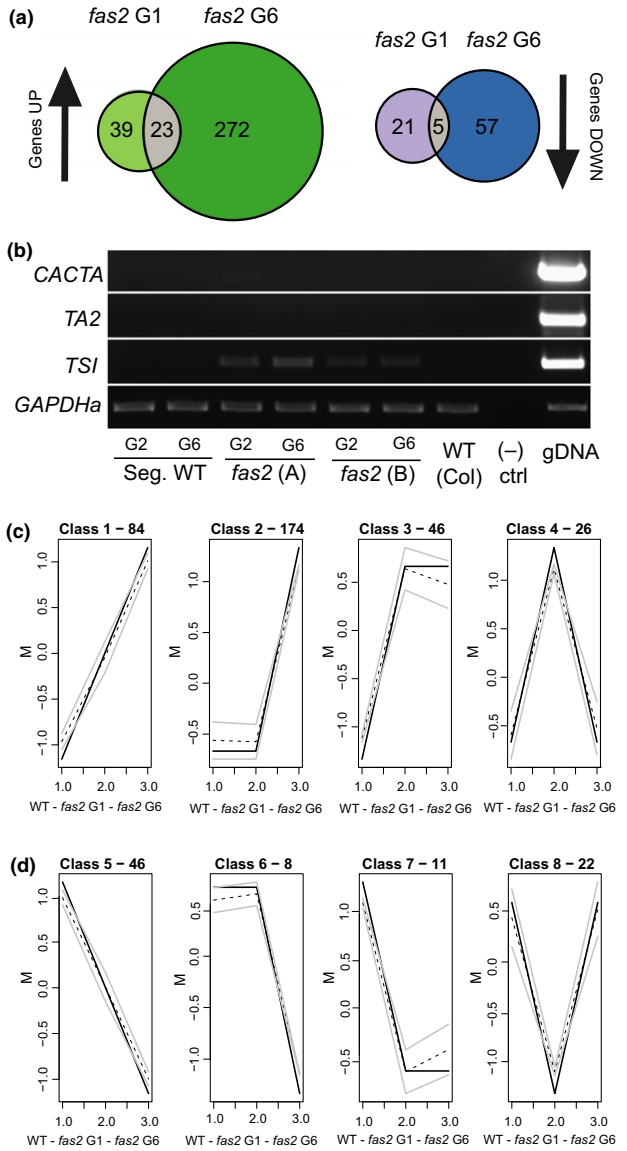

Fig. 3 Gene expression changes in increasing generations of *Arabidopsis thaliana fas2* mutant plants. (a) Venn diagram depicting numbers of genes up- and downregulated in fully expanded rostette leaves of *fas2* G_2 and *fas2* G_6 compared to wild-type (WT). (b) Transposable element expression in wild-type and *fas2* plants assayed by semi-quantitative revese transcription polymerase chain reaction (RT-PCR). WT and mutant plants (*fas2*) were segregated from one *fas2-4*/+ heterozygous parent. WT (Col-0) plants (WT (Col)) served as control. PCR controls were no template control ((-) ctrl) and Col-0 genomic DNA (gDNA). (c) Classes of gene expression changes identified among the genes upregulated in *fas2* G_2 or *fas2* G_6 plants compared to WT. (d) Classes of gene expression changes identified among the genes downregulated in *fas2* G_2 or *fas2* G_6 plants compared to WT. (c, d) *y*-axis represents normalized gene expression change, *x*-axis represents the analysed genotypes (1, WT; 2, *fas2* G_1; 3, *fas2* G_6). Solid black lines in graphs represent the theoretical model, dashed black lines shows means of experimental expression values for all genes assigned to a particular model and gray lines represent 95% confidence intervals. G, generation.

whether DNA methylation undergoes a similar transgenerational change in *fas2* as developmental phenotypes and gene expression. Bisulfite sequencing of WT, and early (G_1) and late (G_6) generations of the *fas2-4* mutant was performed. Plotting the proportion of methylated cytosines (meCs) across all protein coding genes, no global differences in methylation were observed for any of the three sequence contexts CG, CHG and CHH (where H = A, T or C) (Fig. 4a). This established that gene body DNA methylation is not widely affected even in *fas2* G_6. By contrast, averaged DNA methylation on TEs was affected in *fas2*. Global CG DNA methylation on TEs did not differ between WT and *fas2* G1, but was increased in *fas2* G_6 (Fig. 4a). Global CHG DNA methylation on TEs was higher in *fas2* G_1 than in WT and again higher in G_6 than in G_1 (Fig. 4a). Finally, CHH methylation was similar to or slightly lower than in WT in *fas2* G_1 and slightly higher than in WT in G_6 (Fig. 4a). Because CHH contexts are less frequent in the genome than CG or CHG contexts, the CHH methylation profiles are more variable.

Table 1 Classification of differentially expressed genes according to expression patterns

Class	Description	Genes
1	$Expr_{Col} < Expr_{G1} < Expr_{G6}$	84
2	$Expr_{Col} \approx Expr_{G1} < Expr_{G6}$	174
3	$Expr_{Col} < Expr_{G1} \approx Expr_{G6}$	46
4	$Expr_{Col} < Expr_{G1} > Expr_{G6}$	26
5	$Expr_{Col} > Expr_{G1} > Expr_{G6}$	46
6	$Expr_{Col} \approx Expr_{G1} > Expr_{G6}$	8
7	$Expr_{Col} > Expr_{G1} \approx Expr_{G6}$	11
8	$Expr_{Col} > Expr_{G1} < Expr_{G6}$	22

Genes found to be differentially expressed in *Arabidopsis thaliana fas2* generation (G) G_1 or *fas2* G_6 plants were assigned to one of eight main predefined classes (see the Materials and Methods section for details).

Although methylation profiles reveal global trends that can be subtle in amplitude, differentially methylated region (DMRs) reveal larger changes that are locally restricted. We identified 15 522, 2335 and 8703 individual 50-bp DMRs for the CG, CHG and CHH contexts, respectively. Grouping the DMRs in similar classes as the DEGs, revealed that aggravation of differential DNA methylation changes was rare in all sequence contexts (classes 1 and 5) (Table S6). Most CG DMRs were changed in G_1 without major additional changes in G6 (classes 3 and 7). Most CHG DMRs had increased methylation in G_1 without major additional changes in G_6 (class 3). CHH DMRs were likewise abundant in all classes without continuous change (i.e. all except for classes 1 and 5) (Table S6). Together, DMRs in *fas2* G_1 and G_6 do not generally reflect the global trends of methylation changes and rarely show transgenerational aggravation.

Although gene body DNA methylation was not globally altered, most CG DMRs come from gene bodies regardless of the class (Fig. 4b). By contrast, CHG and CHH DMRs often come from TEs. DMRs that map to TEs come most often from intergenic TEs and only rarely from TEs in promoters or gene bodies regardless of the methylation context (Fig. 4b). When TEs are grouped in families, CG DMRs of class 4 are enriched in the Long Terminal Repeat (LTR)-Copia, LTR-Gypsy and Rolling Circle (RC)-Helitron TE families (Fig. 4c). CHG DMRs of classes 1, 2, 4, 6 and 8 also are enriched in the LTR-Copia or

Table 2 Enrichment of gene ontology terms among differentially expressed genes in the eight defined classes

Class	Pattern	GO term	Obs. frequency	Exp. frequency	Enrichment	$-\log_{10}$ (*P*-value)
1	$Expr_{Col} < Expr_{G1} < Expr_{G6}$	Defence response to bacterium	6	0.5	3.7	4.1
2	$Expr_{Col} \approx Expr_{G1} < Expr_{G6}$	Systemic acquired resistance	6	0.1	5.21	7.9
3	$Expr_{Col} < Expr_{G1} \approx Expr_{G6}$	Plant-type cell wall	4	0.4	3.35	2.3
4	$Expr_{Col} < Expr_{G1} > Expr_{G6}$	Anchored to membrane	4	0.2	4.31	4
5	$Expr_{Col} > Expr_{G1} > Expr_{G6}$	Chloroplast	20	4.1	2.28	8.1
6	$Expr_{Col} \approx Expr_{G1} > Expr_{G6}$	*No significant GO terms*				
7	$Expr_{Col} > Expr_{G1} \approx Expr_{G6}$	*No significant GO terms*				
8	$Expr_{Col} > Expr_{G1} > Expr_{G6}$	Cold acclimation	3	0	7.69	7.2
		Response to water deprivation	5	0.1	5.4	7
		Response to cold	5	0.2	4.95	6.2

Shown are observed frequency, expected frequency, enrichment and $-\log_{10}$ (*P*-value) for all gene ontology (GO) terms significantly over-represented ($P < 0.01$) among the genes of a class with differential expression in in *Arabidopsis thaliana fas2* G_1 or *fas2* G_6 plants.

LTR-Gypsy families. CHH DMRs of most classes show the same tendency to be enriched in LTR-Gypsy family but they are also often enriched in RC-Helitrons (Fig. 4c). Together, DMRs in *fas2* affect various TE families but are particularly enriched in LTR TEs. Because LTR TEs are typical for pericentromeric heterochromatin, lack of CAF-1 seems to affect DNA methylation most in pericentromeric heterochromatin. This is consistent with the reported role of CAF-1 in organization of heterochromatin (Kirik *et al.*, 2006) but it does not seem to be reflected by global transcriptional activation of pericentromeric TEs.

Abiotic stress increases the severity of some *fas2-4* phenotypes

Activation of defense-related genes, which are responsive to biotic stress, has been shown to increase the severity of the CAF-1 mutant phenotypes (Mozgova *et al.*, 2015). Here, we asked whether abiotic stress can have a similar effect, contributing to the variable severity of the *fasciata* phenotypes. Wild-type, *fas2-4* G2 and *fas2-4* G4 were cultivated under control conditions or subjected to waterlogging stress – an abiotic stress treatment that could be well controlled even for soil-grown plants. This stress treatment affected plant growth and development and reduced plant vigor (Fig. 5a,b) in all tested genetic backgrounds. Stressed *fas2-4* plants showed an additional aggravation of the phenotype concerning number of adult leaves, rosette diameter and silique length (Fig. 5b). In detail, stress reduced the number of adult leaves from 65% and 40% of WT numbers in control plants of *fas2-4* G_2 and G_4, respectively, to 20% and 0% in stressed plants. Likewise, silique length was reduced from 70% and 45% in control *fas2-4* plants to 35% and 20% in stressed plants. Although the waterlogging stress severely affected rosette size in both WT and *fas2-4* (reducing the rosette size by 70–80% relative to the respective nonstressed control in both genotypes and generations), it had a less severe effect on the number of adult leaves and the silique length in WT (reduced by 40% and 20%, respectively) than in *fas2-4* (reduced by 60% and 70–75%, respectively), suggesting that the latter phenotypic traits are more vulnerable to be affected specifically in the *fas2-4* background than rosette size. These results established that the specific quantitative *fas2-4* phenotype strongly depends on growth conditions, that is, rosette or silique size in *fas2-4* can differ between experiments but the pattern of transgenerational aggravation is consistently observed. Together, stressed *fas2-4* plants of an earlier generation became similar to *fas2-4* plants of a later generation. In other words, waterlogging stress could mimic continuous selfing of *fas2-4* plants.

Next, we asked whether the stress-induced aggravation of the phenotype was heritable. Seeds were harvested from stressed and unstressed plants and progeny was cultivated under control condition. In these plants, rosette size and silique length were assessed (Fig. 5c). There were no significant differences between progeny from stressed and unstressed plants for any tested genotype. In particular, the severity of the *fas2-4* phenotype was determined only by the generation of selfing and not by previous exposure to the abiotic stress.

Alleles causing developmental phenotypes of CAF1 mutants are unstable in the presence of CAF1

The described experiments had established that loss of Arabidopsis CAF-1 leads to a transgenerational aggravation of the mutant phenotype. Next, we asked whether the aggravated phenotype can persist even in the presence of reintroduced CAF-1 function. We had noted that *fas1-4* CAF-1 mutant plants showed a similar transgenerational phenotype aggravation as *fas2-4* plants (Fig. 6a) and compared *fas1-4* G_2, G_4 and G_6 plants to *fas1-4 35S::FAS1* plants. Because the *35S::FAS1* transgene was introduced into *fas1-4* G_4 plants and maintained for two generations to obtain T_2 plants for analysis, three main scenarios could be expected: (1) introduction of the *FAS1* transgene prevents further transgenerational phenotype aggravation; the analyzed *fas1-4 35S::FAS1* T_2 and *fas1-4* G_4 plants will be comparable. (2) Introduction of a *FAS1* transgene does not affect transgenerational phenotype aggravation; the analyzed *fas1-4 35S::FAS1* T_2 and *fas1-4* G_6 plants will be comparable. (3) Introduction of a *FAS1* transgene reverses transgenerational phenotype aggravation; the analyzed *fas1-4 35S::FAS1* T_2 plants will be similar to WT or plants of an

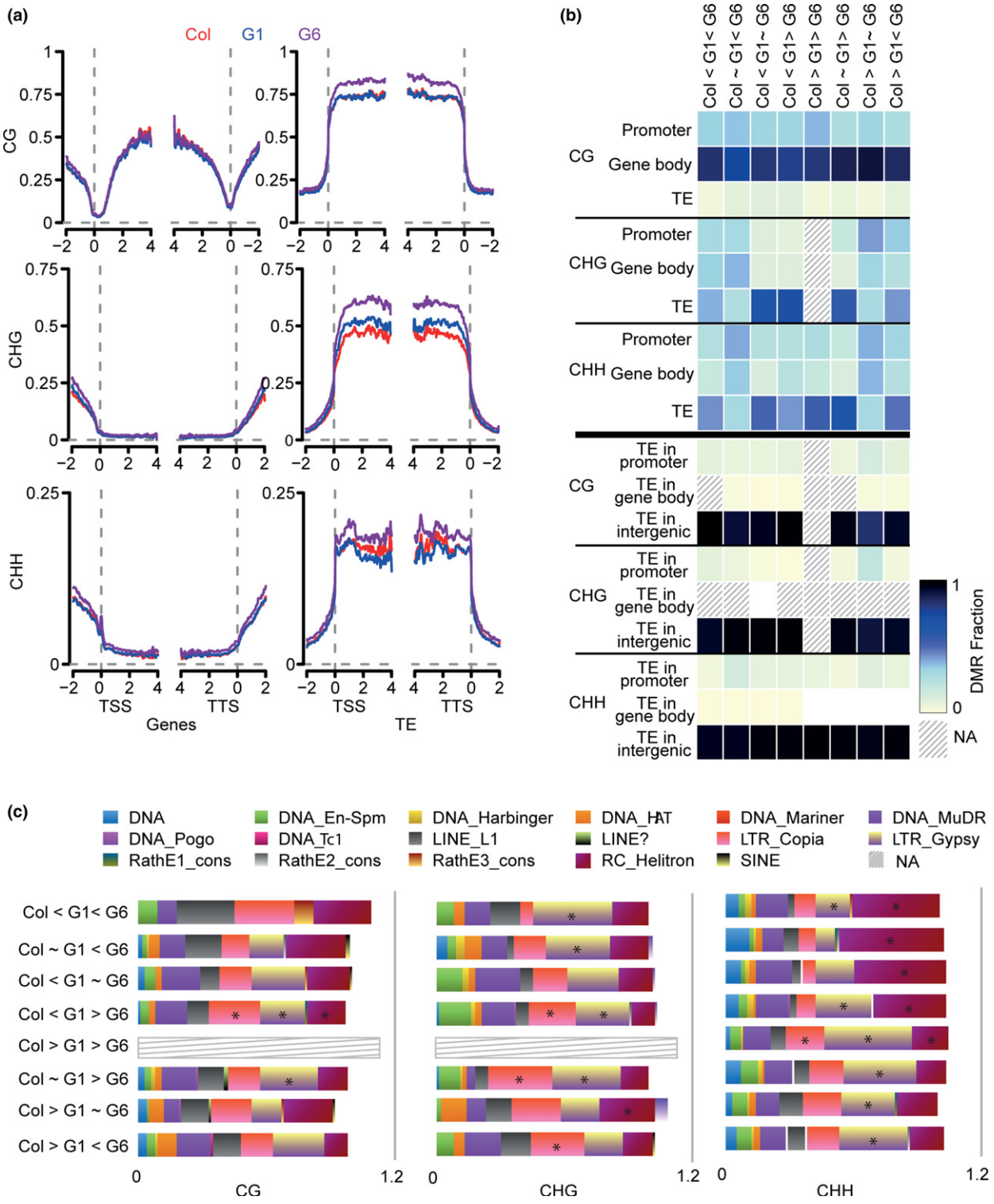

Fig. 4 Transgenerational changes of DNA methylation in *Arabidopsis thaliana fas2* mutant plants. (a) Metagene plots of raw methylation values along genes (left column) and transposable elements (TEs) (right column) across generations. Average values were plotted from 2 kb upstream and downstream of the transcriptional start site (TSS) and the transcriptional termination site (TTS), respectively, and 4 kb into the feature body for wild-type Col (red), *fas2* G_1 (blue) and *fas2* G_6 (purple) in the three methylation contexts. (b) Heatmap of the fraction of differentially methylated regions (DMRs) mapping to promoters, genes or different genomic locations of TEs. The fraction of DMRs from each class of change mapping to promoters (2 kb upstream of TSS), gene body or different genomic locations of TEs was calculated with respect to the total number of significant (Fisher's exact test, *P*-value ≤ 0.01) DMRs following the different trends of changes across generations. Underpopulated classes with ≤ 10 DMRs were not taken into account (shaded). (c) The fraction of DMRs mapping to TEs was further classified into TE families. Different colors represent the fraction of DMRs per class mapping to a given TE family. TE families with a significant overpopulation (hypergeometric test, *P*-value ≤ 0.05) of DMRs are indicated with an asterisk (*). Underpopulated families with ≤ 10 DMRs were not taken into account (shaded). Col, wild-type; G, generation.

early *fas1-4* generation. Using two independent transgenic lines, the results clearly supported scenario (3). The *fas1-4 35S::FAS1* T_2 plants, although G_6 for *fas1-4*, had only a mild mutant phenotype that was even less severe than in *fas1-4* G_2 plants (Fig. 6a). Notably, the presence of the *35S::FAS1* transgene did not complement the telomere length or the 45S rDNA repeat copy number in these transgenic lines (Fig. S3). Together, the aggravated phenotype of *fas1-4* plants after recurrent selfing is unstable in the presence of functional FAS1, despite the fact that the loss of tandem repeats in *fas1* is not fully reverted upon *FAS1* reintroduction (here and (Pavlistova *et al.*, 2016). The reversibility of the phenotype indicates an epigenetic nature of the aggravated phenotype.

The aggravated phenotype in later generations could be caused by different (epi)allelic states at one or more loci. To test whether such allelic states could be characterized genetically, *fas2-4* G_1 and G_4 plants were crossed using the later generation as pollen donor ($G_1 \times G_4$) (Figs S4, 6b). We asked whether the F_2 progeny of such a cross would segregate plants with phenotypes of distinct severity or resemble *fas2-4* G_3 or G_6 plants. However, no clear phenotypic classes of F_2 plants were evident and rosette diameters as well as silique lengths had similar spread (measured as standard deviation) for F_2 plants and regular *fas2-4* G_3 or G_6 plants. F_2 plants had significantly larger rosettes and siliques than *fas2-4* G_6 plants and significantly smaller rosettes and silique length than *fas2-4* G_3 plants. This argues against a single Mendelian locus underlying the analyzed developmental phenotype with transgenerational aggravation in *fas2-4*. For comparison, we also performed a cross of *fas2-4* G_1 and G_4 plants using the earlier generation as pollen donor ($G_4 \times G_1$) (Fig. S4), and analyzed the F_2 progeny. Similar to the reciprocal $G_1 \times G_4$ cross, again no clear phenotypic classes of F_2 plants were evident and rosette diameters as well as silique lengths had similar spread (measured as SD) for F_2 plants from the $G_4 \times G_1$ cross and regular *fas2-4* G_3 or G_6 plants. In contrast to the reciprocal cross, however, average rosette diameters and silique lengths of $G_4 \times G_1$ F_2 and *fas2-4* G_6 plants were similar and significantly smaller than that of *fas2-4* G_3 plants. Thus, the aggravated phenotype of G_4 plants is more efficiently transmitted through the maternal than through the paternal parent. Likewise, the phenotype of F_1 plants was more severely affected in a *fas2-4* $G_4 \times G_1$ than in the reciprocal $G_1 \times G_4$ cross (Fig. S5) supporting the notion of impaired transmission of the aggravated phenotype through the father.

Together, the dependency of the aggravated phenotype of Arabidopsis CAF-1 mutants on continuous lack of CAF-1 function and the unequal parental efficiency to transmit the aggravated phenotype indicate that epigenetic mechanisms underlie the observed transgenerational phenotypic aggravation.

Discussion

Here, we describe a parental-specific transgenerational aggravation of developmental and molecular phenotypes in Arabidopsis chromatin assembly factor 1 (CAF-1) mutants. The developmental phenotypes include reduced rosette size and root length, shortened adult phase and early flowering, reduced silique length, and reduced ovule number. Furthermore, ovule development defects accumulated progressively, correlating with low fertilization efficiency and failure to produce seeds in late generations of the mutants. Molecular phenotypes with transgenerational aggravation were observed for CHG DNA methylation at transposable elements (TEs) and at the transcriptome level. We show that the CAF-1 mutant developmental phenotypes are influenced not only by the level of biotic stress (Mozgova *et al.*, 2015), but also by the abiotic growth conditions (here). Interestingly, ovule development defects in *fasciata 2* (*fas2*) closely resemble those caused by reduced amount of MULTICOPY SUPRESSOR OF IRA 1 (MSI1) in the *MSI1* co-suppression (*msi1-cs*) lines (Hennig *et al.*, 2003). Therefore, we propose that undisturbed CAF-1 activity is needed for normal ovule development in Arabidopsis.

Developmental genes are not enriched among genes misregulated in seedlings or leaves of either early or late generation CAF-1 mutants (Schönrock *et al.*, 2006; Mozgova *et al.*, 2015; this work). Instead, we show here that stress-responsive genes are most enriched among genes that show progressive transgenerational upregulation in *fas2*. Stress-responsive genes also are enriched among genes affected by nucleosome depletion in *fas2* (Munoz-Viana *et al.*, 2017) and lack robust transcriptional repression in *fas1* and *fas2* mutant plants (Mozgova *et al.*, 2015). Stable repression of stress-responsive genes may thus be a general role of Arabidopsis CAF-1. The severity of developmental changes in *fas2* correlates with the amplitude of salicylic acid (SA) signaling, and reduced SA content can partially normalize *fas2* development (Mozgova *et al.*, 2015). In wild-type (WT) Arabidopsis, stress-induced chromatin changes are usually not heritable but rapidly reset (Probst & Mittelsten, 2015; Lamke & Baurle, 2017), whereas in some mutants resetting of stress-induced chromatin states is impaired (Iwasaki & Paszkowski, 2014). Because CAF-1 also is required for efficient resetting (Pecinka *et al.*, 2010), it appeared possible that intensified stress responses together with failure to reset stress-induced chromatin states underlie the transgenerational aggravation of the CAF-1 mutant phenotype. However, stress conditions for parental *fas2* plants did not affect the phenotype severity of the progeny and the additional phenotype aggravation in *fas2* that was induced by stress was not heritable. This suggests that the *fas2* mutant phenotype is determined by two components: a stress-related component that is not heritable and a stress-unrelated component that is heritable and shows transgenerational aggravation. This notion is consistent with earlier reports that impaired SA signaling could only mitigate but not fully suppress the *fas2* mutant phenotype (Mozgova *et al.*, 2015).

Transgenerational aggravation of developmental phenotypes in Arabidopsis mutants also has been reported for telomere maintenance mutants (Riha *et al.*, 2001). Progressive reduction of telomere length in mutants of the catalytic subunit of telomerase (TERT) is associated with phenotype aggravation, which has been hypothesized to be a consequence of increasing genome instability (Riha *et al.*, 2001). The CAF-1 dysfunction in Arabidopsis causes selective loss of tandem repetitive DNA sequences, including the 45S rDNA and the telomeres (Mozgova *et al.*, 2010), which could potentially contribute to the

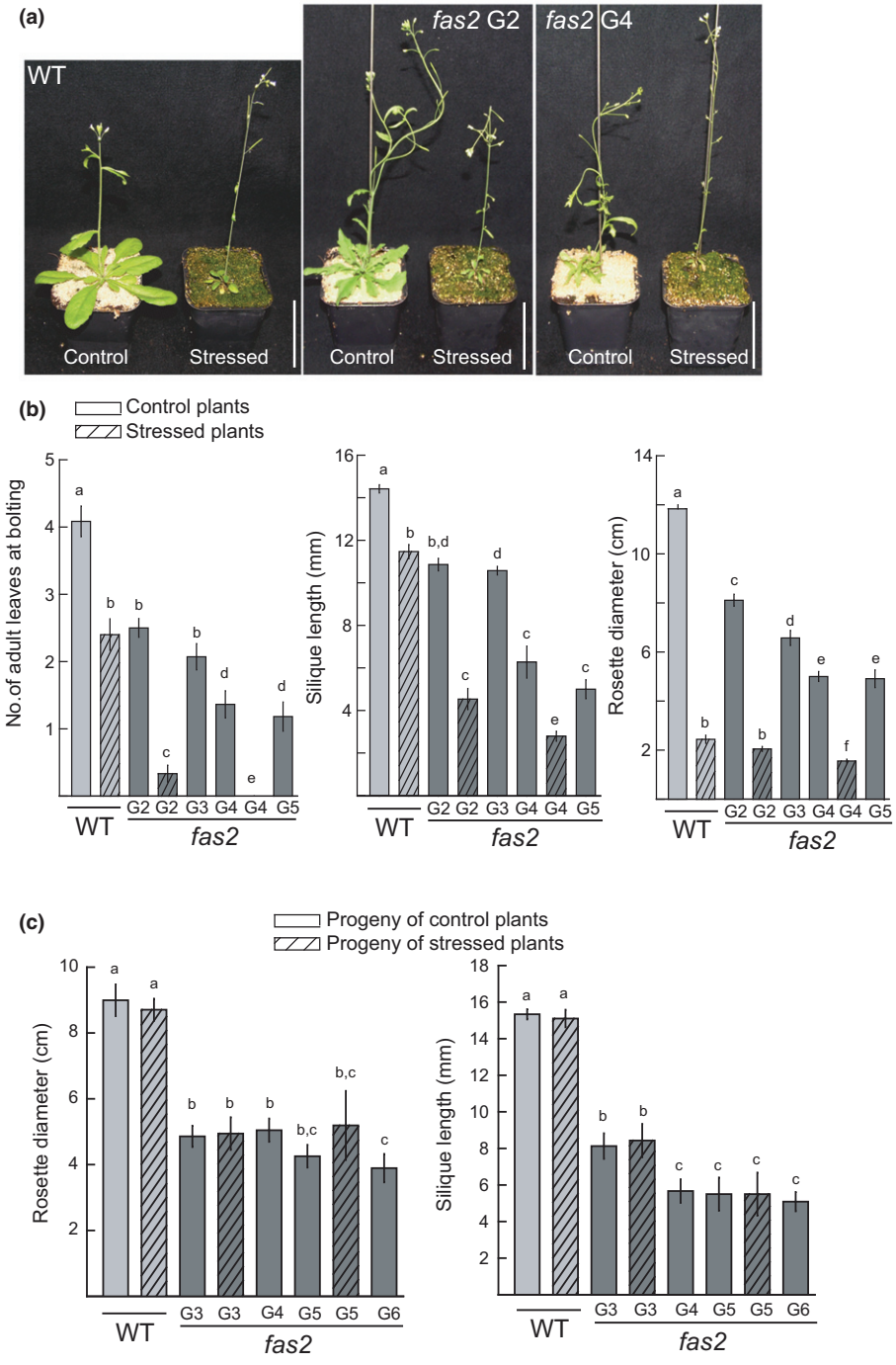

Fig. 5 Abiotic stress enhances the severity of *Arabidopsis thaliana fas2* mutant developmental phenotype but its effect is not heritable. (a) Examples of wild-type (WT) and *fas2-4* phenotypes of plants grown under control conditions (control) and exposed to waterlogging stress (stressed). Bars, 5 cm. (b) Quantification of phenotypes of control and waterlogged plants. ($N_{control}$ = 14 plants, $N_{stressed}$ = 21 plants). (c) Quantification of phenotypes of the progeny of control and waterlogged plants ($N_{control\ progeny}$ = 14 plants, $N_{stressed\ progeny}$ = 14 plants). Bars represent means ± SE. Different letters above bars indicate significant difference ($P < 0.05$ in Student's *t*-test). G, generation.

progressive developmental phenotype severity in the CAF-1 mutants. However, as we show here, late generation *fas1* transformed with *35S::FAS1* display an early generation or even WT developmental phenotype, despite retention of low levels of tandem repetitive sequences in the genome. Neither reduced telomere length nor depletion of 45S rDNA is therefore likely to directly underlie the developmental phenotype or its aggravation in the CAF-1 mutants. The fast reversal of the *fas1* to the WT phenotype upon restoration of CAF-1 activity as also observed in (Pavlistova *et al.*, 2016) furthermore argues against genetic causes of the progressive developmental phenotype aggravation in the CAF-1 mutants. Together with the parent-of-origin effect in the inheritance of alleles determining the early- or late-generation phenotypes, we propose that epigenetic rather than genetic defects underlie the developmental phenotype aggravation in the CAF-1 mutants.

How could lack of CAF-1 activity affect the epigenome? There are several well-established mechanisms that provide plausible scenarios. First, lack of CAF-1 causes locally reduced nucleosome occupancy or nucleosome gaps (Munoz-Viana *et al.*, 2017). This may lead to a more widespread loosening of chromatin packing. Second, lack of CAF-1 shifts the ratio of histone variants H3.1

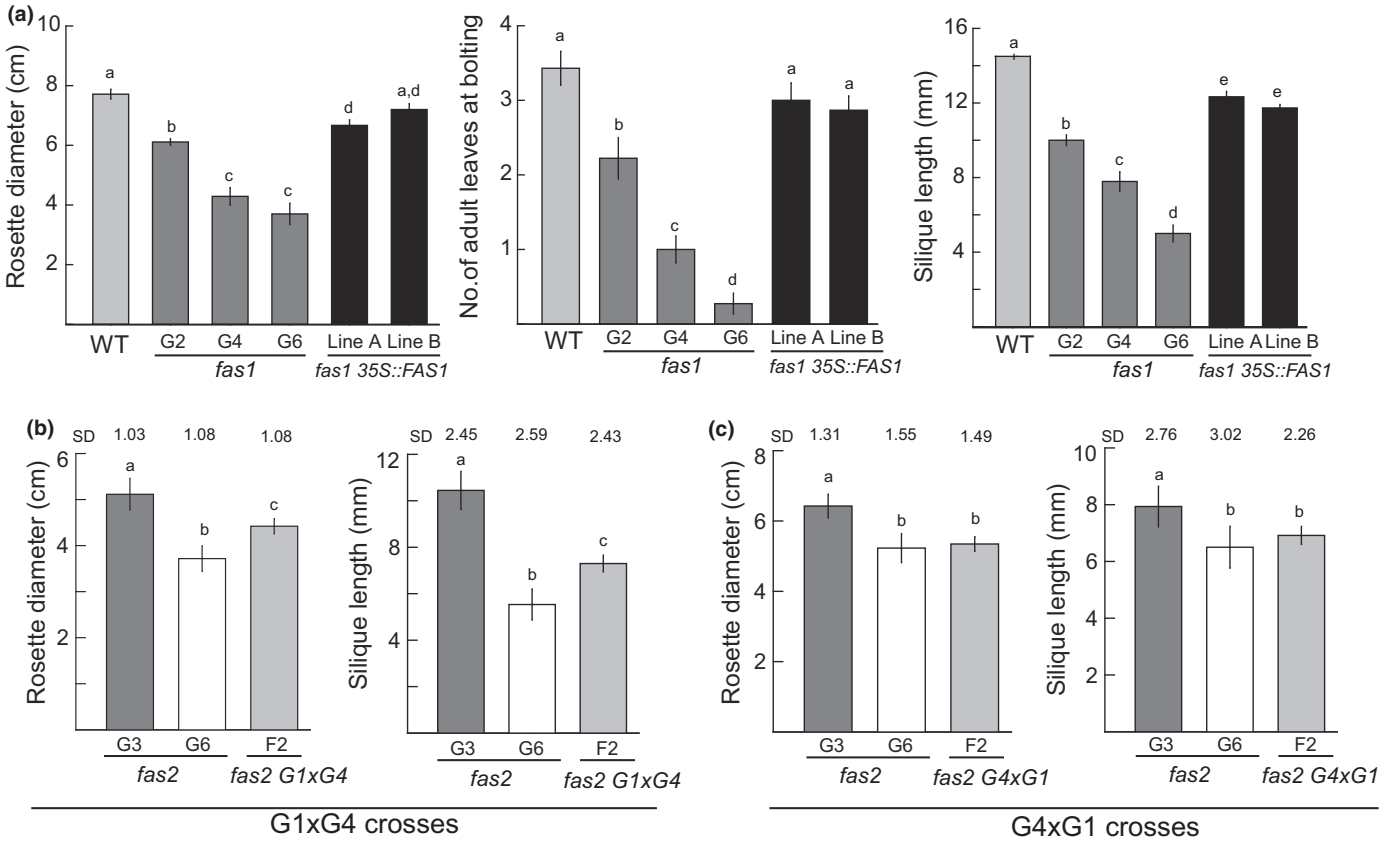

Fig. 6 Epigenetic mechanisms underlie the transgenerational phenotype aggravation in *Arabidopsis thaliana* chromatin assembly factor 1 (CAF-1) mutants. (a) Alleles causing developmental phenotypes of CAF-1 mutants are unstable in the presence of CAF-1. Quantification of phenotypes in two independent transgenic lines (line A, line B) of T_2 generation of *fas1-4 35S::FAS1* transformed in G_4 (i.e. equivalent of G_6 nontransformed plants *fas1-4*). (b) Phenotype quantification of the F_2 generation of plants originating from *fas2-4* G_1 × *fas2-4* G_4 crosses and control progeny of parental plants propagated in parallel (G_3 and G_6) ($N_{F2} = 45$, $N_{G3,G6} = 15$). (c) Phenotype quantification of the F_2 generation of plants originating from *fas2-4* G_4 × *fas2-4* G_1 crosses and control parental plants propagated in parallel (G_3 and G_6) ($N_{F2} = 45$, $N_{G3,G6} = 15$). Bars represent means ± SE. Different letters above bars indicate significant difference ($P < 0.05$ in a one-tailed Student's *t*-test). WT, wild-type; G, generation.

and H3.3 in chromatin, because only CAF-1-dependent chromatin assembly strongly prefers H3.1 over H3.3, whereas CAF-1-independent chromatin assembly, which can partially substitute for CAF-1 function in CAF-1 mutants, works well with H3.3 (Duc *et al.*, 2015, 2017). Third, CAF-1 locates to the site of the replication fork through its interaction with PROLIFERATING CELL NUCLEAR ANTIGEN (Shibahara & Stillman, 1999; Jiang & Berger, 2017) and lack of CAF-1 causes S-phase defects and replication stress, which impairs nucleosome-mediated epigenetic inheritance (Li *et al.*, 2017). Most histone modifications are re-established immediately after S-phase (Alabert *et al.*, 2015), often mediated by CAF-1 interacting with other chromatin proteins such as the mammalian methyl CpG binding protein that recruits a H3K9 methyltransferase (Sarraf & Stancheva, 2004) and HETEROCHROMATIN PROTEIN 1 (Quivy *et al.*, 2004). Plant CAF-1 interacts also with the repressive machinery of Polycomb group (PcG) proteins, thus contributing to maintenance of gene repression by PcG proteins (Jiang & Berger, 2017) at least in seedlings. It is likely that replication stress and impaired recruitment of chromatin proteins together underlie the requirement of CAF-1 for the inheritance of epigenetically determined chromatin states (Monson *et al.*, 1997; Enomoto & Berman, 1998; Tchenio *et al.*, 2001; Ono *et al.*, 2006; Song *et al.*, 2007).

In Arabidopsis, where DNA cytosine methylation is abundant, each of the three described consequences of CAF-1 dysfunction on chromatin can affect DNA methylation. First, chromatin compaction by linker histone H1 and nucleosome density limit the access of DNA methyltransferases to their DNA substrate. This effect is largest for CHROMOMETHYLASE 3 (CMT3), which is responsible for CHG methylation (Zemach *et al.*, 2013). It is thus possible that increased CHG methylation in *fas2* is a consequence of reduced nucleosome occupancy in the absence of CAF-1. Although we did not observe a strong overlap between nucleosome depletion and changes in DNA methylation in the analyzed G0 cells of *fas2* leaves, it is possible that transiently reduced nucleosome occupancy shortly after S-phase suffices to ease access of CMT3. Subsequent activity of CAF-1 independent nucleosome assembly mechanisms re-establishes wild-type-like nucleosome occupancy in most of the genome, as seen in the resting cells of leaves (Munoz-Viana *et al.*, 2017). Second, H3.3 limits H1 recruitment and thus favors DNA

methylation (Wollmann *et al.*, 2017). The shifted H3.1-H3.3 balance in CAF-1 mutants is thus expected to lead to reduced H1 presence and increased CHG methylation, which is consistent with our findings. Finally, replication stress and CAF-1 deficiency may reduce histone H3 lysine 9 dimethylation (H3K9me2) levels (Sarraf & Stancheva, 2004) and affect DNA methylation via the H3K9me2 feed-back loop.

We note that transgenerational phenotype aggravation can also occur when CG DNA methylation is reduced such as in mutants of DECREASED DNA METHYLATION 1 (DDM1), a chromatin remodeler required for DNA methylation (Kakutani *et al.*, 1996), and in mutants for the maintenance DNA methyltransferase MET1 (Mathieu *et al.*, 2007). In contrast to *fas2*, developmental defects in *ddm1* and *met 1* are highly stochastic and can differ greatly between sibling plants. In *ddm1*, particular combinations of phenotypes were found more often than other combinations (Kakutani *et al.*, 1996). In *fas2*, the different aspects of the developmental phenotype consistently occurred together and the quantitative variability among plants of the same generation was generally low. In addition, *fas2* does not show the global reduction of CG methylation found in *ddm1* and *met1*. Because of the nonstochastic nature of the *fas2* phenotype and the lack of globally reduced CG methylation, we consider it unlikely that global CG DNA methylation changes similar to those that occur in *ddm1* or *met1* underlie the phenotype aggravation in CAF-1 mutants.

The inequality of reciprocal crosses between early G_1 and late G_4 generation *fas2* mutants suggests that the epigenetic determinants of phenotype severity are more efficiently transmitted through the egg than the sperm. Which properties make Arabidopsis sperm chromatin particular and could explain transmission differences? Current knowledge suggests two major scenarios:

First, sperm cell chromatin comprises mainly pollen-specific histone H3 variants, especially H3.10 (Borg & Berger, 2015). Although H3.3 and H3.1 differ only at four of 135 positions, H3.10 differs at 13 positions from H3.1. Thus, H3 protein properties such as stability in the nucleosome or efficiency to be targeted by histone modifiers is expected to vary considerably between pollen H3.10 and the sporophytic variants H3.1 and H3.3. In particular, Borg & Berger (2015) suggested that substitutions adjacent to K27 may impair K27 trimethylation by PRC2 for H3.10. Thus, epigenetic information contained in H3K27me3 may not be efficiently transmitted through sperm cells. Another characteristic feature of sperm cell chromatin is a loss of CHH methylation while CG and CHG methylation is maintained. CHH methylation is restored only after fertilization guided by 24-nt small interfering RNAs (Calarco *et al.*, 2012). Thus, epigenetic information contained in CHH methylation may be transmitted efficiently in sporophytic but not through sperm cells. Although only CHG and not CHH methylation showed global transgenerational changes in *fas2* plants, *c.* 8700 regions with locally altered CHH methylation were found that could, in principle, relate to the epigenetic transgenerational aggravation of the *fas2* phenotype. A recent report described that epigenetic memory of abiotic stress may be mediated by epigenetically labile sites (Wibowo *et al.*, 2016). Similar to the determinants of *fas2* phenotype severity, these stress responses are transmitted much more efficiently maternally than paternally, an effect that was attributed to widespread DNA de-methylation in the male germline (Wibowo *et al.*, 2016). Future work will show whether DNA de-methylation in the male germline or the male-specific histone H3.10 forms a more efficient barrier to limit epigenetic inheritance through the paternal side in Arabidopsis. Finally, it is possible that preferred maternal determination of CAF-1 mutant phenotype severity as shown here is not directly related to chromatin properties but to other maternal effects such as steering seed development. However, although stress treatment greatly reduced maternal vigor, it failed to affect the offspring phenotype severity in our system, making it rather unlikely that reduced maternal vigor of *fas2* plants underlies the preferred maternal determination of CAF-1 mutant phenotype severity. In addition, the observed partial paternal transmission argues against an exclusive chromatin-independent maternal effect. Regardless of the molecular mechanism, the preferential maternal determination of Arabidopsis CAF-1 mutant phenotype severity strongly supports the notion that mothers have stronger non-genetic effects on offspring phenotypes than fathers. This is consistent with ecological scenarios that transgenerational phenotype plasticity can be adaptive when responding to maternal growth conditions (Galloway & Etterson, 2007).

Acknowledgements

This work was supported by grants from the Swiss National Science Foundation SNF, the Czech Science Foundation (16-01137S) and the Swedish Science Foundation VR and the Knut-and-Alice-Wallenberg Foundation.

Author contributions

L.H., I.M., T.W., J.F. and C.K. designed the research; T.W., I.M. and P.R. performed research; M.S.T-A., I.M. and L.H. analyzed data; I.M. and L.H. wrote the manuscript; and I.M. and T.W. contributed equally this work.

ORCID

Iva Mozgova http://orcid.org/0000-0002-3815-9223
Lars Hennig http://orcid.org/0000-0002-6645-1862

References

Abe M, Kuroshita H, Umeda M, Itoh J, Nagato Y. 2008. The rice flattened shoot meristem, encoding CAF-1 p150 subunit, is required for meristem maintenance by regulating the cell-cycle period. *Developmental Biology* **319**: 384–393.

Alabert C, Barth TK, Reveron-Gomez N, Sidoli S, Schmidt A, Jensen ON, Imhof A, Groth A. 2015. Two distinct modes for propagation of histone PTMs across the cell cycle. *Genes & Development* **29**: 585–590.

Baerenfaller K, Massonnet C, Walsh S, Baginsky S, Buhlmann P, Hennig L, Hirsch-Hoffmann M, Howell KA, Kahlau S, Radziejwoski A *et al.* 2012.

Systems-based analysis of Arabidopsis leaf growth reveals adaptation to water deficit. *Molecular Systems Biology* **8**: 606.

Borg M, Berger F. 2015. Chromatin remodelling during male gametophyte development. *Plant Journal* **83**: 177–188.

Calarco JP, Borges F, Donoghue MT, Van Ex F, Jullien PE, Lopes T, Gardner R, Berger F, Feijo JA, Becker JD *et al.* 2012. Reprogramming of DNA methylation in pollen guides epigenetic inheritance via small RNA. *Cell* **151**: 194–205.

Chen Z, Tan JL, Ingouff M, Sundaresan V, Berger F. 2008. Chromatin assembly factor 1 regulates the cell cycle but not cell fate during male gametogenesis in *Arabidopsis thaliana*. *Development* **135**: 65–73.

Costa S, Shaw P. 2006. Chromatin organization and cell fate switch respond to positional information in Arabidopsis. *Nature* **439**: 493–496.

Curtis MD, Grossniklaus U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. *Plant Physiology* **133**: 462–469.

Davis MP, van Dongen S, Abreu-Goodger C, Bartonicek N, Enright AJ. 2013. Kraken: a set of tools for quality control and analysis of high-throughput sequence data. *Methods* **63**: 41–49.

Duc C, Benoit M, Detourne G, Simon L, Poulet A, Jung M, Veluchamy A, Latrasse D, Le Goff S, Cotterell S *et al.* 2017. Arabidopsis ATRX modulates H3.3 occupancy and fine-tunes gene expression. *Plant Cell* **29**: 1773–1793.

Duc C, Benoit M, Le Goff S, Simon L, Poulet A, Cotterell S, Tatout C, Probst AV. 2015. The histone chaperone complex HIR maintains nucleosome occupancy and counterbalances impaired histone deposition in CAF-1 complex mutants. *Plant Journal* **81**: 707–722.

Endo M, Ishikawa Y, Osakabe K, Nakayama S, Kaya H, Araki T, Shibahara KI, Abe K, Ichikawa H, Valentine L *et al.* 2006. Increased frequency of homologous recombination and T-DNA integration in Arabidopsis CAF-1 mutants. *EMBO Journal* **25**: 5579–5590.

Enomoto S, Berman J. 1998. Chromatin Assembly Factor 1 contributes to the maintenance, but not the re-establishment, of silencing at the yeast silent mating loci. *Genes & Development* **12**: 219–232.

Exner V, Taranto P, Schönrock N, Gruissem W, Hennig L. 2006. Chromatin assembly factor CAF-1 is required for cellular differentiation during plant development. *Development* **133**: 4163–4172.

Galloway LF, Etterson JR. 2007. Transgenerational plasticity is adaptive in the wild. *Science* **318**: 1134–1136.

Guitton AE, Page DR, Chambrier P, Lionnet C, Faure JE, Grossniklaus U, Berger F. 2004. Identification of new members of *FERTILISATION INDEPENDENT SEED* Polycomb group pathway involved in the control of seed development in *Arabidopsis thaliana*. *Development* **131**: 2971–2981.

Hennig L, Taranto P, Walser M, Schönrock N, Gruissem W. 2003. Arabidopsis MSI1 is required for epigenetic maintenance of reproductive development. *Development* **130**: 2555–2565.

Iwasaki M, Paszkowski J. 2014. Identification of genes preventing transgenerational transmission of stress-induced epigenetic states. *Proceedings of the National Academy of Sciences, USA* **111**: 8547–8552.

Jiang D, Berger F. 2017. DNA replication-coupled histone modification maintains Polycomb gene silencing in plants. *Science* **357**: 1146–1149.

Kakutani T, Jeddeloh JA, Flowers SK, Munakata K, Richards EJ. 1996. Developmental abnormalities and epimutations associated with DNA hypomethylation mutations. *Proceedings of the National Academy of Sciences, USA* **93**: 12 406–12 411.

Kaufman PD, Kobayashi R, Stillman B. 1997. Ultraviolet radiation sensitivity and reduction of telomeric silencing in *Saccharomyces cerevisiae* cells lacking Chromatin Assembly Factor-1. *Genes & Development* **11**: 345–357.

Kaya H, Shibahara K, Taoka K, Iwabuchi M, Stillman B, Araki T. 2001. *FASCIATA* genes for Chromatin Assembly Factor-1 in Arabidopsis maintain the cellular organization of apical meristems. *Cell* **104**: 131–142.

Kirik A, Pecinka A, Wendeler E, Reiss B. 2006. The Chromatin Assembly Factor subunit FASCIATA1 is involved in homologous recombination in plants. *Plant Cell* **18**: 2431–2442.

Köhler C, Hennig L, Bouveret R, Gheyselinck J, Grossniklaus U, Gruissem W. 2003. Arabidopsis MSI1 is a component of the MEA/FIE Polycomb group complex and required for seed development. *EMBO Journal* **22**: 4804–4814.

Krueger F, Andrews SR. 2011. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics* **27**: 1571–1572.

Lamke J, Baurle I. 2017. Epigenetic and chromatin-based mechanisms in environmental stress adaptation and stress memory in plants. *Genome Biology* **18**: 124.

Leyser HM, Furner IJ. 1992. Characterisation of three shoot apical meristem mutants of *Arabidopsis thaliana*. *Development* **116**: 397–403.

Li W, Yi J, Agbu P, Zhou Z, Kelley RL, Kallgren S, Jia S, He X. 2017. Replication stress affects the fidelity of nucleosome-mediated epigenetic inheritance. *PLoS Genetics* **13**: e1006900.

Mathieu O, Reinders J, Caikovski M, Smathajitt C, Paszkowski J. 2007. Transgenerational stability of the Arabidopsis epigenome is coordinated by CG methylation. *Cell* **130**: 851–862.

Monson EK, de Bruin D, Zakian VA. 1997. The yeast CAC1 protein is required for the stable inheritance of transcriptionally repressed chromatin at telomeres. *Proceedings of the National Academy of Sciences, USA* **94**: 13081–13086.

Mozgova I, Mokros P, Fajkus J. 2010. Dysfunction of Chromatin Assembly Factor-1 induces shortening of telomeres and loss of 45S rDNA in *Arabidopsis thaliana*. *Plant Cell* **22**: 2768–2780.

Mozgova I, Wildhaber T, Liu Q, Abou-Mansour E, L'Haridon F, Métraux J, Gruissem W, Hofius D, Hennig L. 2015. Chromatin assembly factor CAF-1 represses priming of plant defence response genes. *Nature Plants* **1**: 15 127.

Müller M, Patrignani A, Rehrauer H, Gruissem W, Hennig L. 2012. Evaluation of alternative RNA labeling protocols for transcript profiling with Arabidopsis AGRONOMICS1 tiling arrays. *Plant Methods* **8**: 18.

Munoz-Viana R, Wildhaber T, Trejo-Arellano MS, Mozgova I, Hennig L. 2017. Arabidopsis Chromatin Assembly Factor 1 is required for occupancy and position of a subset of nucleosomes. *Plant Journal* **92**: 363–374.

Nabatiyan A, Krude T. 2004. Silencing of Chromatin Assembly Factor-1 in human cells leads to cell death and loss of chromatin assembly during DNA synthesis. *Molecular and Cellular Biology* **24**: 2853–2862.

Ono T, Kaya H, Takeda S, Abe M, Ogawa Y, Kato M, Kakutani T, Scheid OM, Araki T, Shibahara K. 2006. Chromatin Assembly Factor-1 ensures the stable maintenance of silent chromatin states in Arabidopsis. *Genes to Cells* **11**: 153–162.

Pavlistova V, Dvorackova M, Jez M, Mozgova I, Mokros P, Fajkus J. 2016. Phenotypic reversion in fas mutants of *Arabidopsis thaliana* by reintroduction of *FAS* genes: variable recovery of telomeres with major spatial rearrangements and transcriptional reprogramming of 45S rDNA genes. *Plant Journal* **88**: 411–424.

Pecinka A, Dinh HQ, Baubec T, Rosa M, Lettner N, Mittelsten Scheid O. 2010. Epigenetic regulation of repetitive elements is attenuated by prolonged heat stress in Arabidopsis. *Plant Cell* **22**: 3118–3129.

Pontvianne F, Blevins T, Chandrasekhara C, Mozgova I, Hassel C, Pontes OM, Tucker S, Mokros P, Muchova V, Fajkus J *et al.* 2013. Subnuclear partitioning of rRNA genes between the nucleolus and nucleoplasm reflects alternative epiallelic states. *Genes & Development* **27**: 1545–1550.

Probst AV, Mittelsten Scheid O. 2015. Stress-induced structural changes in plant chromatin. *Current Opinion in Plant Biology* **27**: 8–16.

Quivy JP, Roche D, Kirschner D, Tagami H, Nakatani Y, Almouzni G. 2004. A CAF-1 dependent pool of HP1 during heterochromatin duplication. *EMBO Journal* **23**: 3516–3526.

Ramirez-Parra E, Gutierrez C. 2007a. E2F regulates *FASCIATA1*, a chromatin assembly gene whose loss switches on the endocycle and activates gene expression by changing the epigenetic status. *Plant Physiology* **144**: 105–120.

Ramirez-Parra E, Gutierrez C. 2007b. The many faces of Chromatin Assembly Factor 1. *Trends in Plant Science* **12**: 570–576.

Rehrauer H, Aquino C, Gruissem W, Henz SR, Hilson P, Laubinger S, Naouar N, Patrignani A, Rombauts S, Shu H *et al.* 2010. AGRONOMICS1: a new resource for Arabidopsis transcriptome profiling. *Plant Physiology* **152**: 487–499.

Reinholz E. 1966. Radiation induced mutants showing changed inflorescence characteristics. *Arabidopsis Information Service* **3**: 19–20.

Riha K, McKnight TD, Griffing LR, Shippen DE. 2001. Living with genome instability: plant responses to telomere dysfunction. *Science* **291**: 1797–1800.

Sarraf SA, Stancheva I. 2004. Methyl-CpG binding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin assembly. *Molecular Cell* **15**: 595–605.

Schönrock N, Exner V, Probst A, Gruissem W, Hennig L. 2006. Functional genomic analysis of CAF-1 mutants in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **281**: 9560–9568.

Shibahara K, Stillman B. 1999. Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. *Cell* **96**: 575–585.

Smyth GK. 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology* **3**: 1–26.

Song Y, He F, Xie G, Guo X, Xu Y, Chen Y, Liang X, Stagljar I, Egli D, Ma J *et al.* 2007. CAF-1 is essential for Drosophila development and involved in the maintenance of epigenetic memory. *Developmental Biology* **311**: 213–222.

Storey JD, Tibshirani R. 2003. Statistical significance for genomewide studies. *Proceedings of the National Academy of Sciences, USA* **100**: 9440–9445.

Stroud H, Greenberg MV, Feng S, Bernatavichute YV, Jacobsen SE. 2013. Comprehensive analysis of silencing mutants reveals complex regulation of the Arabidopsis methylome. *Cell* **152**: 352–364.

Takeda S, Tadele Z, Hofmann I, Probst AV, Angelis KJ, Kaya H, Araki T, Mengiste T, Scheid OM, Shibahara K *et al.* 2004. BRU1, a novel link between responses to DNA damage and epigenetic gene silencing in Arabidopsis. *Genes & Development* **18**: 782–793.

Tchenio T, Casella JF, Heidmann T. 2001. A truncated form of the human CAF-1 p150 subunit impairs the maintenance of transcriptional gene silencing in mammalian cells. *Molecular and Cellular Biology* **21**: 1953–1961.

Verreault A, Kaufman PD, Kobayashi R, Stillman B. 1996. Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. *Cell* **87**: 95–104.

Wibowo A, Becker C, Marconi G, Durr J, Price J, Hagmann J, Papareddy R, Putra H, Kageyama J, Becker J *et al.* 2016. Hyperosmotic stress memory in Arabidopsis is mediated by distinct epigenetically labile sites in the genome and is restricted in the male germline by DNA glycosylase activity. *eLife* **5**: e13546.

Wollmann H, Stroud H, Yelagandula R, Tarutani Y, Jiang D, Jing L, Jamge B, Takeuchi H, Holec S, Nie X *et al.* 2017. The histone H3 variant H3.3 regulates gene body DNA methylation in *Arabidopsis thaliana*. *Genome Biology* **18**: 94.

Yu Z, Liu J, Deng WM, Jiao R. 2015. Histone chaperone CAF-1: essential roles in multi-cellular organism development. *Cellular and Molecular Life Sciences* **72**: 327–337.

Zemach A, Kim MY, Hsieh PH, Coleman-Derr D, Eshed-Williams L, Thao K, Harmer SL, Zilberman D. 2013. The Arabidopsis nucleosome remodeler DDM1 allows DNA methyltransferases to access H1-containing heterochromatin. *Cell* **153**: 193–205.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Scheme of plant propagation and cultivation.

Fig. S2 CAF-1 deficiency does not cause widespread activation of H3K27me3-positive genes.

Fig. S3 Characterization of two independent T_2 *fas1 35S::FAS1* lines complemented in the fourth mutant generation (G4).

Fig. S4 Scheme of reciprocal crosses between early (G1) and late (G4) generation *fas2-4* mutants.

Fig. S5 Reciprocal crosses between early (G1) and late (G4) generation *fas2-4* result in different phenotype severity in populations of F_1 progeny.

Table S1 Primers used in this study

Table S2 Read statistics of the Bisulphite sequencing

Table S3 Estimation of the Bisulphite conversion rate

Table S4 List of up- and downregulated genes

Table S5 List of genes in defined classes

Table S6 Classification of differentially methylated regions expressed genes according to patterns of change

Methods S1 Supporting information on production of successive *fas1* and *fas2* mutant generations, characterization of ovule development and gene ontology analysis.

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.