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Stress-induced DNA methylation changes and their heritability in asexual dandelions

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Summary

- DNA methylation can cause heritable phenotypic modifications in the absence of changes in DNA sequence. Environmental stresses can trigger methylation changes and this may have evolutionary consequences, even in the absence of sequence variation. However, it remains largely unknown to what extent environmentally induced methylation changes are transmitted to offspring, and whether observed methylation variation is truly independent or a downstream consequence of genetic variation between individuals.
- Genetically identical apomictic dandelion (*Taraxacum officinale*) plants were exposed to different ecological stresses, and apomictic offspring were raised in a common unstressed environment. We used methylation-sensitive amplified fragment length polymorphism markers to screen genome-wide methylation alterations triggered by stress treatments and to assess the heritability of induced changes.
- Various stresses, most notably chemical induction of herbivore and pathogen defenses, triggered considerable methylation variation throughout the genome. Many modifications were faithfully transmitted to offspring. Stresses caused some epigenetic divergence between treatment and controls, but also increased epigenetic variation among plants within treatments.
- These results show the following. First, stress-induced methylation changes are common and are mostly heritable. Second, sequence-independent, autonomous methylation variation is readily generated. This highlights the potential of epigenetic inheritance to play an independent role in evolutionary processes, which is superimposed on the system of genetic inheritance.

Introduction

Epigenetic mechanisms, such as DNA methylation, can cause stable alterations in gene activity without changes in the underlying DNA sequence. DNA methylation is associated with silencing of transposons, imprinting and silencing of both transgenes and endogenous genes (Kooter *et al.*, 1999; Grossniklaus *et al.*, 2001; Miura *et al.*, 2001; Lippman *et al.*, 2004; Shiba *et al.*, 2006; Zilberman *et al.*, 2007). In mammals, resetting of DNA methylation takes place during early embryonic development (Santos *et al.*, 2002). In plants, by contrast, a considerable proportion of DNA methylation marks can be stably transmitted from parents to offspring (Kakutani *et al.*, 1999; Vaughn *et al.*, 2007; Johannes *et al.*, 2009), and many examples exist of methylation epi-alleles that cause segregating phenotypes (Cubas *et al.*, 1999; Kalisz & Purugganan, 2004; Richards, 2006).

The combination of heritability and phenotypic consequences of DNA methylation suggests that the mechanism could play a role in natural selection and adaptation, in ways that may not be explained by DNA sequence variation (Rapp & Wendel, 2005; Grant-Downton & Dickinson, 2006; Richards, 2006; Bossdorf *et al.*, 2008; Boyko & Kovalchuk, 2008; Jablonka & Raz, 2009). In order to evaluate an evolutionary role of epigenetic inheritance, it is important to first gain a better insight into the processes that generate methylation variation between individuals.

These processes are currently poorly understood. Although microarray and bisulfite sequencing studies provide a detailed but static picture of the genomic methylation landscape in plants (Cokus *et al.*, 2008; Lister *et al.*, 2008; Zhang, 2008), it largely remains to be determined how responsive the methylation code is to internal and external cues.

Major genomic events, such as hybridization and polyploidization (Adams & Wendel, 2005; Dong *et al.*, 2006; Chen, 2007; Paun *et al.*, 2007), and also environmental stresses (Chinnusamy & Zhu, 2009), can trigger DNA methylation changes in plants. Stress-induced methylation changes may be targeted specifically to stress-related genes. Alternatively, methylation changes may generate nonspecific (random) differences between individuals, which may have adaptive significance during times of stress (Rapp & Wendel, 2005), because they increase the range of variation that natural selection can act upon. Whether stress-targeted or random, environmentally induced methylation variation may add an interesting epigenetic component to population responses to natural selection. It is therefore relevant to establish whether environment-induced methylation modification is a common phenomenon, and whether induced methylation changes are stably transmitted to next generations.

The evolutionary relevance of epigenetic variation requires that it is not simply a direct downstream consequence of genetic (DNA sequence) variation. Only when epigenetic and genetic variation are independent, or at least not fully dependent, can epigenetic inheritance affect evolutionary processes in ways that cannot be explained by sequence variation (Richards, 2006, 2008; Bossdorf *et al.*, 2008). Unraveling epigenetic from genetic variation can be a difficult task in genetically diverse populations (Johannes *et al.*, 2008). This is one of the main obstacles to evaluating the evolutionary relevance of epigenetic inheritance. However, detecting independent epigenetic variation is considerably less complicated in populations that lack genetic variation (Johannes *et al.*, 2009; Verhoeven *et al.*, in press). In this study, we explored stress-induced methylation variation in apomictic dandelion plants. Apomictic dandelions reproduce through unfertilized seeds, and offspring are genetic copies of the mother plant (van Dijk, 2003). The dandelion system thus has the advantage that epigenetic alterations can be studied in the absence of genetic variation.

The system is also interesting to study from an evolutionary perspective. The evolutionary potential of apomictic lineages is severely limited because of the absence of genetic variation that is normally associated with sexual reproduction. There are indications that apomictic dandelions may have compensatory mechanisms to generate heritable variation, for instance via increased transposon activity or somatic recombination (Richards, 1989; King & Schaal, 1990). A similar enhanced role for epigenetic variation might be hypothesized.

In this study, we exploited the genetic identity of apomictic clone members by exposing the same genotype to different environments and evaluating the methylation consequences in stressed plants and in their unstressed offspring. This can detect stress-induced and heritable epigenetic variation that does not directly reflect genetic variation. Using methylation-sensitive amplified fragment length polymorphisms (MS-AFLPs) to assess methylation variation at genome-wide, anonymous marker loci, we specifically asked: do salt stress, nutrient stress and chemical induction of anti-herbivore and anti-pathogen defenses promote methylation changes? And, if so, are these changes transmitted to offspring?

Materials and Methods

Plant material and growing conditions

Asexual variants of the common dandelion, *Taraxacum officinale* Weber ex Wigg., are polyploid (usually triploid, $3x = 24$) obligate apomicts that produce clonal seeds in a process that involves unreduced egg cell formation (diplospory), parthenogenic embryo development and autonomous endosperm formation (van Dijk *et al.*, 1999). We used progeny from a single apomictic plant (AS34) that was produced in an experimental cross between a sexual diploid mother and diploid pollen from a triploid father (Verhoeven *et al.*, in press). Seeds from AS34 were germinated on water-saturated filter paper in Petri dishes for 10 d (10 h dark : 14 h light; 15°C : 20°C), and seedlings were transplanted to individual pots and raised in a climate chamber (10 h dark : 14 h light at *c.* 275 photosynthetically active radiation (PAR); 15°C : 20°C) where they were exposed to different environmental treatments (see Experimental treatments). Plants were watered several times per week with half-strength Hoagland nutrient solution. Pots were weighed and reset to the same weight every week by adding demi-water in order to maintain a constant soil moisture level across the entire experiment.

Experimental treatments

Generation 1 Plants were randomly assigned to one of five experimental treatments: low nutrients, salt stress, jasmonic acid (JA) application, salicylic acid (SA) application and control treatment ($n = 8$ plants per treatment). JA and SA are plant hormones involved in herbivore and pathogen defenses (Durner *et al.*, 1997; McConn *et al.*, 1997; Glazebrook, 2005; Howe & Jander, 2008), and their application is often used to experimentally mimic biotic attack and to induce defense pathways. There is extensive crosstalk between JA and SA pathways but, generally speaking, SA-induced

defenses are often associated with biotrophic pathogens and JA-induced defenses are often associated with herbivorous insects (Pieterse & Dicke, 2007). Nutrient stress was applied by five-fold dilution of the Hoagland nutrient solution relative to control plants throughout the experiment. Salt stress was applied by adding NaCl to the Hoagland nutrient solution to a concentration of 150 mM. JA was applied twice, when plants were 5 and 7 wk old; 0.25 ml (week 5) and 0.75 ml (week 7) of a 10 mM JA solution (Sigma J-2500, dissolved in ethanol and diluted to the desired concentration with a 0.1% Triton X-100 surfactant solution) was applied and manually distributed over the surface of two leaves (week 5) or four leaves (week 7). SA application followed the same protocol as JA application, using 10 mM SA (Sigma S-7401, dissolved in 0.1% Triton X-100 surfactant solution). The low-nutrient and high-salt treatments resulted in 71% and 27% reduction in flower production, respectively, relative to control plants (with further reductions in the number of seeds produced per flower), whereas the JA and SA treatments did not decrease flower production (data not shown). In all treatments, plants started to flower after 10–13 wk and seeds were collected from each plant.

Generation 2 From each plant, one offspring individual was raised in a common control environment (fully randomized positions in the climate chamber). The germination and growing conditions were as described for the control treatment in generation 1.

AFLP and MS-AFLP analysis

Leaf tissue was collected from 8-wk-old plants in generation 1 and from 4-wk-old plants in generation 2 and stored at −80°C until DNA isolation. Total DNA was isolated using the hexadecyl-trimethyl-ammonium-bromide procedure (Doyle & Doyle, 1990). AFLP fingerprinting (AFLP®, patent and registered trademark owned by Keygene N.V., Wageningen, the Netherlands) was performed according to Vos *et al.* (1995). Four *Eco*RI/*Mse*I primer combinations were used for fragment amplification: *Eco*RI + TAA/*Mse*I + CCA, *Eco*RI + TAA/*Mse*I + CTA, *Eco*RI + TAC/*Mse*I + CAA and *Eco*RI + TTC/*Mse*I + CTA. For restriction-ligation reactions, 250 ng of DNA were used per sample. Fragments were separated on 4.5% denaturing polyacrylamide gels. MS-AFLP fingerprinting followed the same general protocol as the AFLP analysis described above, but the *Eco*RI restriction enzyme was replaced by the methylation-sensitive restriction enzyme *Hpa*II. *Hpa*II cleaves CCGG sequences, but cleaving is blocked when either or both cytosines are fully methylated, and may be impaired or blocked when one or both of the cytosines are hemi-methylated (McClelland *et al.*, 1994; Roberts *et al.*, 2007). Thus, in the absence of genetic (sequence) variation among samples, MS-AFLPs arises as a result of variation among samples in the methylation status of marker loci. We used five *Hpa*II/*Mse*I primer combinations, each with two (*Hpa*II) or three (*Mse*I) selective nucleotides: *Hpa*II + CT/*Mse*I + CTA, *Hpa*II + CC/*Mse*I + CAC, *Hpa*II + CA/*Mse*I + CAG, *Hpa*II + AT/*Mse*I + CAG and *Hpa*II + AC/*Mse*I + CT. Sample positions on gels were randomized (within primer combinations) and gels were inspected visually for the presence/absence of fragments. Visually poor-quality samples were excluded from scoring, and all scoring was performed by the same person in the absence of information on sample identities. The sample randomization and scoring procedure prevented systematic biases in fragment scores, and any scoring errors that may have occurred will be distributed randomly over the dataset. We excluded singleton observations from the dataset, i.e. markers with only one nonconsensus sample. In addition, for 115 MS-AFLP markers, fragment intensities were estimated from gel images using AFLP-Quantar® software (registered trademark of Keygene N.V.). AFLP and MS-AFLP analyses were carried out by Keygene Laboratories.

Data analysis

MS-AFLP variation can be interpreted as methylation variation (not sequence polymorphism) when samples are genetically identical. Genetic identity of offspring is expected under apomictic reproduction, and we have confirmed previously that the offspring of the AS34 apomict used in this study lack detectable AFLP variation (Verhoeven *et al.*, in press). In the current study, we AFLP genotyped all individuals from generation 2 to confirm the genetic similarity of the samples, and then, conditional on genetic similarity, we interpreted MS-AFLPs as methylation polymorphisms. One caveat is that the *Eco*RI enzyme used in (normal) AFLP analysis is not completely insensitive to methylation and may show somewhat reduced cleavage when its restriction site is methylated (Roberts *et al.*, 2007). However, the absence of appreciable AFLP variation observed in our material (see Results) indicates that this is not an important source of AFLP variation. To identify the direction of methylation change at detected MS-AFLPs, we inferred a consensus epigenotype based on the MS-AFLP marker scores in the control group of generation 1. This was considered as the starting state of all plants in the experiment; any deviations were assumed to have arisen during the experiment. The consensus state (present or absent) was determined for 19 markers that were either monomorphic or had only one deviating observation (among all replicate individuals) in the control group; one polymorphic marker was excluded from the analysis because its consensus state could not be determined with confidence (marker X15M49-308.24; Fig. S1, see Supporting Information). It should be noted that the consensus state is based only on control plants because we might expect treatment-induced

methylation changes in other groups; however, the exact same consensus epigenotype would have been produced by considering all plants from generation 1 (including stress treatment) and taking the majority state at each marker.

The frequencies of methylation changes were compared between treatment groups using *G* tests and Fisher's exact tests, as implemented in PROC FREQ, SAS 9.1 for Windows (SAS Institute Inc., Cary, NC, USA). To account for marker differences and a random individual plant effect, we also modeled the effect of treatment on the probability of methylation change using generalized linear mixed models (PROC GLIMMIX add-on to SAS 9.1).

Multivariate analysis

Patterns of variation in MS-AFLP present/absent profiles were explored by performing classical multidimensional scaling (MDS; see Kruskal & Wish, 1978) using the Jaccard distance measure. This provides a representation of the largest variation in all markers among the plants in a series of scores, which can be visualized and interpreted. Separate analyses were performed on generations 1 and 2. Subsequently, the among-replicate variance in the MDS scores was calculated per treatment group and summed over the first three MDS component axes, in order to obtain a quantitative measure of the variation in MS-AFLP profiles within treatment groups. Confidence intervals on these variances were determined by jackknifing: from each treatment group, one randomly selected plant was left out and the variances were calculated again on the scores of models fitted on the reduced dataset. This was repeated 1000 times, resulting in confidence intervals of the variance in every treatment group (Efron, 1982). All multivariate analyses were conducted using MATLAB R2007b (Mathworks Inc., Natick, MA, USA) using the Statistics Toolbox.

As a result of the dominant nature of AFLP markers, part of the variation between individuals is not captured in presence/absence scores. In the triploid samples, a methylation change is only exposed if it makes the difference between zero and ≥ 1 visible fragment copies, but changes within the range of 1–3 copies go undetected. Also, within-individual heterogeneity in methylation patterns (for instance, because of cell type-specific methylation) may be present that goes undetected in presence/absence scoring. However, these factors might contribute to meaningful variation in gel fragment intensities. The relationship between fragment copy number and intensity is not linear (for instance, because of PCR steps in the AFLP protocol) and the interpretation of AFLP fragment intensities is disputed because it is unclear to what extent variation between individuals is caused by technical noise rather than meaningful biological variation. However, intensity data may contain at least some biological information on dosage variation that can be captured using quantitative analysis (Castiglioni *et al.*, 1999; Klahr *et al.*, 2004). We therefore also analyzed quantitatively a larger set of 115 MS-AFLP markers (mostly labeled monomorphic in presence/absence scoring) for which gel fragment intensity scores were obtained using AFLP-Quantar® software (registered trademark of Keygene N.V.). Raw intensity scores were normalized by dividing each fragment score by the mean value of all fragments in the gel lane. This normalization accounts for overall differences in intensity scores between samples, for instance as a result of slight differences between samples in initial DNA concentrations. Normalized intensities were subjected to principal component analysis using Simca-P 10.5 software (Umetrics, Kinnelon NJ, USA).

Results

Genetic variation

AFLP genotyping of all individuals from generation 2 using *Eco*RI/*Mse*I, revealed no polymorphisms among 216 scorable fragments. Some singletons were observed (contributed by three samples; Fig. S1, see Supporting Information), representing either true genetic variation, technical/scoring artifacts or, perhaps, low-level sensitivity of the AFLP *Eco*RI enzyme to methylation. The data provide strong evidence that plants in the experiment lacked appreciable genetic variation, conforming with the expectation under apomictic reproduction, and it follows that most or all MS-AFLPs polymorphisms can be interpreted as methylation (not genetic) variation.

Epigenetic variation – generation 1

Across the entire experiment, MS-AFLP genotyping revealed a subset of 20 polymorphic markers among 359 scorable fragments. These 20 markers were often polymorphic among replicates in multiple environments, including the control environment (Fig. S1). Comparing individual samples with the consensus epigenotype reveals that more methylation changes occurred in each of the nutrient, salt, JA and SA groups than in the control group in generation 1 (Table 1). When loci were considered as independent observations, the proportion of changed loci differed significantly between groups (*G* test, likelihood ratio $\chi^2 = 24.5$, d.f. = 4, $P < 0.0001$), with pairwise comparisons between the control group (proportion of changed loci, 7.6%) and individual treatments indicating higher rates of methylation change caused by JA (proportion of changed loci, 17.9%; $P < 0.01$), SA (29.6%; $P < 0.0001$) and NaCl (14.6%; $P = 0.06$) treatments. A statistical model that accounts for correlated responses, arising because multiple markers in the same individual are not independent, showed a significant treatment effect on the probability that a change in methylation status occurs only for the SA treatment (Table 2). This analysis also showed that individual loci differed in their probability to change methylation status.

Table 1 Methylation changes observed in common dandelion, *Taraxacum officinale*, plants that were exposed to different experimental treatments (generation 1) and in offspring of treated plants (generation 2, raised in a common control environment)

		Generation 1				Generation 2				
		Methylation changes		Not changed	**% changed**	Methylation changes from generation 1		Not changed in generation 1		
Group	Total cases (markers × samples)[1]	0 > 1[2]	1 > 0[2]			**Transmitted**	Reverted	0 > 1	1 > 0	Not changed
Control	133	4	6	123	**7.5**	**8**	2	0	1	121
JA	152	16	11	125	**17.8**	**23**	4	1	3	120
NaCl	152	11	11	130	**14.5**	**18**	4	0	8	121
Nutrient	152	12	8	132	**13.2**	**14**	5	2	5	125
SA	125	19	18	88	**29.6**	**34**	3	1	4	83

In Generation 1, the presence/absence scores of 19 methylation-sensitive amplified fragment length polymorphism (MS-AFLP) loci were evaluated against the consensus epigenotype in seven to eight individuals per treatment; the percentage of cases in which a locus had deviated from consensus is highlighted in bold. In Generation 2, changes that had occurred in generation 1 were either stably transmitted (highlighted in bold) or reverted to consensus; additional methylation changes were observed at loci that were not affected in Generation 1.
[1], Nineteen MS-AFLP markers scored in eight replicate plants per group yields 152 cases; missing data result in total cases < 152.
[2], Direction of observed methylation change: 0 > 1, change from absent to present *Hpa*II/*Mse*I fragment; 1 > 0, change from present to absent fragment.
JA, jasmonic acid; SA, salicylic acid.

Epigenetic variation – generation 2

Most methylation changes observed in generation 1 were faithfully transmitted to offspring (between 74% and 92% of changes in each group; Table 1). Only a small proportion reverted to the consensus epigenotype. In generation 2, an additional one to eight methylation changes per group were observed at loci that had not changed in generation 1 (Table 1). It should be noted that these 'generation 2' changes might include changes that actually occurred in generation 1 between the moment of leaf tissue collection for DNA analysis and seed production, or that occurred in generation 1 reproductive tissue but not in leaf tissue. As a result of generally faithful transmission of methylation changes from generation 1, and because in generation 2 less additional methylation changes occurred in the control group than in the treatment groups (Table 1), the association between treatment and methylation change showed better statistical support in the offspring of treated plants than in the treated plants themselves (Table 2).

Table 2 Treatment effects on the probability that a methylation-sensitive amplified fragment length polymorphism (MS-AFLP) marker deviates from the consensus epigenotype (based on presence/absence scores of 19 MS-AFLP markers)

Fixed effect	d.f. (num, den)	*F*	*P* value
Generation 1			
Marker[1]	14, 514	4.3	< 0.0001
Treatment	4, 30.3	1.8	0.16
Contrast 'control vs JA'	1, 35.0	1.9	0.18
Contrast 'control vs NaCl'	1, 35.4	1.1	0.30
Contrast 'control vs nutrient'	1, 36.7	0.6	0.44
Contrast 'control vs SA'	1, 33.5	6.3	0.02
Generation 2			
Marker[1]	18, 679	5.1	< 0.0001
Parental treatment	4, 30.0	2.1	0.11
Contrast 'control vs JA'	1, 38.6	2.8	0.10
Contrast 'control vs NaCl'	1, 38.9	2.6	0.12
Contrast 'control vs nutrient'	1, 39.3	1.8	0.19
Contrast 'control vs SA'	1, 36.8	8.1	< 0.01

Fixed effect results from generalized linear mixed model analysis with the individual plant as random factor. A separate analysis was performed for each generation. Note that the treatment effect in generation 2 refers to the parental treatments; generation 2 was raised in a common control environment.
[1], Four markers that were polymorphic only in generation 2 were excluded from the generation 1 analysis.
JA, jasmonic acid; SA, salicylic acid.

To evaluate whether treatments caused targeted methylation changes at specific loci or random methylation changes, classical MDS was used to describe and visualize the variation contained in the 20 polymorphic MS-AFLP loci. If a treatment causes targeted methylation changes at specific loci, which occur consistently in different replicate plants, group-level divergence in methylation patterns will be visible between control and treatment groups. We tested for group-level divergence by subjecting MDS sample scores on each of the first three component axes to analysis of variance (ANOVA). This revealed significant differentiation between control and SA treatment groups on the first axis in both generations (Fig. 1 and Table 3; the control and SA group mean MDS scores differed on axis 1, which explained the majority of variation in the data). Alternatively, treatment-induced methylation changes might be nonspecific (random). In that case, no group-level divergence may be

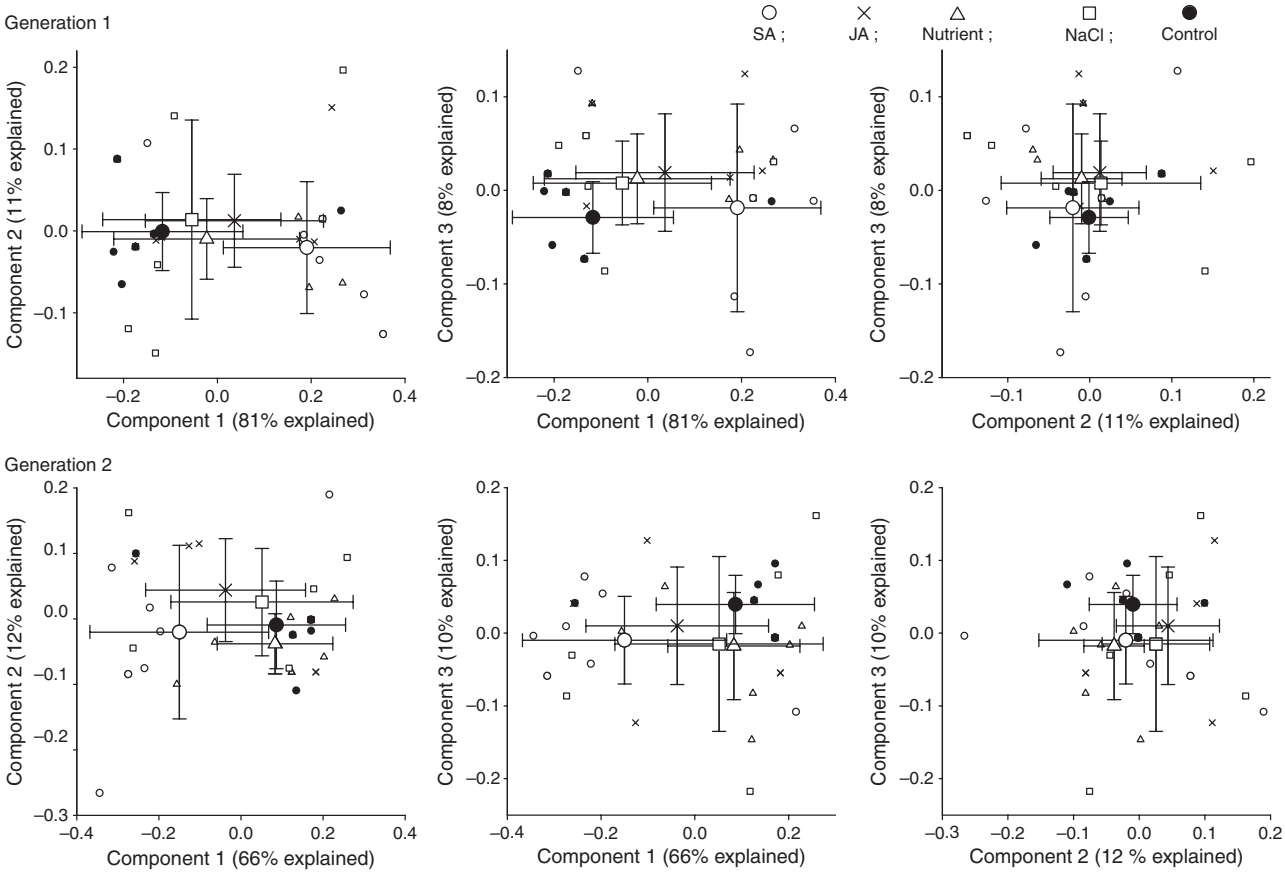

Fig. 1 Multidimensional scaling representation of variation in methylation epigenotypes between samples, based on presence/absence scores of 20 polymorphic methylation-sensitive amplified fragment length polymorphism (MS-AFLP) markers. The first three components were extracted and plotted against each other (separate analysis per generation; top panels, generation 1; bottom panels, generation 2). Small symbols are individual plants (common dandelion, *Taraxacum officinale*) and large symbols indicate treatment group mean ± 1SD. Individuals with missing data at one or more markers were excluded from the models (n = 6–8 plants per treatment group). JA, jasmonic acid; SA, salicylic acid.

present, but replicate plants from the same group will develop dissimilarities in their methylation pattern, leading to increased methylation variation within treatment groups. We tested for increased levels of methylation variation within treatment groups by comparing group variances in MDS scores between control and treatment groups on each of the first three component axes. Although no clear pattern was visible in generation 1, in generation 2 the offspring of control plants showed less methylation variability than the offspring from SA, JA and NaCl plants, as indicated by marginal or no overlap in the 95% confidence intervals for the variance estimates (Fig. 2).

A comparable pattern of larger methylation variation among replicate plants in the offspring of stressed plants relative to the offspring of control plants was also detected in principal component analysis of MS-AFLP marker intensity data (Fig. S2, see Supporting Information). Fragment intensity variation between individuals arises as a result of presence/absence polymorphisms, but also because of variation contained in monomorphic bands, as several types of methylation heterogeneity can lead to the expression of a visible MS-AFLP fragment (see Materials and Methods). The analysis of fragment intensities (Fig. S2) was based on many more fragments than the presence/absence analysis (Figs 1,2), and yielded very similar results.

Discussion

Our results show that environmental stresses readily induce DNA methylation changes at a genome-wide scale and demonstrate that most of the induced changes are faithfully transmitted to offspring. Because we used an apomictic study species, the observed methylation variation in the offspring of stressed plants is associated with parental environments and not with genetic variation among plants. Thus, the results reflect transgenerational epigenetic plasticity of a single genotype in response to environmental stress. We found some evidence that specific stresses can trigger specific methylation changes, leading, for instance, to epigenetic divergence between control and salicylic acid-treated plants. However, there was also general evidence for a stress-induced increase in methylation variation within

Table 3 Treatment effects on group-level divergence in DNA methylation patterns

	Component axis 1		Component axis 2[1]		Component axis 3[1]	
	F	*P* value	*F*	*P* value	*F*	*P* value
Generation 1						
Treatment	2.5	0.06	0.3	0.88	1.4	0.31
Contrast 'control vs JA'	2.5	0.12	0.2	0.63	3.3	0.10
Contrast 'control vs NaCl'	0.4	0.52	0.1	0.76	2.9	0.11
Contrast 'control vs nutrient'	1.0	0.34	0.1	0.72	3.4	0.09
Contrast 'control vs SA'	8.8	< 0.01	0.3	0.61	0.1	0.84
Generation 2						
Parental treatment	2.0	0.12	1.0	0.41	0.6	0.69
Contrast 'control vs JA'	1.3	0.26	1.2	0.29	0.4	0.51
Contrast 'control vs NaCl'	0.1	0.75	0.5	0.48	1.5	0.23
Contrast 'control vs nutrient'	0.0	0.98	0.3	0.56	1.7	0.21
Contrast 'control vs SA'	5.2	0.03	0.1	0.82	1.3	0.26

Analyses of variance (ANOVAs) were fitted to multidimensional scaling sample scores as plotted in Fig. 1 (separate analyses per axis and per generation). Axes 1–3 explained 81%, 11% and 8% of the total variation in generation 1, and 66%, 12% and 10% in generation 2.
[1], In generation 1, the homogeneity of variances assumption for ANOVA was not met for axes 2 and 3. These two analyses were therefore performed using unequal variances ANOVA (PROC MIXED in SAS) in which a separate error variance was estimated for each of the five groups.
JA, jasmonic acid; SA, salicylic acid.

treatment groups, especially in generation 2, with replicate plants showing limited consistency in their methylation changes. Thus, ecological stresses promote autonomous, heritable epigenetic variation and, depending on phenotypic effects, this variation is available for natural selection to act upon. With genetic inheritance, the role of the environment in evolutionary processes is essentially to select among heritable variation but, with epigenetic inheritance, the environment may have an additional role of generating heritable variation at the moment at which it is most required, i.e. during times of stress.

Stress effects on methylation patterns were statistically detectable, even in this relatively small experiment, but it was not typically observed that individual loci showed a consistent methylation change as a result of stress (shared among the majority of replicates), whereas control plants remained unchanged. Rather, there was a subset of inherently unstable loci that were often also polymorphic within the control group, and the effect of stresses was to increase the likelihood that methylation changes occurred at these loci. The functional interpretation of this pattern is unclear. Consistent methylation changes that are controlled by, for instance, hormone signals may occur at specific genes (Kim *et al.*, 2009), but highly localized and specific responses will not generally be captured by AFLPs. In order to gain a better insight into the functional significance of stress-induced methylation changes, it will be important to evaluate the sequence context of AFLPs and, more generally, to take a gene-level approach to the evaluation of stress effects on methylation patterns.

There has been considerable speculation about the evolutionary implications of stress-induced epigenetic variation (e.g. Rapp & Wendel, 2005; Grant-Downton & Dickinson, 2006; Richards, 2006; Bossdorf *et al.*, 2008; Boyko & Kovalchuk, 2008; Jablonka & Raz, 2009). However, current evidence in plants that ecological stresses induce methylation repatterning is limited to a few examples, and the heritability of such induced methylation changes has remained largely unknown. Using methylation-sensitive AFLPs, methylation changes at anonymous marker loci have been reported previously in response to heavy metal stress in hemp and clover (mostly hypomethylations; see Aina *et al.*, 2004), water deficit stress in pea roots (mostly hypermethylations; Labra *et al.*, 2002) and viral infection in tomato (Mason *et al.*, 2008). In tobacco, viral infection (Wada *et al.*, 2004) and several abiotic stresses (Choi & Sano, 2007) caused demethylation and the associated upregulated expression of stress-related genes. A rare example of transgenerational methylation effects has been documented in tobacco, in which virus-infected plants produced offspring with globally hypermethylated genomes, but with hypomethylated defense-related *R* loci (Boyko *et al.*, 2007). Our results show that environment-induced methylation changes, when they occur, are generally transmitted faithfully to the next generation.

The independence between genetic and epigenetic variation is a key feature of our experiment. However, it is likely that some low-level genetic variation is generated by stresses, via stress-induced transposon activity (Capy *et al.*, 2000; Slotkin & Martienssen, 2007) or increased somatic recombination rates (Molinier *et al.*, 2006). Both processes can, in fact, result from stress-induced demethylation, because DNA methylation functions to suppress transposons (Miura *et al.*, 2001) and might also shield genomic

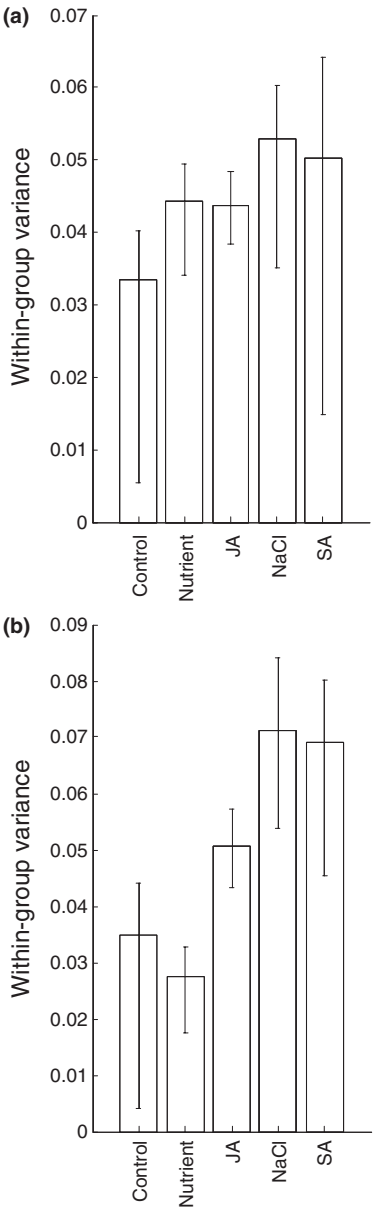

Fig. 2 Within-group variances in methylation epigenotypes among replicate plants (common dandelion, *Taraxacum officinale*), based on the presence/absence scores of 20 polymorphic methylation-sensitive amplified fragment length polymorphism (MS-AFLP) markers. For each group, the among-replicate variance in component scores from multidimensional scaling analysis (as plotted in Fig. 1) was calculated per component axis, and subsequently summed over the first three components. (a) Generation 1, grown in different environments. (b) Generation 2, grown in a common control environment. Error bars indicate 95% confidence intervals as obtained by jackknifing. JA, jasmonic acid; SA, salicylic acid.

regions from somatic recombination (Boyko *et al.*, 2007). It is therefore possible that some MS-AFLP variation in our experiment is associated with induced genetic variation. However, if such genetic modifications occurred, they were clearly not sufficiently pronounced to cause detectable AFLP variation, and thus it seems unlikely that induced genetic variation is responsible for the large MS-AFLP variation that was observed.

Within a single apomictic dandelion genotype, as in our experiment, the observed epigenetic variation is not associated with genetic variation. In natural populations that consist of multiple apomictic lineages, however, there could very well be a genetic component to environmentally induced epigenetic plasticity, because different genotypes may express different levels of plasticity. This is often observed with transgenerational phenotypic plasticity (Sultan, 1996; Galloway, 2001; Holeski, 2007). Genotypes with higher propensity to methylation alterations will show higher within-genotype epigenetic variability. In such situations, the relationship between genetic and epigenetic polymorphisms may be weak, if stress-induced methylation changes are random rather than targeted to specific loci. We detected stress-induced random methylation changes, but only within a subset of susceptible loci. The majority of MS-AFLP loci remained unaffected across generations and treatments. Variation in methylation stability between loci may result from differences in the underlying mechanisms that generate and maintain DNA methylation in different genomic contexts (Chan *et al.*, 2005). Some regions, notably some transposable elements and other repeats, are under the control of RNAi-guided DNA methylation, and these regions remain very stably methylated, but methylation in other contexts can be less strictly controlled (Richards, 2006; Henderson & Jacobsen, 2007; Teixeira *et al.*, 2009).

In comparing the number of methylation changes per experimental group, we found strong statistical evidence that more incidences of methylation change occurred in several treatment groups than in the control group. However, some individuals showed higher overall propensity to express methylation changes than others, and only the SA–control comparison stood up to a more conservative test that accounts for the fact that markers scored in the same individual are not independent. In this analysis, all other comparisons at best approached the subsignificance level in generation 2. Therefore, a cautious overall interpretation of these statistical results is that it is very probable that treatments other than SA trigger heritable methylation changes; however, this awaits confirmation in follow-up studies.

Our study demonstrates the fundamental point that ecological stresses cause autonomous DNA methylation variation, at a genome-wide scale, that is transmitted to offspring. This highlights the potential of epigenetic inheritance to play a role in evolutionary processes. However, important questions remain to be addressed. First, our study does not provide an insight into the stability of stress-induced methylation changes beyond the first generation.

Methylation changes that are caused by 5-azacytidine demethylation or by mutants that are deficient in methylation enzymes are often stable for many generations (Stokes *et al.*, 2002; Fieldes *et al.*, 2005; Akimoto *et al.*, 2007; Johannes *et al.*, 2009), but this remains to be demonstrated for changes that are induced by ecological stresses. Second, and importantly, the phenotypic consequences of the observed methylation changes are unknown. In our experiment, we observed that offspring traits were significantly affected by each of the four stress treatments, and these transgenerational effects were not simply attributable to treatment-related differences in seed biomass (KJF Verhoeven, unpublished). In some cases, maternal stress exposure enhanced the offspring responses when exposed to the same stress. For instance, all plants responded to nutrient stress by increasing the root : shoot biomass ratio (this allocation to root tissue is a well-known strategy to capture more of the limiting nutrient resources; Gedroc *et al.*, 1996), but the increase was significantly larger in offspring of nutrient-stressed mothers relative to the offspring of control mothers. Such seemingly adaptive transgenerational effects can persist for multiple generations (Whittle *et al.*, 2009) and our data are certainly consistent with an underlying epigenetic mechanism for this phenomenon. However, from our current data, it cannot be established whether the observed methylation effects and observed phenotypic effects are in fact causally related.

Apomictic reproduction in *Taraxacum* involves the production of unreduced egg cells via a modified process of female meiosis that circumvents normal meiotic I reductional division, and is further characterized by embryo and endosperm development without fertilization (Vijverberg & van Dijk, 2007). It is possible that these deviations from normal sexual reproduction affect the inheritance of methylation patterns, as epigenetic regulation may take place during these phases in sexual reproduction (Santos *et al.*, 2002; Slotkin *et al.*, 2009). It is currently unknown whether or not apomictic reproduction is unusually permissive to transgenerational inheritance of methylation marks, and thus whether our observations are specific to asexual reproduction or are more general. This issue could be addressed in future studies that also include sexual *T. officinale*, which co-occur with apomictic conspecifics in nature (van Dijk, 2003).

Striking heritable phenotypic variation is sometimes observed in the absence of detectable genetic variation, and epigenetic variation is a candidate mechanism to account for such observations (Richards *et al.*, 2008). Our work demonstrates that heritable DNA methylation variation is readily generated in apomictic dandelions. Depending on long-term stability and phenotypic effects, such variation might add to the heritable plasticity and to the evolutionary potential of apomictic lineages that have limited genetic variation.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Presence/absence of methylation-sensitive amplified fragment length polymorphisms (MS-AFLPs) detected in the experiment.

Fig. S2 Principal component analysis of gel fragment intensity scores of 115 methylation-sensitive amplified fragment length polymorphism (MS-AFLP) markers.

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