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Epialleles via DNA methylation: consequences for plant evolution

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In plants, naturally occurring methylation of genes can affect the level of gene expression. Variation among individuals in the degree of methylation of a gene, termed epialleles, produces novel phenotypes that are heritable across generations. To date, ecologically important genes with methylated epialleles have been found to affect floral shape, vegetative and seed pigmentation, pathogen resistance and development in plants. Currently, the extent to which epiallelic variation is an important common contributor to phenotypic variation in natural plant populations and its fitness consequences are not known. Because epiallele phenotypes can have identical underlying DNA sequences, response to selection on these phenotypes is likely to differ from expectations based on traditional models of microevolution. Research is needed to understand the role of epialleles in natural plant populations. Recent advances in molecular genetic techniques could enable population biologists to screen for epiallelic variants within plant populations and disentangle epigenetic from more standard genetic sources of phenotypic variance, such as additive genetic variance, dominance variance, epistasis and maternal genetic effects.

Heritable phenotypic variation within populations is the raw material for adaptive MICROEVOLUTION (see Glossary). The type and structure of the genetic variation underlying phenotypic traits determines the potential for, and rate of, an adaptive response to natural selection. Genetic causes of phenotypic variance are attributable to many sources. Mutations create allelic variation and recombination alters the genetic background or architecture in which alleles are expressed, creating changes in epistatic and pleiotropic interactions among alleles. More subtle sources of genetic variation that alter phenotypic variation also exist. Genes in the parental generation can affect offspring phenotypes, especially those genes that are associated with maternal provisioning of offspring [1,2]. Recently, variation among individuals in the degree of METHYLATION of genes has also been found to produce heritable, altered states of gene expression [3] and novel phenotypes. Genes with different degrees of methylation are termed EPIALLELES. Described plant epialleles affect ecologically important traits, including floral symmetry [4] plant and seed pigmentation levels [5] and pathogen resistance [6], as well as several developmental and phenological traits [3]. Phenotypes produced by such epialleles could have substantial fitness effects in the wild. The control, stability, genetic basis and phenotypic effects of epialleles are expected to have important implications for phenotypic variation and microevolution in natural plant populations, although these implications are currently unexplored.

A few evolutionary, developmental and quantitative genetic models have included epigenetic effects on the rate of adaptive phenotypic evolution (e.g. [7–9]). In these models, the term 'epigenetics' is used in a broad sense, subsuming many types of non-nuclear genetic phenomenon, genetic interactions during development and/or some types of maternal genetic effect into a single term. Epigenetic effects are either partitioned into within-generation (i.e. within individual genome or development) and across-generation (maternal) effects (e.g. [9]) or have combined spatial and temporal epigenetic effects that are expressed during development into a generalized epigenetic effect (e.g. [8]). In spite of these broad definitions of epigenetics, models of phenotypic trait evolution within populations consistently reveal that evolutionary trajectories are both sensitive to, and complicated by, epigenetic effects [7–9], suggesting that evolution in natural populations can have significant epigenetic influences.

By contrast, molecular genetics recognizes a narrow definition of epigenetics – the heritable modification of gene expression without a change in its nucleotide sequence, in which the modification is attributable to patterns of DNA methylation and/or histone modification associated with the gene (Box 1) [10]. Both methylation and/or histone modification can alter chromatin structure and yield a range of specific epigenetic consequences that

Glossary

Endogenous methylation: methylation resulting from the activity of cellular enzymes
Epialleles: alleles that differ from each other in the patterns of methylation of DNA nucleotides of the gene, rather than stable nucleotide mutations
Hemimethylation: methylation of one strand of the DNA double helix, usually as a result of DNA replication of a double helical molecule in which both DNA strands are methylated
Hypermethylation: dense methylation of nucleotides in a DNA sequence
Methylation: the addition of methyl groups as a cellular means of chemical marking of biological macromolecules, including DNA nucleotides and amino acids in proteins
Microevolution: small-scale evolutionary changes, such as changes in gene frequencies within populations that can result in changes in the average phenotype

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Box 1. The molecular basis of methylation

The two major types of methylation associated with epigenetic changes are detailed below. In this article, we discuss DNA methylation exclusively.

DNA methylation

Although all DNA nucleotides can be methylated, the most prevalent form of DNA methylation is cytosine methylation [12]. This is catalyzed by cytosine methyltransferase enzymes, which add methyl groups to the nucleotide cytosine. Ten genes that encode methyltransferases and that are involved in the initial establishment and maintenance of methylation patterns have been identified in the thale cress *Arabidopsis thaliana* [12,40]. These genes include those that encode the MET (methyltransferase), CMT (chromomethylase) and DRM (domain rearranged methylase) classes of enzymes [12,40].

Histone methylation

Methylation of the lysine at amino acid position 9 (K9) on the histone H3 protein is associated with gene repression, whereas methylation of lysine at position 4 (K4) on the same protein can activate repressed genes [41]. Recent work indicates that methylation of cytosine nucleotides at the DNA level is correlated with changes in histone H3 protein methylation states, suggesting that there is a strong relationship between these two types of methylation process at the cellular level [21,22].

can profoundly alter phenotypes, including paramutation [5], genomic imprinting [11] and gene silencing [12]. Here, we focus on DNA methylation changes in which the expression level of the gene is altered, which have important phenotypic effects and which have implications of for understanding microevolution in plant populations [13]. We do not discuss the effects of other methylation changes, such as histone methylation, which, unlike DNA methylation, cannot be stably inherited across generations There is no reference for this case. All the inheritance studies have focused on DNA methylation.

Epigenetics, methylation and epialleles in plants

Methylation

In some eukaryotic genomes, methylation of the nucleotides of genes is a major mechanism for epigenetic change [12]. In plants, cytosine is the most common base that is methylated, particularly that within the trinucleotide motif CXG (where G is guanine and X is any nucleotide). Studies of ENDOGENOUSLY METHYLATED DNA [14–16] and transgene silencing [17] both indicate that methylation is associated with repeated sequences.

Differences in methylation levels can lead to differences in gene expression, and include variation in transcriptional levels that confer phenotypic effects [18,19]. The gene expression variation associated with methylation could include stable variations in chromatin structure [20], and these constitute a mechanism for gene regulation [21,22]. Cytosine HYPERMETHYLATION of genes, for example, is primarily associated with gene silencing [12]. The genetic consequences of methylation are demonstrated in demethylation studies using *Arabidopsis thaliana*. In these experiments, plants were used that are unable to establish or maintain methylation patterns (either as a result of mutation or chemical induction). These studies reveal that the loss of methylation typically results in developmental aberrations, including changes in leaf structure, flowering time and floral structure [18,19]. An increase in transposon activity can also arise from the loss of methylation [23], increasing the rate of stable, insertional mutations.

Epialleles

Epigenetic alleles or epialleles differ in the number or distribution of methylated nucleotides at specific gene sequences, and plants with different epialleles can exhibit distinct phenotypes. Plant epialleles are usually mitotically and, in many instances, meiotically stable [3,19]. Replication of methylated DNA sequences results in HEMIMETHYLATION (i.e. only one strand of the DNA double helix is methylated). Full methylation of both strands is restored because cells can methylate nucleotide sequences based on the methylation status of the other DNA strand (Figure 1). This provides a mechanism for the inheritance of methylation patterns across both cell and organismal generations [3,19].

Several stable epialleles have been found in plants during the past few years, primarily as by-products of artificially induced mutagenesis screens (Table 1). The phenotypic effects of epialleles are best illustrated by studies of the *A. thaliana SUPERMAN* [14] and *FWA* [16] genes. *SUPERMAN* (*SUP*) encodes a transcriptional activator that defines inner floral whorl boundaries. Seven independently isolated *clark kent* (*clk*) alleles of *SUP* were identified by mutant screens and are relatively stable alleles of this locus [16]. DNA sequence analysis, however, revealed no nucleotide changes between these *clk* and wild-type *SUP* alleles [16]. The *clk* alleles were shown to be hypermethylated, and were designated as epialleles [16]. The case of the *FWA* homeodomain gene is similar to *SUPERMAN*. Here, mutational analysis identified a stable dominant *FWA* allele that confers a late-flowering phenotype. This allele is a gain-of-function epiallele, with stable demethylation of the *FWA* promoter resulting in ectopic expression of the gene [16]. In these and other cases

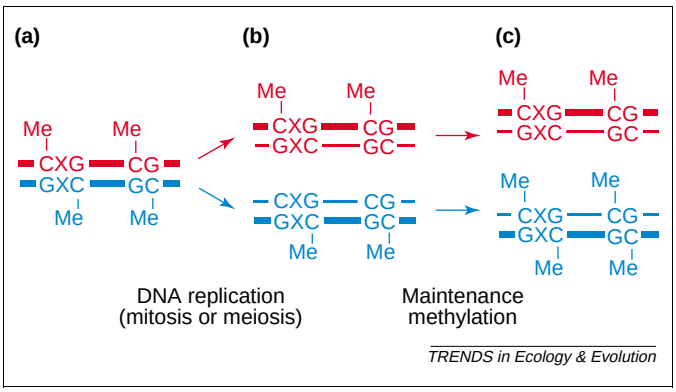

Figure 1. Inheritance of methylation states according to the classic Riggs–Holliday model of methylation state inheritance via maintenance methylation [45,46]. Replication of methylated DNA **(a)** results in hemimethylated progeny DNA. **(b)** Maintenance methylation enzymes, such as maintenance DNA methyltransferase, methylate cytosine **(c)** based on the hemimethylated states of symmetric CG or CXG motifs. The blue and red colors differentiate the two strands in the methylated DNA molecule and their respective replication products. The thick versus thin lines represent the original and replicated strands, respectively, after mitosis or meiosis. C and G represent cytosine and guanine, respectively, whereas X is any nucleotide. Other mechanisms of methylation inheritance are also known [47].

Table 1. Examples of plant epialleles

Species	Gene	Nature of change	Phenotypic trait	Refs
Arabidopsis thaliana	*SUPERMAN*	Mutagen	Floral morphology	[14]
	FWA	Mutagen	Flowering time	[16]
	PAI2	Spontaneous	Gene expression[a]	[15]
	BAL1	Hypomethylated line[b]	Disease resistance	[6]
Zea *mays*	*PI*	Spontaneous (paramutation)	Pigmentation	[42]
	B	Spontaneous (paramutation)	Pigmentation	[43]
	P	Spontaneous (paramutation)	Pigmentation	[44]
Linaria vulgaris	*Lcyc*	Spontaneous	Floral symmetry	[4]

[a]Differences in gene expression assayed for epialleles.
[b]Derived from a *ddm1* hypomethylation line.

of stable methylation-associated epiallelic inheritance, there is no evidence of gender bias in transmission.

Duplications and repeat sequences

Several examples of epigenetic silencing via methylation are associated with gene duplications and repeated sequences in the genome. For example, the *PAI* tryptophan biosynthetic gene family of many *Arabidopsis* ecotypes includes three unlinked genes (*PAI1–PAI3*). Natural inactive variants of *PAI2* were identified and are associated with hypermethylation throughout the locus [15]. However, hypermethylated *PAI2* epialleles are associated with a stable duplication polymorphism in the genome: only *Arabidopsis* ecotypes that have an inverted duplication of the gene at *PAI1* (referred to as the *PAI1–PAI4* locus) display the hypermethylation and associated transcriptional repression of *PAI2* [15]. The association of epigenetic changes with repeated sequences is also illustrated by epiallele formation and gene silencing accompanying polyploidization [24–26].

Naturally occurring epialleles

Several naturally occurring epialleles have also been described. In toadflax *Linaria vulgaris*, radially symmetric floral mutants of the wild-type bilaterally symmetric flowers exist in nature [4]. Molecular genetic studies revealed that the radial forms result from epialleles of the *CYCLOIDEA* gene, which encodes a transcriptional activator causing the development of floral asymmetry [4]. In corn *Zea mays*, naturally occurring epiallelic changes are described in the *R, B, Pl* and *P* pigmentation genes that result in altered seed and vegetative tissue pigmentation [5]. Several of these pigmentation epialleles are associated with altered methylation states that are produced by paramutation [5].

Finally, a recent genetic mapping study of *A. thaliana* demonstrated natural variation in methylation levels at rDNA loci found in nucleolar organizing regions (*NOR*) [27]. Additionally, accessions of *A. thaliana* show considerable among-accession methylation-sensitive polymorphism, with up to 34% differences in methylation-sensitive variation of amplified fragment length polymorphism (AFLP) markers [28]. This variation is not due to nucleotide sequence variants, but to the amount of methylation. Although all the epialleles described above are naturally occurring, there are currently no data available about the frequency of epiallelic variants from natural populations.

Population genetic consequences of epialleles

If the data from model plants are indicative of the possible phenotypic effects of epialleles in nature, then epialleles are expected to have ecologically significant fitness effects in the wild. They can directly contribute to heritable variation within populations and, when stably inherited across generations, will behave in a similar way to sequence-based allelic variation with respect to phenotypes and fitness effects. If present in wild populations, described epiallelic phenotypes could influence mating system or pollination syndromes (floral symmetry [4]), physiology, herbivory and seed predation (plant and seed pigmentation levels [5]), patterns of disease (pathogen resistance [6]) and life-history trait evolution (developmental and phenological traits [3]). Therefore, heritable epialleles will influence evolution in wild plant populations through their effects on both phenotypic trait distributions and fitness. Because methylation changes can regulate the amount or degree of gene expression, epialleles can produce continuous variation in phenotypes rather than producing discrete phenotypic classes. For example, continuous variation exists in the degree of radial symmetry of *L. vulgaris* flowers, where the degree of radial symmetry increases with increasing methylation density of the *CYCLOIDEA* gene [4]. Radial phenotypes of toadflax are likely to have substantial negative fitness effects with respect to pollinator efficiency and seed set in the field. Interestingly, radial phenotypes similar to those first collected by Linnaeus remain in low frequency in the original wild populations [4].

Do epialleles contribute to heritable variation in nature? Current methods of estimating genetic variation underlying phenotypic variation in natural populations [e.g. nucleotide sequence variation, quantitative genetic analyses, and quantitative trait loci (QTL) mapping approaches] differ in the extent to which their estimates could be affected by, or can even distinguish, the effect of epialleles. Molecular population genetic studies of single nucleotide polymorphisms (SNPs) will not identify methylation variants, because methylated nucleotides cannot be detected by standard DNA sequencing technologies. By contrast, quantitative genetic analyses [1,2] use phenotypic trait correlations among relatives to calculate heritabilities, h^2, and methylated epialleles could play a role here. By definition, $h^2(V_a/V_p$, where V_a is additive genetic variance and V_p is total phenotypic variance. If epialleles contribute to the variance in trait value, they will affect V_a; if they contribute to variance in developmental stability, they will affect V_p. Phenotypic variance

caused by epialleles could alter one or both variance components, affecting h^2 estimates. In QTL studies, estimates of gene location from associations between marker alleles and phenotype are made to determine the number of genes contributing to a phenotype. Unless the identified genes are subsequently cloned, sequenced and tested for methylation states, one cannot distinguish the effects of epialleles from those resulting from base sequence changes between parents. Clearly, currently applied methods of estimating genetic variance within plant populations do not shed much light on the magnitude or frequency of the contribution of epialleles to heritable phenotypic variation.

Evolutionary dynamics of epialleles

To assess the importance of epialleles in the evolutionary process, their frequency and stability in natural populations must be addressed. The stability of epialleles and hemimethylation changes remain unknown. Although it is clear that these changes can be inherited over several generations in the laboratory [4,14,16], it is unclear whether they are stable over large numbers of generations that correspond to evolutionary time. If loss of methylation occurs at a higher rate than that of reverse nucleotide substitution rates over evolutionary time, this should result in a higher reversion rate for epiallele-associated phenotypes.

We propose that the evolutionary dynamics of epialleles could differ from those of sequence-based variation in two ways: (i) If the rate of formation of new epialleles differs from rate of stable nucleotide mutations, μ, and/or the average effects of an epiallele or the distribution of these effects differs from those of stable mutations, then rates of evolutionary change could be affected; and (ii) By contrast, if epialleles persist for multiple generations, but are not permanent, then they could play a transitory role in adaptation if the rate of formation of new methylated epialleles is (μ. If h^2 is generated faster by epialleles than through stable nucleotide mutations, and the sizes of the two heritable effects are equal, then phenotypic evolution could proceed more quickly through epiallelic variation than through stable mutation, although both will occur simultaneously. However, if epialleles decay with generational time, then sequence variation will eventually replace epiallelic variation as the heritable basis of the adaptation.

Even if epialleles only persist for tens of generations, they could still have significant evolutionary effects. First, because epiallelic phenotypes can be less extreme than those caused by mutations in nuclear genes, epiallelic variants that modulate the degree of gene expression might experience less intense selection than do sequence mutations variants that result in loss-of-function alleles [29]. Thus, the phenotypes produced by different epiallelic could explore novel habitats and could facilitate co-adaptation with other genes in the short term. Second, if epialleles are segregating in a population, they could temporarily 'protect' the genome during periods of rapid environmental change if methylation can modulate the phenotypic effect of a gene whose historical phenotype becomes deleterious in a novel environment. Alternatively, the methylated form of the gene could have higher fitness by decreasing the penetrance of the gene in the phenotype (i.e. damping the phenotypic expression). Finally, it has been proposed that, across generations, mutations can accumulate in methylated sequences. Thus, sequence mutants with novel phenotypic effects can arise and eventually be uncovered if demethylated [29], functioning in a manner analogous to heat shock proteins (e.g. *HSP90*), which buffer the expression of morphological variation and mask cryptic genetic variation [30,31].

Interestingly, even in plant populations that are fixed for a single nuclear gene, individuals could express phenotypic variation for traits associated with the monomorphic gene if methylation produced epiallelic variants with phenotypic effects. These epimutations could be expressed in populations that undergo little outcrossing, are completely selfing or clonal. Epialleles could also explain a portion of the observed among-line phenotypic variation in large-scale mutation accumulation experiments with *A. thaliana* [32,33]. Thus, epialleles can contribute to the standing phenotypic variance within plant populations on several levels, but supporting data are currently lacking.

Control of methylation in natural populations

These speculations about the population-level effects of epialleles beg the question of whether the propensity to methylate is optimized in natural populations. In contrast to its possible positive fitness effects via modulation of the dosage of particular genes, uncontrolled methylation of the genome would have obvious negative fitness effects. Similarly, methylation of a gene might not produce an optimal phenotype, resulting in an epimutational load – the epiallelic equivalent of mutational load [34,35]. There is likely to be genetic variation in the propensity to methylate within populations and natural selection can be expected to favor the methylation level that provides the highest fitness in the long term. However, the methylation optimum might change as environmental conditions change. How quickly methylation propensity or the ability to demethylate can and will evolve to match the environmental optimum within populations is also unexplored. Mutant alleles of methylation genes such as *DDM1* are found in viable individuals, suggesting that variation in genes controlling methylation is possible.

Research directions

Assessing the importance of methylated epialleles in plant population genetics and evolution requires the determination of: (i) the extent of variation in methylation patterns among individuals within a population; (ii) the degree to which methylation patterns affect phenotypes; and (iii) the extent to which natural methylation variants are stably inherited. In laboratory studies, focusing on genes that already have known epialleles, such as *FWA* or *PAI*, or in loci that have repeat sequence structures that are prone to methylation, might be a good starting point for this exploration. If conducted in conjunction with more general screens for the presence of other methylated genes, such studies could provide the foundation for

understanding the extent to which epialleles underlie ecologically and evolutionarily relevant variation.

Several techniques are currently available for assaying gene-specific methylation differences and could be useful in population-level studies. Restriction fragment length polymorphism (RFLP) and methylation-sensitive AFLP analyses [28] using methylation-sensitive and insensitive restriction enzyme isoschizomers can establish whether RFLPs and AFLPs are the result of nucleotide polymorphisms or methylation differences at restriction sites [27]. Additionally, bisulfite sequencing, in which genomic DNA is treated with bisulfite to convert unmethylated cytosines to uracil, could provide detailed methylation sequences of genes [36,37]. Several assays that utilize high-throughput genomics technologies enable the simultaneous assay of multiple genes [38,39]. However, there are pitfalls to many of these approaches. None are particularly easy to use, compared with current assays of sequence variation, and methylation patterns might differ in different tissues. However, the technical potential exists to assess methylation pattern differences between individuals and, thus, estimate the levels of methylation-associated epiallelic diversity [27,28] and its related phenotypic diversity. Understanding the phenotypic effects of epialleles and their genetic control and stability will provide a platform for the development of new evolutionary models and fruitful, collaborative research between molecular geneticists and plant population biologists.

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