

## RESEARCH ARTICLE

# Transgenerational plasticity is sex-dependent and persistent in yellow monkeyflower (*Mimulus guttatus*)

Kayla C. Akkerman<sup>1</sup>, Arash Sattarin<sup>2</sup>, John K. Kelly<sup>2</sup> and Alison G. Scoville<sup>1,\*</sup><sup>1</sup>Department of Biology, Central Washington University, Ellensburg, WA 98926, USA and <sup>2</sup>Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence, KS 66045, USA

\*Correspondence address. Department of Biology, Central Washington University, Ellensburg, 400 E University Way, Ellensburg, WA, 98926. Tel: 509-963-2802. Fax: 509-963-2730 E-mail: scoville@cwu.edu

## Abstract

Transgenerational phenotypic plasticity, whereby environmental cues experienced by parents alter the phenotype of their progeny, has now been documented in diverse organisms. Transmission of environmentally determined responses is known to occur through both maternal and paternal gametes, but the underlying mechanisms have rarely been compared. In addition, the persistence of induction over multiple generations appears to vary widely, but has been characterized for relatively few systems. Yellow monkeyflower (*Mimulus guttatus*) is known to exhibit transgenerational induction of increased glandular trichome production in response to simulated insect damage. Here, we test for differences between maternal and paternal transmission of this response and examine its persistence over five generations following damage. Maternal and paternal damage resulted in similar and apparently additive increases in progeny trichome production. Treatment of germinating seeds with the genome-wide demethylating agent 5-azacytidine erased the effect of maternal but not paternal damage. The number of glandular trichomes remained elevated for three generations following damage. These results indicate that transgenerational transmission occurs through both maternal and paternal germ lines, but that they differ in the proximate mechanism of epigenetic inheritance. Our results also indicate that a wounding response can persist for multiple generations in the absence of subsequent damage.

**Key words:** epigenetic inheritance, transgenerational plasticity, induced defense, *Mimulus*, trichome

## Introduction

Transgenerational phenotypic plasticity occurs when environmental cues experienced by parents alter the phenotype of their progeny. This phenomenon has been documented in diverse organisms, including bacteria [1], yeast [2], plants [reviewed by 3, 4], and mammals [reviewed by 5, 6]. A number of studies provide evidence that transgenerational plasticity can be adaptive [reviewed by 7–9]. For example, maternal light [10] and parental soil moisture [11] conditions induce adaptive responses in plant offspring. Parental temperature induces adaptive life history

responses in fish [12]. Attack by predators, herbivores, or pathogens can cause transgenerational induction of defenses in both plant and animal species, resulting in progeny that are better defended than offspring from unthreatened parents [reviewed by 13–15].

The adaptive potential of transgenerational plasticity depends on the probability that parental environmental cues accurately predict conditions experienced by their descendants [16–19]. Differences in the dispersal of male and female gametes may therefore place different selective pressure on

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transgenerational inheritance through the male and female germline [20]. In addition, fitness benefits of transgenerational plasticity are expected to be highest when the persistence of an induced effect across generations matches the temporal periodicity of environmental change [18, 21–23]. Some authors argue that prediction is likely to be poor over multiple generations, and single-generation inheritance is therefore most apt to produce adaptive effects [18], whereas others point out that stably inherited states could provide long-term adaptation to changing environmental conditions [24–26]. The precise mechanisms and resultant patterns of transgenerational plasticity may affect the adaptive potential of this phenomenon and may also be shaped by past selection. Characterizing sex-dependent patterns, proximate mechanisms, and persistence of transgenerational plasticity is thus of prime importance.

The unequal nature of maternal and paternal contributions to zygote cytoplasm, organelles, and offspring provisioning have long prompted investigation into environmentally determined maternal effects. Most studies have focused on traits such as offspring size, seed size, nutrient provisioning, and accumulation of defensive secondary metabolites [3, 19, 27, 28]. Nevertheless, environmentally determined paternal effects are now well-documented, even in species without paternal care [reviewed by 29, 30, 31]. They are often qualitatively different from maternal effects [32, 33] and may be transmitted even more effectively than maternal effects over multiple generations [34]. The existence of both maternal and paternal transgenerational effects makes sense in the light of recent evidence that three interrelated epigenetic mechanisms may be involved: DNA methylation, histone modification and production of small RNA (sRNA), all of which may be stably inherited through meiosis [35–38].

Environmental conditions are associated with changes in DNA methylation [35] and patterns of DNA methylation are often inherited from one generation to the next, particularly in plants [25, 39–41]. Environmental cues are also associated with histone modification [reviewed by 42], which can act as a signal integration and storage platform [43, 44] and influence transcription by changing the local chromatin structure [45]. In many cases, DNA methylation and histone modification act together to regulate gene expression [46–50]. A variety of biotic and abiotic environmental stressors, such as infection, mechanical stress, cold, heat, salt, and drought have also been linked to expression of sRNA, including small interfering RNA (siRNA) and microRNA (miRNA) [47, 51]. In plants, environmentally induced phytohormones are known to effect changes in expression of sRNA [52–54], which is mobile between cells and throughout the vasculature [55–57]. sRNA molecules are potentially capable of entering the germline [reviewed in 47, 58, 59] and have been associated with transgenerational transmission [34, 47, 60, 61]. In addition to post-transcriptional regulation, sRNA is involved in recruitment of epigenetic modifiers to specific loci and alteration of chromatin through mechanisms such as RNA-directed DNA methylation [62–64, reviewed by 65]. In some cases, sRNA is known to be triggered by stress signaling through phytohormones [52] and involved in transmission of induced states to progeny [54, 66]. sRNA may thus play a role in transgenerational plasticity by acting to initiate and/or maintain targeted alterations to chromatin in response to environmental conditions [47].

*Mimulus guttatus* (Phrymaceae; [67]) is known to exhibit transgenerational induction of increased glandular trichome density in offspring in response to simulated insect damage administered prior to the development of reproductive tissue [68]. Using

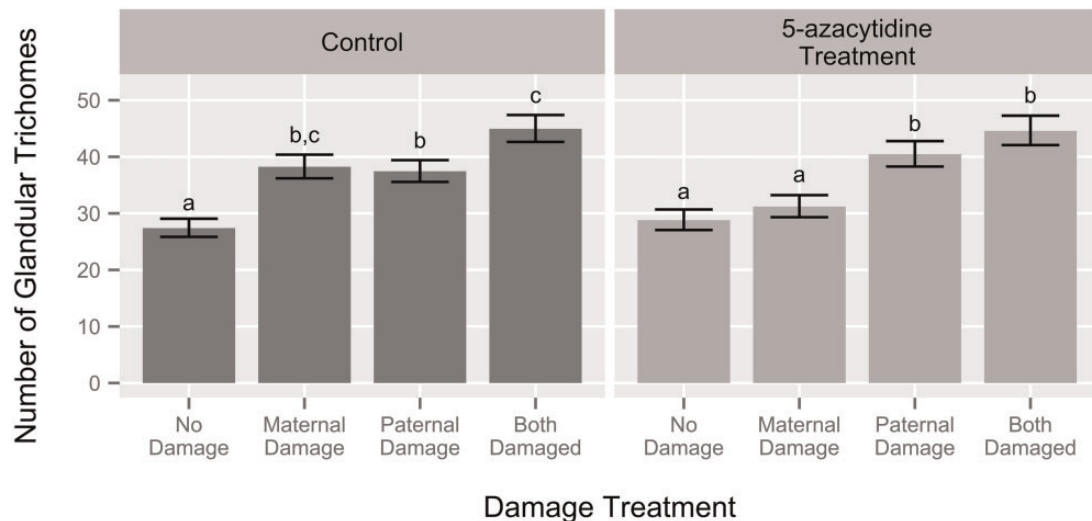
a panel of recombinant inbred lines (RILs) derived from a cross between a high-alpine annual population (Iron Mountain) and a perennial coastal population (Point Reyes), Holeski [68] and Scoville *et al.* [69] demonstrated genetic variation in both within-generation and between-generation induction of this response. Studies on one of these RILs showed that transgenerational induction of increased trichome density was associated with reproducible differential expression in over 900 genes. These genes were associated with four functional categories related to trichome formation and clustered into four putative co-regulatory groups, suggesting targeted modification of particular developmental pathways [70]. The putative defensive function of glandular trichomes [71–74] makes this system a potential example of adaptive transgenerational plasticity. If parental damage correctly predicts the level of herbivory experienced by progeny, transgenerational induction of trichomes can confer a fitness advantage [16, 17, 19]. However, the adaptive potential of this trait depends on dispersal in seeds and pollen and whether the epigenetic signal is transmitted through the maternal or paternal line, the degree to which this signal persists over multiple generations, and the spatial and temporal dynamics of herbivore populations.

This study represents a first step in comparing the maternal and paternal contributions to transgenerational plasticity, testing for involvement of particular epigenetic mechanisms, and characterizing the persistence of induction across multiple generations in *M. guttatus*. Specifically, we use a single RIL known to exhibit transgenerational induction (RIL 85) to test for sex-dependent differences in the transmission of increased trichome production due to simulated insect damage. In addition, we treat a subset of germinating seeds with the nucleoside analogue 5-azacytidine, which incorporates into the genome of proliferating cells during DNA synthesis and traps DNA methyltransferases, targeting them for degradation and resulting in genome-wide demethylation [75]. This allows us to test for a role of chromatin modification in transgenerational transmission through either the maternal or paternal gamete. Finally, we track the persistence of induction over five generations produced by self-pollination.

## Results

### Sex-Dependent Epigenetic Inheritance

Maternal and paternal damage resulted in significant and comparable increases in the number of glandular trichomes (Fig. 1 and Supplementary data S1). The lack of significant interaction between maternal and paternal damage (Table 1), and the magnitude of increase in glandular trichomes among plants receiving both types of ancestral damage (Fig. 1) are consistent with an additive effect of maternal and paternal damage. The interaction between maternal damage and treatment with 5-azacytidine was significant, with 5-azacytidine largely erasing effect of maternal damage. In contrast, the interaction between paternal damage and treatment with 5-azacytidine was only marginally significant, with 5-azacytidine increasing the effect of paternal damage. Other effects and interactions were not significantly different from zero. *Post hoc* pairwise comparisons of marginal means reveal a significant effect of maternal damage and paternal damage among plants without 5-azacytidine treatment (Table 2). In plants treated with 5-azacytidine, however, the effect of maternal damage is no longer significant whereas paternal damage remains highly significant.



**Figure 1:** Number of glandular trichomes produced along a mid-leaf transect across the underside of both leaves in the 5th leaf pair. Bars represent marginal means for each combination of maternal and paternal damage, for control plants and plants treated with 5-azacytidine at germination. Letters indicate significant differences measured via pairwise comparisons within control or 5-azacytidine treated plants ( $\alpha=0.05$ ). Error bars show  $\pm 1$  SE. N = 1314.

**Table 1:** results from generalized linear mixed-model predicting number of glandular trichomes as a function of all two-way interactions involving maternal damage, paternal damage, and treatment with 5-azacytidine

Factor	Effect Size (SE)	DF	T	P
Maternal damage	0.29 (0.08)	21	3.88	<b>0.0009*</b>
Paternal damage	0.27 (0.07)	21	3.72	<b>0.0013*</b>
5-Azacytidine treatment	-0.00 (0.05)	1284	-0.04	0.9687
Maternal damage $\times$ 5-azacytidine interaction	-0.16 (0.05)	1284	-2.92	<b>0.0036*</b>
Paternal damage $\times$ 5-azacytidine interaction	0.12 (0.05)	1284	2.12	<b>0.0338*</b>
Maternal damage $\times$ paternal damage interaction	-0.08 (0.10)	21	-0.81	0.4279

Effect sizes and standard errors are reported on the natural log scale. Significance is denoted by bold type ( $P < 0.05$ ) and \* ( $P < 0.005$ ). N = 1314.

## Persistence of Transgenerational Induction

The number of glandular trichomes remained elevated for at least three generations following damage, demonstrated by non-overlapping 95% credible intervals for control and damaged lineages (Fig. 2 and Supplementary Table S1 and data S2). Generation 4 showed no evidence of increased trichome production in response to ancestral damage. The results from Generation 5 are inconclusive: damaged lineages produced a higher mean number of trichomes, but there is no clear separation between credible intervals. Residual variance (i.e. overdispersion) varied among combinations of generation and damage treatment, although no clear pattern was evident with respect to generation or treatment (Supplementary Table S2). Generation 2 plants grown after 6 months of seed storage (during the production of Generation 3 seeds) showed a similar response to damage compared with plants grown after 31 months of seed storage (Block A  $\times$  treatment interaction = 0.40; 95% credible interval = -1.81 to 2.82), or 56 months of storage (Block B  $\times$  treatment interaction = -0.40; 95% credible interval = -2.31 to 1.63; Supplementary data S3).

## Discussion

### Maternal versus Paternal Effects

Although the existence of maternal [3, 19, 27, 28], paternal [29–31], and biparental [e.g. 12, 53] transgenerational plasticity

is well-established, very few studies to date explicitly compare maternal and paternal contributions within a single system [but see Ref. 32]. Our results indicate that transgenerational transmission of increased glandular trichome production occurs through both the maternal and paternal gamete. The effects of maternal and paternal damage are similar in magnitude and apparently additive. This is consistent with a scenario in which both parents transmit the same type of epigenetic change that contributes to a continuous, rather than a threshold, response. Alternatively, maternal and paternal transmission could be accomplished through different but complementary modes of action.

Data from *Arabidopsis* show that patterns of DNA methylation can be stably inherited for many generations and are associated with changes in gene expression and phenotype [25]. DNA methyltransferases are active during plant gametogenesis and embryogenesis [reviewed by 47] and functional activity of gametophytic cytosine-DNA-methyltransferase 1 (MET1), which maintains CG methylation, is necessary for epigenetic inheritance during gametogenesis [41]. These results lend support to the notion that faithful reproduction of DNA methylation patterns through meiosis is the causal mechanism for transgenerational epigenetic inheritance [reviewed by 40]. Treatment with 5-azacytidine results in genome-wide demethylation via destruction of methyltransferases [75, 76]. Recently, 5-azacytidine has also been shown to affect the integrity of histone methylation complexes and change genomic histone patterns in

complex ways, such as erasing repressive histone marks from promoters but increasing them in other parts of genome, or switching histone variants [77]. Importantly, treatment with 5-azacytidine erased most if not all of the maternal contribution but none of the paternal contribution to transgenerational induction of increased trichome production. The marginally significant paternal damage  $\times$  5-azacytidine interaction indicates that the 5-azacytidine treatment may actually have increased the effect of paternal damage, although these results should be interpreted with caution, given the approximate nature of *P*-values obtained from generalized linear mixed-models.

### Potential Mechanisms

Persistence of the effect of paternal damage but not maternal damage after treatment with 5-azacytidine indicates that the two germ lines differ in the proximate mechanism of epigenetic inheritance through meiosis. Erasure of the effect of maternal damage via treatment with 5-azacytidine is consistent with maternal epigenetic inheritance via faithful reproduction of methylation patterns. This pattern may also be consistent with epigenetic inheritance via persistence of histone modifications rather than methylation changes.

In contrast, paternal inheritance in this system is accomplished via a mechanism that is apparently resistant to 5-

**Table 2:** results for *post hoc* pairwise comparisons isolating the effects of maternal and paternal damage on the number of glandular trichomes under control conditions and after treatment with 5-azacytidine

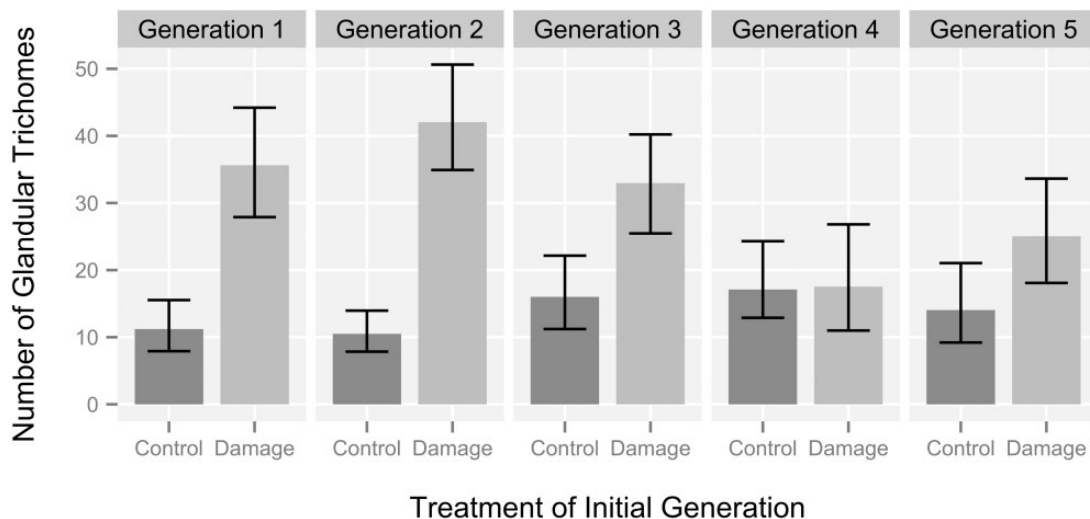
Treatment	Factor	Effect Size (SE)	T	P
Control	Maternal damage	0.25 (0.05)	-4.67	<b>0.0001*</b>
	Paternal damage	0.23 (0.05)	-4.31	<b>0.0003*</b>
5-Azacytidine	Maternal damage	0.10 (0.06)	-1.67	0.1107
	Paternal damage	0.35 (0.06)	-5.97	<b>0.0000*</b>

Effect sizes and standard errors are reported on the natural log scale, and *P*-values are adjusted using the Tukey method. Significance is denoted by bold type (*P* < 0.05) and \* (*P* < 0.005). Degrees of freedom = 21 for each comparison. *N* = 1314.

azacytidine treatment of seeds during germination. Because each seed contains a multicellular plant embryo resulting from multiple rounds of mitosis, maternal and paternal DNA should be equally susceptible to the effects of this treatment. Histone modifications could thus be responsible for paternal inheritance [78], depending on their susceptibility to alteration by 5-azacytidine. However, these data are also consistent with involvement of sRNA, a prime candidate for transgenerational epigenetic inheritance [38, 65, 79]. Critical components of sRNA pathways, including those mediating miRNA and siRNA, show microsporophyte-specific expression patterns throughout pollen development and in the sperm [31, 80–82]. Developing pollen shows accumulation of mature miRNAs [81], and there is evidence that sRNAs derived in the vegetative nucleus migrate to sperm cells as the pollen matures, coinciding with silencing of transposable elements [59, 82]. Data on compromised pollen tube growth in dicer mutants indicates that the transcriptional activity of mature pollen may be regulated by siRNAs [82]. In *Arabidopsis*, some methylation states that are erased in the absence of functional DNA (cytosine-5)-MET1 are restored in later generations, once *met1* mutations are complemented with wild-type alleles [83]. This indicates that methylation at a subset of sites can be re-initiated by another mechanism, such as the continued production of sRNAs [47]. These lines of evidence suggest that male-specific sRNA might be produced in the microspore or microgametophyte, packaged with sperm and inherited by the zygote [81, 84] where it could initiate *de novo* DNA methylation in the developing embryo and thus contribute to transgenerational inheritance of DNA methylation patterns [84].

### Persistence of Transgenerational Effects

Current studies document a wide range of persistence patterns for transgenerational epigenetic inheritance [18, 25, 34, 50, 85] and the reason for differences in persistence remain unclear [86]. Most examples of adaptive transgenerational plasticity involve just a single generation, although many studies do not explicitly test for persistence beyond that [reviewed by 7]. Here, we show that a significant effect of parental damage on trichome production persists for at least three generations.



**Figure 2:** Number of glandular trichomes along a mid-leaf transect for 5 generations of plants originating from either control or damaged Generation 0 ancestors and produced by self-pollination. Bars represent marginal means for each combination of generation and ancestral damage treatment. Error bars show 95% credible intervals. *N* = 670.

Persistence beyond the first generation demonstrates that this phenomenon is truly an example of transgenerational inheritance to offspring whose cells were not exposed to the initial environmental cue [42, 87], or even the somatic response to that cue. In addition, the damage response remained similar for Generation 2 plants, whether they were grown during production of Generation 3 seeds, 1 year later (Block A) or 2 years later (Block B), indicating no detectable change due to storage of seeds at 4°C.

### Future Studies

The use of a single RIL in this study allowed us to isolate epigenetic transmission of an environmentally induced signal within a uniform genetic background. This was critical, as it is often difficult to disentangle epigenetic and genetic variation [25, 88]. However, numerous studies indicate the existence of genetic variation in transgenerational effects [e.g. 50, 89–91], which is necessary for evolution of this phenomenon. Other lines from our panel of RILs show greater or lesser amounts of transgenerational induction [68, 69], and may exhibit different patterns of maternal transmission, paternal transmission, or persistence over generations. Studies of additional RILs will help elucidate the nature of genetic variation in patterns of epigenetic inheritance in our panel. In addition, our panel of RILs was derived from a cross between two populations from disparate ecological settings and does not, therefore, represent a natural population. Expanding this investigation to natural populations of *M.guttatus* and its predators will be a next step in evaluating whether or not the induction of increased trichome production is a case of adaptive transgenerational plasticity, shaped by natural selection, and understanding the ecological and evolutionary consequences of sex-specific patterns and persistence of this response. Finally, the effects of treatment with 5-azacytidine are complex, and have been associated with both increases and decreases in gene expression, as well as changes in both DNA methylation and histone modification [77]. Tissue and developmental stage-specific studies of chromatin structure and sRNA production will be needed to reveal the molecular mechanisms underlying differences in maternal and paternal transmission, as well as patterns of persistence, indicated by our results.

## Methods

### Experiment 1: Sex-dependence

Fifty plants were grown from a single RIL (RIL 85; [69]). Half were randomly assigned to a damage treatment that involved punching two holes of roughly 3 mm diameter in each leaf of the second to fifth leaf pair as soon as the subsequent leaf pair expanded [modified from 68]. Plants were then randomly paired and intercrossed to create a full factorial experiment involving maternal and paternal damage. Each combination of treatments, including no damage, only maternal damage, only paternal damage, and damage of both parents, was represented by 6–7 independent pairs of plants that were unilaterally crossed to produce seeds that were stored at 4°C until germination. Progeny germinated from these seeds were raised together in standard greenhouse conditions in three successive blocks. Plants were grown in 10cm pots that were placed randomly into flats. Flats were bottom watered and rotated daily on the greenhouse bench. Natural light was supplemented with a 16 h light/8 h dark cycle with Sylvania Lumalux LU1000 high pressure

sodium bulbs. Plants received fertilizer (2.6 ml Jack's Professional® 10-30-20 Blossom Booster Water-Soluble Fertilizer/1l water) every week, plus Marathon® II Liquid Insecticide and Subdue Maxx® Fungicide (2 ml/l water each) every other week.

The first block of plants included eight replicate progeny per parent pair, totaling 200 plants. In order to test for a role of DNA methylation, the second and third blocks included 12 replicate progeny per parent pair and an additional 12 replicate progeny per parent pair that were treated with 5-azacytidine, totaling 576 plants per block. For these blocks, seeds were soaked in ultra-purified water in the dark for 48 h (control plants) or for 24 h, followed by 24 h in a 1mM solution of 5-azacytidine (treatment plants). This concentration was chosen to equal or exceed treatments shown to result in measurable genome-wide demethylation in other plants [92, 76, 93] without causing increased mortality in preliminary experiments. All seeds were then rinsed with ultra-purified water, transplanted into pots, and raised in standard greenhouse conditions. When progeny reached expansion of the 6th leaf pair, we measured trichome production on the underside of the 5th leaf pair by folding the tip of the leaf to the base and counting the total number of trichomes visible above the fold across both leaves together.

### Experiment 2: Persistence

Eight plants were grown from the same RIL (RIL 85; [69]). Half were randomly assigned to the same damage treatment described above. Each plant was used to establish an independent lineage that was propagated by self-pollination each generation for five subsequent generations. Seeds were pooled from multiple plants within each generation of each lineage and stored at 4°C prior to germination. Finally, seeds from all generations and lineages were grown together in two replicate blocks (Generations 1–4 in block A and Generations 1–5 in block B) and measured for trichome production on the underside of the fifth leaf pair, as described above. A total of 365 plants were measured in block A and 305 in block B. In each generation and each block, plants were grown together in standard greenhouse conditions, randomized in location, and rotated around the bench daily. By growing plants from multiple generations together, we controlled for variation due to block-level effects. However, seeds from earlier generations experienced a longer time at 4°C prior to germination, compared to seeds from later generations. In order to test for an effect of storage time on transgenerational transmission, we also grew and phenotyped a subset of Generation 2 plants during production of Generation 3 seeds (planted January 2013), and compared these with Generation 2 plants grown in Block A (planted February 2014) and Block B (planted March 2015).

### Statistical Analysis

To analyze the data for experiment 1, we applied a generalized linear mixed-model, executed with the `glmmPQL` function from the `MASS` package in R [94]. The number of glandular trichomes was modeled as a function of block, all two-way interactions involving maternal damage, paternal damage, and treatment with 5-azacytidine, and parent pair, with parent pair treated as a random effect. We used a log-link function and a Poisson distribution of error terms, allowing for overdispersion. This model appropriately represents unique parent pairs, which are nested within each combination of parental treatment, as the unit of independent replication [94, 95]. Following 'best practices' [96], estimation was performed via penalized quasi-likelihood and

hypothesis testing of fixed effects was performed using Wald  $t$  statistics, which account for uncertainty in the estimates of overdispersion. We performed specific *post hoc* pairwise comparisons using the `lsmeans` function from the `lsmeans` package in R [97], with  $P$ -values adjusted using the Tukey method and degrees of freedom calculated using the 'between-within' rule [98]. To probe the robustness of our results, we fit the same model using maximum likelihood estimation based on Laplace approximation, executed with the `glmer` function from the `lme4` package in R [99], as well as Bayesian Markov chain Monte Carlo (MCMC) simulations, executed with the `MCMCglmm` function from the `MCMCglmm` package in R [100]. We confirmed that all three analyses yielded closely matched estimates, confidence/credible intervals, and  $p/pMCMC$ -values.

For the second experiment, we again applied generalized linear mixed-models with a log-link function and a Poisson distribution of error terms, allowing for overdispersion. First, we used data from all generations (Block A and B) to model the number of glandular trichomes as a function of block, damage treatment of the initial generation, number of generations since damage, damage treatment  $\times$  generation interaction, and lineage, with lineage treated as a random effect. Second, we analysed all Generation 2 data, including plants grown in an additional block during production of Generation 3 seeds, by modeling the number of glandular trichomes as a function of block, damage treatment of the initial generation, block  $\times$  damage treatment interaction, and lineage, with lineage treated as a random effect. We used the block  $\times$  damage treatment interaction in order to assess the effect of seed storage time on transgenerational induction. Both models appropriately represent lineages founded by unique Generation 0 plants, which are nested within damage treatment of the initial generation, as the unit of independent replication [94, 95].

In our Experiment 2 analyses, residual variance was heterogeneous among combinations of damage treatment and generation (Analysis 1) and damage treatment and block (Analysis 2). We therefore exploited the flexibility of Bayesian MCMC simulations (executed with the `MCMCglmm` function in R; [100]) to fit models with four different variance structures: (i) our original model, with a single among-line variance; (ii) a separate line-level variance within each combination of damage treatment and generation/block; (iii) a separate residual variance (i.e. overdispersion) within each combination of damage treatment and generation/block; and (iv) separate line-level variances and residual variances within each combination of damage treatment and generation/block. In each case, we used weak proper priors (a multivariate Gaussian distribution with mean=0 and variance =  $I \times 1+e10$  for fixed effects, and an inverse Wishart with  $V = 1$  and  $\nu = 0.002$  for random effects) and a burnin period of 10 000 draws, followed by 500 000 iterations with a thinning interval of 25. We confirmed convergence from different starting values, as well as adequate mixing and absence of autocorrelation in the resultant chains.

For both analyses, we compared model fits based on deviance information criterion (DIC) score, averaged between two runs. For the first analysis, Models 3 and 4 yielded comparable DIC values ( $\Delta DIC < 1$ ), which were superior to Model 1 ( $\Delta DIC = 67$ ) and Model 2 ( $\Delta DIC = 60$ ). For parsimony, and based on highly overlapping 95% credible intervals for all lineage-level variances estimated from Model 4, we present results derived from Model 3. We also confirmed that Model 4 yields qualitatively similar results. For analysis of all Generation 2 data, Model 4 yielded a better average DIC score than Model 1 ( $\Delta DIC = 45$ ), Model 2 ( $\Delta DIC = 34$ ), or Model 3 ( $\Delta DIC = 2$ ). Model 4 also yielded non-

overlapping 95% credible intervals for both line and residual-level variances, indicating the importance of including this structure in our analysis. We thus present results from Model 4, but also confirmed that Model 3 yields qualitatively similar results.

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*Conflict of interest statement.* There are no conflicts of interest to report.

## Supplementary data

Supplementary data is available at *EnvEpig* online.

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