Individual variation in size and fecundity is correlated with differences in global DNA cytosine methylation in the perennial herb *Helleborus foetidus* (Ranunculaceae) [1]

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ABSTRACT

- *Premise of the study* Few studies have examined how epigenetic modifications of DNA may influence individual plant phenotypes and ecological processes in wild plant populations. We investigated natural variation in global DNA cytosine methylation and its phenotypic correlates in the perennial herb *Helleborus foetidus*.
- *Methods* We focused specifically on individual differences in size- and fecundity-related traits and use HPLC to quantify percent of total cytosines in the genome of young full-grown leaves that were methylated.
- *Key Results* About one third of all cytosines in *H. foetidus* genome were methylated. Methylation level differed significantly among individual plants (range = 26.4–36.6%; *n* = 60 plants), and this variation was significantly related to most size- and fecundity-related traits considered. Relatively hypomethylated plants bore more vegetative, reproductive and total ramets, produced more flowers, larger inflorescences and more seed-bearing follicles, and their ramets remained vegetative for fewer years before reproducing sexually, than relatively hypermethylated ones. Taken together, results revealed that individual differences in size and reproductive output were inversely related to global cytosine methylation.
- *Conclusions* Results confirm, in a natural scenario, the association between DNA methylation and size- and fecundity-related traits previously found by experimental studies. Variations in global cytosine methylation were predictably related to individual differences in sexual reproduction through significant effects on flower and fruit production, which might ultimately influence patterns of selection and population dynamics in this species. This study provides novel insights on the potential ecological significance of epigenetic heterogeneity in wild plant populations.

Key Words: DNA cytosine methylation; epigenetics; HPLC; *Helleborus foetidus*; individual variation; plant fecundity; plant size

INTRODUCTION

Epigenetic modifications may affect gene expression and individual phenotype without changes in the genome sequence. Validation of recent hypotheses postulating ecological or evolutionary roles for epigenetic processes (Bossdorf *et al.*, 2008; Jablonka and Raz, 2009) requires a deeper understanding of the magnitude, patterns and consequences of epigenetic variation in natural environments (Richards *et al.*, 2010; Richards, 2011). In the case of plants, inferences on the evolutionary and ecological significance of epigenetic processes have been mostly drawn from investigations in artificial environments on agricultural crop species or model species. A handful of studies on non-model plants in natural environments, however, support the notion that epigenetic processes may be important in ecology and evolution. Epigenetic modifications of DNA may influence ecological processes at the individual and population levels, including community productivity and stability, plastic responses to herbivory, colonization ability, population recruitment and inbreeding depression levels (Herrera and Bazaga, 2011, 2013; Latzel *et al.*, 2012, 2013; Richards *et al.*, 2012; Vergeer *et al.*, 2012; Herrera *et al.*, 2014). Nevertheless, considerable research effort on natural populations is still needed before the actual ecological significance of epigenetic variation can be confidently assessed.

Investigations on the ecological and evolutionary significance of epigenetic variation in plants have nearly always focused on cytosine methylation, the chief mechanism for epigenetic modification of DNA in plants (Finnegan *et al.*, 1998; Grant-Downton and Dickinson, 2005, 2006). Methylated cytosines occur at variable local densities throughout genic and intergenic spaces (including transposable elements) of nuclear plant genomes, and cytosine methylation plays significant roles in the regulation of gene expression, control of genomic integrity, and plant growth and development (Richards, 1997; Finnegan *et al.*, 2000; Cokus *et al.*, 2008; Lister *et al.*, 2008). Considerable evidence indicates that plant

exposure to biotic or abiotic agents may induce broad changes in the pattern (distribution across specific sites or regions in the genome) and level (proportion of total cytosines that are methylated) of cytosine methylation (Steward *et al.*, 2002; Chinnusamy and Zhu, 2009; Lira-Medeiros *et al.*, 2010; Verhoeven *et al.*, 2010; Herrera and Bazaga, 2013; Michalak *et al.*, 2013; Yanez Barrientos *et al.*, 2013). Next to nothing is known, however, on individual variation in global cytosine methylation level and its possible phenotypic correlates in wild plant populations. For example, treatment with methylation inhibitors often induces alterations in plant size, fecundity or time to flowering (Sano *et al.*, 1990; Finnegan *et al.*, 1996; Fieldes and Amyot, 1999; Tatra *et al.*, 2000; Kondo *et al.*, 2006), but it is unknown if the variation in these latter parameters that customarily occurs in wild plant populations bears some relationship to individual differences in cytosine methylation levels. This paper evaluates this possibility for the perennial herb *Helleborus foetidus*.

Previous studies exploring intraspecific variation in cytosine methylation level have usually relied on methylation-sensitive restriction endonucleases to assess cytosine methylation in anonymous 5'-CCGG-3' tetranucleotides randomly distributed over the genome (methylation-sensitive AFLP, or MSAP technique; Cervera *et al.*, 2002). Nevertheless, since the MSAP method probes an unknown, presumably small fraction of CCGG motifs in the genome, and methylated cytosines frequently occur also in other nucleotide contexts (particularly in plants; Gruenbaum *et al.*, 1981), cytosine methylation estimates obtained with restriction endonucleases might not satisfactorily reflect global cytosine methylation in the genome as a whole. This latter magnitude can be accurately estimated by chromatographic methods (Rozhon *et al.*, 2008; Kouidou *et al.*, 2010), but these techniques do not seem to have been applied so far to investigate DNA methylation in natural plant populations. We use here high-performance liquid chromatography (HPLC) to investigate natural intraspecific variation in global cytosine methylation and its possible

correlation with size- and fecundity-related traits in wild-growing plants of *Helleborus foetidus*. Results show that wild-growing individuals of this species differ significantly in cytosine methylation levels, and that individual differences are predictably related to variation in size- and fecundity-related traits of ecological relevance.

MATERIALS AND METHODS

Helleborus foetidus L. (Ranunculaceae) is an evergreen understory herb widely distributed in Western Europe. Plants consist of 1-20 distinct ramets, each of which eventually produces a single terminal inflorescence after 2-7 seasons of vegetative growth and dies after fruit maturation. Only a small number of each plant's ramets (range = 1–5) flower on a given season. Flowering mostly takes place during February-April, each inflorescence producing 25-75 flowers which are predominantly bumble bee-pollinated. Fruit maturation and seed shedding take place in June-early July.

In the spring of 2012, 20 widely spaced, inflorescence-bearing plants of *H. foetidus* were randomly selected in each of three sites in the Sierra de Cazorla, a forested mountain area in Jaén province, southeastern Spain. These 60 plants were the same studied by Herrera *et al.* (2013, 2014), where further details can be found. A sample of young leaves at full-grown stage was collected from each plant in similar dates and dried at ambient temperature in silica gel. The following fecundity- and size-related plant traits were recorded at the time of leaf collection: number of vegetative and reproductive ramets, diameter of inflorescences at the base, number of flowers per inflorescence, and age in years of flowering ramets as determined from counts of the characteristic annual scars left on stems by late-season leaves. The number and proportion (relative to initial ones, 'fruit set' hereafter) of follicles that developed into ripe fruits was later determined for each plant shortly before fruit maturation. Samples of ripe seeds were also collected, weighed individually, and a mean value for seed mass obtained for each plant.

Total genomic DNA was extracted from dry leaf samples using Qiagen DNeasy Plant Mini Kit. A 100 ng aliquot was digested with 3 U of DNA Degradase Plus™ (Zymo Research, Irvine, CA), a nuclease mix that degrades DNA to its individual nucleoside components. Digestion was carried out in a 40 µL volume at 37°C for 3 h, and terminated by heat inactivation at 70ºC for 20 minutes. Digested samples were stored at -20ºC until analysed. To allow for a statistical test of the significance of individual differences, two independent replicates of DNA hydrolyzate were prepared for each plant, and the 120 samples (60 plants x 2 replicates) were processed in randomized order. DNA cytosine methylation was determined for each sample by reversed phase HPLC with a spectrofluorimetric detection technique modified after Lopez Torres *et al.* (2011). Derivatization with 2-bromoacetophenone was conducted under anhydrous conditions, and HPLC quantification of 2'-deoxycytidine and 5-methyl-2'-deoxycytidine was accomplished in a Waters equipment (Waters 2695 Separations Module, Waters 2475 FDL) with a SunFire™ C18 column controlled by Empower™ software (Waters Corporation, Milford, Massachusetts, USA). Equipment and detector were stabilized for >3h, column temperature was maintained at 30ºC, and each derivatized sample was automatically diluted (1:1) with deionized water immediately prior to injection. Fluorimetric detection was carried out at excitation/emission wavelengths of 306/378 nm, respectively. Global cytosine methylation was estimated for each sample as 100 x 5mdC/(5mdC + dC), where 5mdC and dC are the integrated areas under the peaks for 5-methyl-2'-deoxycytidine and 2'-deoxycytidine, respectively. The position of each nucleoside was determined using commercially available standards (Sigma Aldrich), the method allowing also distinction from 5-hydroxi-methylcytosine, which was not detected in our samples. Repeatability of independent measurements for the same plant, estimated with the intraclass correlation coefficient, was 0.95 (95% confidence interval = 0.92–0.97).

To determine whether individual variation in global DNA cytosine methylation explained a statistically significant fraction of individual differences in size- and fecundity-related traits, separate linear mixed-effect models were fit for each trait. Plant traits were treated in these models as dependent variables, and global cytosine methylation as the single predictor. The 60 plants sampled were genetically structured, falling into one of two genetically distinct clusters as determined by applying Bayesian clustering to individual AFLP fingerprints (see Herrera *et al.*, 2014, for details). Since the heterogeneous genetic background of the sample might contribute to individual differences in genomic methylation (Herrera *et al.*, 2014), the possible confounding effect of genetic structuring on the trait-global methylation association analyses was corrected by incorporating genetic cluster as a random effect in the models (Price *et al.*, 2010; Herrera *et al.*, 2014). One plant yielded an unusually high cytosine methylation estimate (4.96 standard deviations greater than the sample mean), which persisted after two runs of independent analytical repetitions. To avoid spurious effects and conform with the normality assumptions of linear models, this extreme outlier (which otherwise was not an outlier for any of the traits considered) was omitted from trait-global methylation association analyses. Fecundity-related traits exhibiting non-normal distributions (flower and fruit production) were log-transformed to achieve normality. All statistical analyses were carried out using the R environment (R Development Core Team, 2012). The lme function from the nlme package was used to fit linear mixed-effect models.

RESULTS

Mean global cytosine methylation (± s.e.) in the sample of 60 *H. foetidus* plants studied was 29.5 ± 0.18 %. As depicted in Fig. 1, there was significant spread of individual values around the population mean (range = 26.4–36.6%; interquartile range = 28.9–30.2%). Between-plant heterogeneity in cytosine methylation level was statistically significant (chi-squared = 114, df = 59, $p << 0.001$, Kruskal-Wallis rank sum test). Statistical significance of

individual differences was unaffected by removal of the extreme outlier at the right tail of the distribution (Fig. 1) (chi-squared = 112, df = 58, $p << 0.001$).

Plants sampled differed broadly in all size- and fecundity-related traits considered, as denoted by the broad ranges of variation (Table 1). After statistically accounting for the different genetic backgrounds represented in the sample, individual variation in percent cytosine methylation explained significant proportions of individual variance in seven of the nine plant traits considered, namely number of total, vegetative and reproductive ramets, age of flowering ramets, inflorescence diameter, total number of flowers and total number of seed-bearing follicles produced (Table 1). The relationships with fruit set and mean seed mass were statistically nonsignificant (Table 1). *Helleborus foetidus* plants characterized by comparatively high cytosine methylation levels tended to be smaller (i.e., consisted of fewer vegetative, reproductive, and total ramets), had smaller inflorescences, produced fewer flowers and seed-bearing follicles (Fig. 2), and ramets remained more years in a vegetative state before entering the reproductive stage and producing an inflorescence.

DISCUSSION

DNA cytosine methylation is a common phenomenom in plants, able to alter gene expression in the absence of DNA sequence change. Global cytosine methylation measurements do not provide information on the genomic positions at which methylation occurs. Its variation, however, is commonly associated with modifications in the methylation status of specific genic and intergenic regions, which has functional consequences in terms of altered gene expression or genomic instability (McClintock, 1984; Messeguer *et al.*, 1991; Steward *et al.*, 2002; Feschotte and Pritham, 2007; Bonchev and Parisod, 2013). Mean global cytosine methylation for *H. foetidus* found in this study falls near the upper limit of the range previously reported for species of angiosperms (5–37%) (Messeguer *et al.*, 1991). Methylation level differed significantly among conspecific plants, and the range of

individual differences in *H. foetidus* was as broad or broader than the differences between some species (Messeguer *et al.*, 1991). To our knowledge, results for *H. foetidus* presented here provide the first direct demonstration for a wild-growing plant of significant individual heterogeneity in global DNA cytosine methylation. Since leaf samples were collected from all plants at similar dates and identical developmental stage, individual differences in cytosine methylation level can be interpreted as an indication that *H. foetidus* plants sampled were intrinsically heterogeneous in their epigenetic characteristics.

Observed variation among *H. foetidus* plants in global cytosine methylation level may reflect the combined action of ecological factors acting either on the plants themselves or on their immediate ancestors. Experimental studies have often documented short-term alterations in genomic methylation levels following plant exposure to pathogens, herbivores, low temperatures or water stress (Steward *et al.*, 2002; Lukens and Zhang, 2007; Peng and Zhang, 2009; Verhoeven *et al.*, 2010; Grativol *et al.*, 2012). Differential exposure of *H. foetidus* plants during their lifetimes to a variable constellation of stressing agents could therefore have led to observed intraspecific heterogeneity in cytosine methylation levels. For example, drought stress and low nitrogen availability are known to increase global cytosine methylation level in some species (Labra *et al.*, 2002; Bian *et al.*, 2013), hence the variable methylation level of *H. foetidus* plants might reflect the variable quality of their respective growing microsites with regard to water stress or nitrogen availability. The inverse relationship found here between global cytosine methylation and reproductive output is compatible with this interpretation, but the environmental data required to evaluate the hypothesized connection between global cytosine methylation and environmental characteristics of the plants' growing microsites are not available. Individual differences in global methylation might also reflect variable 'stressful memories' (Bruce *et al.*, 2007), or the differential exposure of the plants' ancestors to biotic and abiotic stressors operating in

the past. As in many other plants (Kakutani, 2002; Jablonka and Raz, 2009), most cytosine methylation marks are transgenerationally heritable in *H. foetidus* (Herrera *et al.*, 2013), and maternal differences in global cytosine methylation are probably preserved in the progeny.

Individual variation in global cytosine methylation was correlated with fitness-related, ecologically relevant traits in *H. foetidus*. Plants with relatively hypomethylated genomes were at a comparative size and fecundity advantage over those with relatively hypermethylated ones. In the long run, these individual differences in size- and fecundity-related traits should eventually translate into differential survival, longevity and seed production of individuals differing in methylation level. Cytosine methylation level was not found significantly related to fruit set and individual seed mass. In the study area, fruit set and seed mass of *H. foetidus* are often limited by pollen quantity and quality (Herrera *et al.*, 2001; Herrera, 2002), hence individual variation in these reproductive parameters is likely to depend more on frequency and quality (i.e., relative proportion of self and cross pollen) of pollination than on the amount of resources allocated by plants to sexual reproduction. Demonstrating that observed correlations between cytosine methylation and individual traits stem from a causal link between epigenetic and phenotypic features will require experimentation, e.g., through artificial manipulation of methylation level by application of methylation inhibitors (Fieldes and Amyot, 1999; Vergeer *et al.*, 2012). Nevertheless, the frequent experimental demonstration under artificial conditions of a causal association between global cytosine methylation and plant size and sexual reproduction (Sano *et al.*, 1990; Finnegan *et al.*, 1996; Fieldes and Amyot, 1999; Tatra *et al.*, 2000; Kondo *et al.*, 2006) strongly supports the view that differences in global cytosine methylation may contribute to generate the intraspecific heterogeneity in size and fecundity that characterizes populations of *H. foetidus* and many other plants (Herrera and Jovani, 2010, and references therein). If this causal link were proven by future experiments, the implications of epigenetic

variation in natural plant populations would be considerably broadened, since variance in fecundity may decisively influence patterns of selection, population recruitment and the temporal dynamics of populations (Gillespie, 1974; Harper, 1977). In conclusion, this study confirms in a natural scenario the association between global cytosine methylation and fitness-related traits previously found by experimental investigations. Individual variation in cytosine methylation was correlated with differential sexual reproduction, which might eventually lead to epigenetically-based patterns of selection and population dynamics in *H. foetidus*. These findings provide novel insights on the potential ecological significance of cryptic epigenetic heterogeneity in wild plant populations.

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TABLE 1. Statistical tests of the relationship between individual variation in global DNA cytosine methylation (proportion of total cytosines that are methylated) and size- and fecundity-related traits in the sample of *Helleborus foetidus* plants studied.

		Linear mixed model fit		
Individual trait	Range	Parameter estimate (± s.e.)	$F_{1,56}$	*p*-value
Number of vegetative ramets	0-21	–1.021 ± 0.460	4.93	0.030
Number of reproductive ramets	1-5	–0.261 ± 0.097	7.20	0.0096
Number of total ramets	1-24	–1.285 ± 0.487	6.95	0.011
Age of flowering ramets (yr)	2-6	+0.374 ± 0.109	11.71	0.0012
Basal diameter of inflorescence (mm)	7.2-18.0	–0.728 ± 0.257	8.03	0.0064
Flower production (total number of flowers) *	16-255	–0.080 ± 0.026	9.64	0.003
Fruit production (total number of seed-bearing follicles) *	19-440	–0.105 ± 0.030	12.06	0.001
Fruit set (percent of initial follicles eventually bearing seeds)	34.1-98.7	–0.017 ± 0.015	1.30	0.26
Mean seed mass (mg)	7.6-16.5	+0.270 ± 0.158	2.93	0.093

* Log_{10} transformed for the analysis

Figures

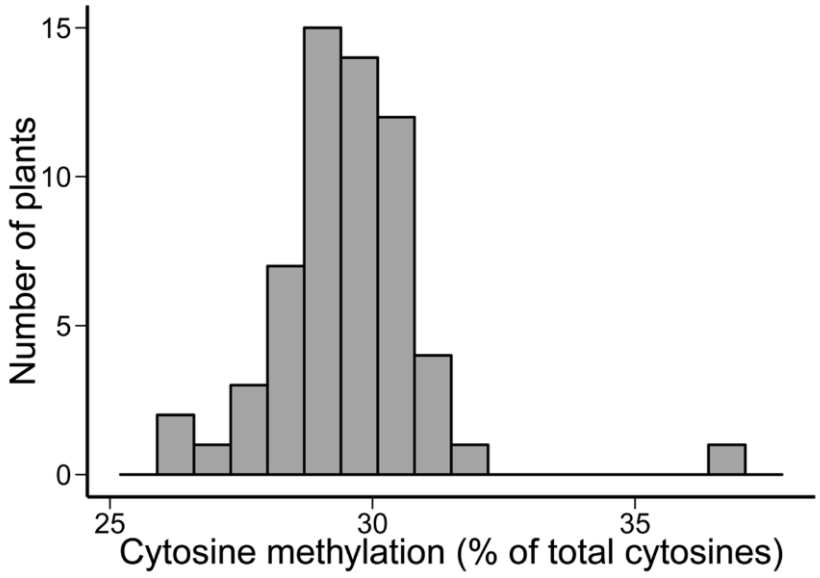

FIG. 1. Frequency distribution of global DNA cytosine methylation (percent of all genomic cytosines that are methylated) in the sample of *Helleborus foetidus* plants studied. Each plant was characterized by the mean value of two independent determinations.

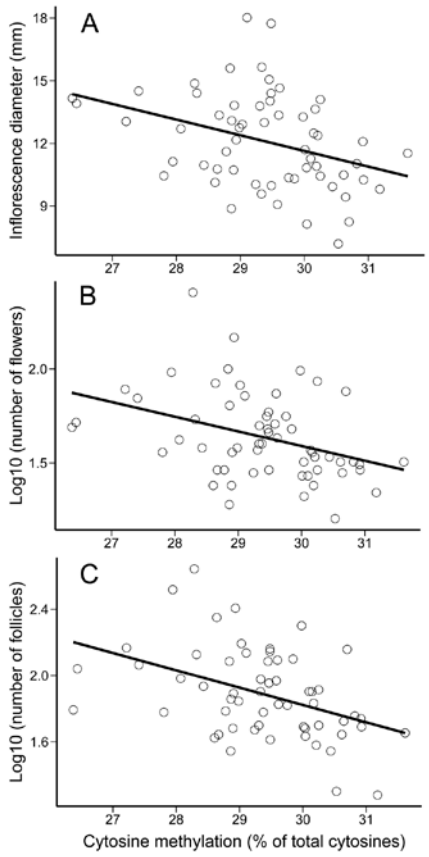

FIG. 2. Relationship between global DNA cytosine methylation (percent of all genomic cytosines that are methylated) and basal diameter of inflorescence (A), flower production (B) and follicle production (C), in the sample of individual *Helleborus foetidus* plants studied. Least-squared fitted regressions are shown only for reference, see Table 1 for results of significance tests based on linear mixed model fits to the data.