Research review

Epigenetics and plant evolution

Author for correspondence:
Jonathan F. Wendel
Tel: +1 515 294 7172
Fax: +1 515 294 1337
Email: jfw@iastate.edu

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Ryan A. Rapp and Jonathan F. Wendel

Department of Ecology, Evolution and Organismal Biology, Iowa State University, Ames, Iowa 50011, USA

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Summary

A fundamental precept of evolutionary biology is that natural selection acts on phenotypes determined by DNA sequence variation within natural populations. Recent advances in our understanding of gene regulation, however, have elucidated a spectrum of epigenetic molecular phenomena capable of altering the temporal, spatial, and abundance patterns of gene expression. These modifications may have morphological, physiological, and ecological consequences, and are heritable across generations, suggesting they are important in evolution. A corollary is that genetic variation *per se* is not always a prerequisite to evolutionary change. Here, we provide an introduction to epigenetic mechanisms in plants, and highlight some of the empirical studies illustrative of the possible connections between evolution and epigenetically mediated alterations in gene expression and morphology.

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Introduction

One of the central tenets of biology is that the evolutionary process requires genetic variation upon which to act. This fundamental neoDarwinian notion permeates all of biological thought as well as biology education. Probably, most biologists assume that the power and primacy of natural selection is dependent on the existence, amount, and structuring of standing genetic variation. Moreover, most evolutionary theory derives from this fundamental assumption. If genetic variation forms the fodder for natural selection, it logically follows that genetic uniformity will severely constrain the evolutionary potential of a given population or species. Yet recent insights into epigenetic phenomena have revealed how heritable variation need not be sequence-based; instead, novel permutations of spatial and temporal patterns of gene expression may be achieved via a suite of epigenetic mechanisms, even in the complete absence of genetic variability. This realization suggests that we re-examine basic assumptions and consider what is meant by 'variation' and whether it really needs to be 'genetic' for there to be phenotypic change that is potentially visible to natural selection.

The famous concept of 'inheritance of acquired characteristics', so intimately associated with Lamarck, was also recognized by Charles Darwin himself:

> Some authors use the term 'variation' in a technical sense, as implying a modification directly due to the physical conditions of life; and 'variations' in this sense are supposed not to be inherited; but who can say that the dwarfed condition of shells in the brackish waters of the Baltic, or dwarfed plants on Alpine summits, or the thicker fur of an animal from far northwards, would not in some cases be inherited for at least a few generations? (Darwin, 1859)

These comments were written nearly a century and a half ago, yet the scale and scope of the problem and the mechanistic underpinnings of 'acquired characteristics' have only recently

begun to be illuminated. The purpose of the present review is to draw attention to these epigenetic possibilities, commenting on areas of active research that are altering our views of the evolutionary process. One of the key points we make is that epigenetic alterations appear to be especially prevalent during the formation of interspecific hybrids and polyploids (Liu & Wendel, 2003; Levy & Feldman, 2004; Pires *et al.*, 2004; Wang *et al.*, 2004a). These organism-level processes are exceptionally common in angiosperms (> 70%) as well as pteridophytes (> 95%) (Rieseberg, 1997; Cronn & Wendel, 2004; Hegarty & Hiscock, 2005; Wendel & Doyle, 2005). In his seminal volume *Plant Speciation*, published a quarter of a century ago, Verne Grant devoted 13 of 35 chapters to hybridization and polyploidy (Grant, 1981). Modern plant biologists may place even more emphasis on genomic mergers, as evidenced by the recent special edition of this journal, where 10 of 14 papers focused on polyploidy or hybridization as prominent players during speciation (Rieseberg & Wendel, 2004). From the standpoint of the present discussion, hybridization and polyploidy are significant not only because of conventional explanations involving novel allelic and genic combinations resulting from the fortuitous merger of divergent genomes, but also because of the bewildering array of unexpected epigenetic outcomes and possibilities.

Definitions and mechanisms

The term 'epigenetics' generally is used in reference to a class of heritable molecular events involving a wide variety of protein complexes and regulatory mechanisms that do not involve change in the DNA sequence (Bender, 2002). Jablonka & Lamb offer an etymological history of the term 'epigenetics', from its original coining by Conrad Waddington in the 1940s as a descriptor of protein-by-gene developmental interaction, to its current use as 'epigenetic inheritance' (Jablonka & Lamb, 1994). In the context of this article, we take the broad view of the application of the term 'epigenetics' to mean the alteration of phenotype, morphological or molecular, *without* change in either the coding sequence of a gene or the upstream promoter region.

This definition is operational as opposed to mechanistic, in that it doesn't require *a priori* demonstration of methylation, histone deacetylation, or any other epigenetic mark. Instead, it is an umbrella application of the term 'epigenetic', accommodating not only these molecular mechanisms at some target site under discussion, but also stoichiometric phenomena that underlie and may alter gene expression and various types of transcription factor interactions of protein elements in complexes with DNA regions (Birchler *et al.*, 2005; Veitia, 2005). The advantage of this more encompassing definition is that, in most cases of epigenetic inheritance, the actual underlying mechanism is not known, yet there is a very real phenomenon (altered expression or phenotype) worthy of description. The alternative, traditional application is narrower, referring usually to a single genetic locus and one or more locally operating molecular mechanisms that suppress gene expression. What falls into this narrowly circumscribed molecular window is a class of well-described and not-so-well-described machineries regulating both normal and aberrant mRNA expression in eukaryotic cells.

DNA methylation

The best-described epigenetic mechanism is DNA hypermethylation, or the predominant marking of CpG, CpCpG, CpHpHp, and CpNpG motifs in DNA (Finnegan *et al.*, 1998; Robertson & Wolffe, 2000; Fulnecek *et al.*, 2002). Methylation of the 5′-carbon of the cytosine aromatic ring results in transcriptional silencing within the methylated promoter region, and is strongly tied to another phenomenon, the modification of histones and the condensation of chromatin (Chiang *et al.*, 1996). The sole methyl donor for all eukaryotes, *S*-adenosylmethionine (Chiang *et al.*, 1996; Rocha *et al.*, 2005), provides the methyl group essential for such *Arabidopsis thaliana* enzymes as *CHROMOMETHYLASE 3* (*CMT3*) and *DOMAINS REARRANGED METHYLASE 2* (*DRM2*), responsible for marking of CpNpG motifs, and *METHYLTRANSFERASE 1* (*MET1*), the marker of CpG islands (Kankel *et al.*, 2003). The methylation marking system facilitates not only the silencing of genes, but also the silencing of transposons otherwise capable of genomic proliferation, as evidenced by double knockouts of the *CMT3* and *MET1* loci, which release from suppression the CACTA family of transposable elements (Kato *et al.*, 2003). Studies using *Arabidopsis* show that knockouts of helicases involved in methylation (*DOMAINS REARRANGED 1*, *DDM1*) as well as methyltransferase knockouts slowly release genes from silencing as mitotic and meiotic divisions progress, but that, otherwise, phenotypes generated by methylation or methylation lesions themselves segregate in a Mendelian fashion during crosses (Finnegan *et al.*, 1998). Although a demethylating enzyme is known from animal systems (Bhattacharya *et al.*, 1999), no homolog has been discovered in plants.

From an evolutionary perspective, methylation is relevant because of its local, immediate effects on gene expression and its longer-term, more indirect consequences resulting from suppression and release of transposable elements (Kumar & Bennetzen, 1999; Bennetzen, 2000; Kashkush *et al.*, 2003; Levy & Feldman, 2004; Madlung *et al.*, 2005). In addition, cytosine methylation, an epigenetic phenomenon, may itself be mutagenic, in that methylated cytosines have a high rate of spontaneous deamination to thymidines (Gonzalgo & Jones, 1997).

Histone modifications

Covalent modification of histone proteins is another primary mechanism of controlling gene expression. Histones comprise a family of highly conserved globular proteins whose N-terminal tails reside on the surface of the nucleosome octomer, exposed

for chemical modifications. Histones provide the primary packing structure for chromosomal DNA in eukaryotes with each histone wrapped in ~146 bp of DNA to form the nucleosome, and are structured from two copies each of four different subunits: H2A, H2B, H3, and H4, whose residues are subjected to an array of covalent modifications. In fact, the variety of covalent histone modifications is so extensive that researchers have suggested a histone code capable of specifying the chromatin state, and thus the transcriptional state, of genes and chromosomal stretches (Turner, 2000; Jenuwein & Allis, 2001).

The best-studied histone modification is N-terminal tail acetylation, where ε-amino groups of phylogenetically conserved lysine residues are acetylated, thereby reducing the positive charge of the histone surface. It was originally postulated that this decrease in positive charge reduces the affinity of the histone for DNA, thereby increasing access of the transcriptional machinery to enhance transcription; the currently favored hypothesis suggests that these additions act as signals for silencing in the histone code (Jenuwein & Allis, 2001). Conversely, hypoacetylation (= deacetylation) results in suppression of expression as the chromatin is condensed from euchromatin to heterochromatin (Turner, 2000). Tian *et al.* have demonstrated that overexpression of a deacetylase in *Arabidopsis* results in ectopic expression of certain pathways, and repression in others, in a tissue-dependent, promoter-governed manner, showing that acetylation states can serve as regulatory elements across various developmental stages (Tian *et al.*, 2005).

Other histone modifications appear to be common and are epigenetic marks for silencing, such as methylation of H3 lysine9 residues (Tariq & Paszkowski, 2004). The elucidation of a 'histone code' has been driven by the observation that one epigenetic mark begets another. Enzymatic proteins catalyzing histone modifications have binding domains capable of recognizing modified histone residues. Most prominent are the *bromo* domain for acetylation and the *chromo* domain for methylation. These recognition motifs, when coupled to enzymatic domains, seek out epigenetically marked histones and target deacetylase or methyltransferase activity to enable a cascade of histone modifications up and down the chromosome region (Rusche *et al.*, 2003). Such reinforcement is necessary for chromatin to be effectively condensed from euchromatin to heterochromatin (Turner, 2000; Jenuwein & Allis, 2001; Rice & Allis, 2001; Grewal & Moazed, 2003); it is unclear whether DNA methylation drives histone methylation or the reverse, or if there is generalized cross-talk between the two (Jenuwein & Allis, 2001; Grewal & Moazed, 2003). It is clear, however, that there is a link between the two. For example, non-CG methylation requires an intact histone methyltransferase, and loss of CG methyltransferase activity can entail demethylation of histones from silenced tracts of DNA (Johnson *et al.*, 2002; Tamaru *et al.*, 2003).

Additional histone modifications have been discovered and described as having epigenetic implications (Jenuwein & Allis, 2001), such as phosphorylation and ubiquitination, yet their roles and regulation are less clear. It is worth emphasizing that these various epigenetic modifications, methylation, acetylation, phosphorylation, ubiquitination and DNA methylation, are interconnected at the regulatory level and comprise a set of mechanisms whose concerted efforts control the condensation level of chromatin. Moreover, the next phenomena described, involving small RNA molecules, may be involved in DNA and histone modifications under certain conditions (Grewal & Moazed, 2003).

Micro RNA (miRNA) and small interfering RNA (siRNA)

The journal *Science* hailed small RNAs as the breakthrough of 2002 (Couzin, 2002) and, since then, our appreciation of the significance of these cryptic oligonucleotides has gone from negligible to panoramic. These tiny RNAs, 21–24 nucleotides (nt) in length, span all eukaryotic kingdoms in their distribution and in some cases have been shown to control conserved homologous pathways. They also serve as molecular signposts to identify targets of silencing: retroviruses, retrotransposons, aberrantly expressed genes, and normal developmental loci.

Small RNAs are divided into two classes in plants (miRNA and siRNA), based on their structure and origin, and the pathways in which they participate. First discovered in *Caenorhabditis elegans*, micro RNAs (miRNA) comprise a class of small RNA molecules encoded by eukaryotic genomes as key regulators of development (Lee *et al.*, 1993). In plants, these molecules control polarization during abaxial/adaxial leaf determination and organ ontogeny during floral development, regulate heterochronic shifts, and influence many other developmental pathways (Kidner & Martienssen, 2005). Micro RNA loci are transcribed and processed into pre-miRNA transcripts 80–100 bp long, which contain a stretch of self-complimentarity capable of forming hairpin loops, with two or three mismatches in the stem portion. The Dicer-like (*DCL*) family of proteins (e.g. *DCL1*) (Ketting *et al.*, 2001) cleaves these hairpins into final miRNAs 21–24 nt in length suitable for binding to the *PAZ* single-stranded binding domain (Lingel *et al.*, 2003) of the protein *ARGONAUTE* (Carmell *et al.*, 2002). As the miRNA–protein complexes hybridize to their complimentary targets in the transcript pool, they facilitate degradation of the target transcript.

The second class of small RNA is termed 'small interfering RNA' (siRNA). These RNAs arise from cleavage (described below) of a diverse pool of double-stranded RNAs, and appear to inhibit processing of 'foreign' DNA, such as retroviruses and endogenous retrotransposons. For siRNAs, RNA perfect match duplexes form between homologous target mRNAs and the siRNA, thus serving to direct gene silencing. Such perfect match duplexes of these DNA elements are generated by RNA-dependent RNA polymerases. *ARGONAUTE*-type proteins bind the siRNA-target, double-stranded RNA fragments and, along with the RNA-induced silencing complex (RISC), degrade the target mRNA pool. The proliferation of siRNA elements

from the degradation pool allows targeting and methylation of the genomic loci responsible for their transcription (Gendrel & Colot, 2005).

Location, location, location: chromosomal territories and matrix/scaffold attachment regions (M/SARs or MARs)

Spatial location within the nucleus is increasingly being recognized as an important determinant of expression (Taddei *et al.*, 2004). Classical positional effects have been recognized for decades, but only recently have finer-scale cytogenetic studies revealed the importance of spatial location within eukaryotic nuclei for gene expression (Fransz *et al.*, 2002; Bode *et al.*, 2003). These studies have shown that regions of chromosomes are spatially arranged in a nonrandom fashion, with nongenic DNA and repetitive elements preferentially localized to the interior, and euchromatic, genic DNA biased toward the periphery (Fransz *et al.*, 2002). The regions in the nucleus occupied by chromosomes in this biased arrangement are termed 'chromosomal territories'. In *Arabidopsis*, it has been shown that the centers of chromosomal territories contain hypermethylated DNA and deacetylated histones – hallmarks of silencing (Fransz *et al.*, 2002). Also confined to the center of chromosomal territories are the 45S loci of the nucleolar organizing region (NOR) for chromosomes 4 and 2 and the 5S loci marking the centromeres of chromosomes 4 and 5 – transcriptionally inactive regions. Thus, the relative expression level of any given gene *may* be influenced by its physical location with respect to nongenic and repetitive DNA, as opposed to a model wherein gene expression is exclusively determined by local promoter and enhancer effects. Intra- and interspecific variation in the physical location of genes and repetitive DNAs, and their interspersion patterns, thus are likely to profoundly impact gene expression in evolutionarily significant ways.

In addition to dispersion patterns of genic and repetitive DNA along individual chromosomes, the spatial arrangement of chromosomes within the nucleus has effects on gene expression. Tanabe *et al.* (2002) demonstrated relative conservation of the positions of chromosomes 18 and 19 within the nucleus among eight different primates representing roughly 30 Myr of higher primate evolution (Tanabe *et al.*, 2002). Also noted was the higher expression of loci closer to the center of the nucleus, with chromosome 19 being gene-dense and interior to the gene-poor chromosome 18. Others have similarly noted a high correlation between the nuclear location of genes and their expression levels (Kozubek *et al.*, 2002).

Other components of nuclear architecture, such as matrix/scaffold attachment regions (M/SARs, here referred to simply as MARs), may also influence gene expression (Rudd *et al.*, 2004). MARs are specific DNA sequences shown to bind the nuclear matrix (a proteinaceous scaffold in the nucleus) *in vitro*. In living systems, these sequences can protect transgenes from transcriptional silencing, and may help mediate the expression of genes (Allen *et al.*, 2000). These positional effects of genes relative to MARs further add to the complexities of chromosome–nucleus interactions and gene expression (Bode *et al.*, 2003). Additionally, it should be noted that some studies have revealed no positional effects. In these cases, truncated insertions, tandem transposons or otherwise deficient insertions were the primary reason for expression variation. This may suggest that different plant lineages have different mechanisms, or respond differentially to the same mechanism when positional cues are involved in transcription (Schubert *et al.*, 2004).

The foregoing condensed overview introduces an impressive diversity of regulatory mechanisms that may directly impact gene expression but which do not involve changes in the genic DNA sequence or promoter regions. Thus, the evolutionary process entails more than the steady but stochastic progression of nucleic acid substitutions in protein-coding regions. We have come a long way from the neoDarwinian conceptualization of genes as 'beads on a string', and have arrived at a point where this immensely powerful framework can be enriched by incorporating epigenetic phenomena and our blossoming understanding of heritable epigenetic variation. To illustrate several of the evolutionarily relevant dimensions of this undertaking, we offer in the following a synopsis of salient evolutionary studies where evolution, molecular or morphological, has been mediated by the epigenetic phenomena described above.

Highpoints in the epigenetic, evolutionary landscape

Tools of the epigenetic trade

Techniques routinely used in evolutionary surveys to assay variation, such as amplified fragment length polymorphisms (AFLPs), random fragment length polymorphisms (RFLPs), isozymes, DNA sequencing, microsatellites, and simple sequence repeats (SSRs), do not routinely assess epigenetic variation. Epigenetic variation 'slips under the radar' of these molecular tools, surfacing only when specifically assayed by other tools, such as methylation-sensitive AFLP (MS-AFLP) analysis or studies of gene expression. Despite the relatively recent realization that epigenetic variation may be important, existing studies document interindividual epigenetic variation in many different plant groups (Ashikawa, 2001; Knox & Ellis, 2001; Cervera *et al.*, 2002; Riddle & Richards, 2002; Liu & Wendel, 2003; Wang *et al.*, 2004b). These studies collectively demonstrate that epigenetic variation is common in plants, increasing the likelihood that it has effects potentially visible to natural selection.

Polyploidy and gene expression

Studies of the potential evolutionary significance of epigenetic phenomena have been of two kinds, one involving genomic

surveys of epigenetic marks and expression patterns, and the other focused on specific phenotypes, such as flowering time and floral symmetry. These two types of studies are often interconnected, of course, in that global epigenetic repatterning may have overt phenotypic consequences.

Chief among these global repatternings is change of DNA methylation upon hybridization and/or polyploidization. Methylation patterns can be radically altered by these repatterning processes, as exemplified by studies in *Brassica*, *Arabidopsis*, *Triticum*, and *Oryza*. In these species, methylation-sensitive AFLP analysis documented widespread changes in genomic methylation, including changes in genes, as indicated by methylation-sensitive AFLP analysis using only cDNAs (Song *et al.*, 1995; Liu *et al.*, 1998a,b, 1999; Shaked *et al.*, 2001; Liu & Wendel, 2002, 2003; Madlung *et al.*, 2002).

Interestingly, Liu *et al.* found that methylation reprogramming does not always accompany allopolyploidization, as MS-AFLPs in synthetic *Gossypium* (cotton) tetraploids and hexaploids showed additive methylation when compared to the diploid and tetraploid progenitors (Liu *et al.*, 2001). Why it is that differences in methylation reprogramming should exist among different plant orders is unclear, but this observation underscores the general point that different groups of plants may have varied responses to similar genomic challenges.

One of the more recent lessons about epigenetic responses to hybridization and polyploid formation comes from the genus *Spartina*. Hybridization of a European native hexaploid *Spartina maritima* and the American hexaploid *Spartina alterniflora* has occurred twice in the last century, resulting in two F_1 hybrids that are genetically highly uniform, *Spartina* × *townsendii* and *Spartina* × *neyrautii*. MS-AFLP analysis of these hybrids demonstrated that the genomes of both hybrids have experienced massive methylation repatterning compared to their ancestors (Salmon *et al.*, 2005). Moreover, a remarkably high percentage of newly methylated fragments were shared between the two hybrids, demonstrating in some sense that the epigenetic reprogramming was 'directed' or at least not stochastic. At present, these altered methylation states have not been connected to morphological, physiological, or ecological phenotypes, but the sheer magnitude of the phenomenon strongly suggests that connections remain to be discovered.

This indication that some epigenetic changes are directed by one or more underlying molecular mechanisms, and hence are repeatable in independent hybridizations or polyploidizations, has also been found in *Arabidopsis* (Wang *et al.*, 2004a) and in *Gossypium* (Adams *et al.*, 2004). In the latter two studies, changes first inferred from AFLP analysis were subsequently verified using reverse transcriptase–polymerase chain reaction (RT-PCR) or other molecular tools. Moreover, by using AFLP analysis on cDNAs instead of genomic DNAs, the epigenetic changes accompanying hybridization/polyploidization were shown to represent changes in actual gene expression. That some of these changes reflect altered methylation was most elegantly demonstrated in a recent study in *Arabidopsis* (Wang *et al.*, 2004b). Using a transgenic technology involving RNA interference, Wang *et al.* (2004a) created lines that were defective for two genes involved in DNA methylation, and showed that the expression of two previously silenced genes was reactivated in these lines. Because other genes that were silenced in polyploid *Arabidopsis* were not similarly reactivated, it is evident that cytosine methylation is responsible for only a portion of gene silencing.

At present there is little understanding of the mechanistic underpinnings of most gene expression modulation in hybrids and allopolyploids, but it is likely that the full spectrum of mechanisms discussed in the first part of this review play some role. For example, one can imagine that the spatial arrangement of chromosomes in the nucleus of a polyploid plant differs in many respects from that of its diploid progenitors; this difference may be reflected in gene expression alterations that are epigenetically mediated through spatial repositioning of chromosomal territories and chromosomes or variation in matrix attachment regions, as discussed above. Alternatively, in some cases transposable element activity may be implicated, as in the study by Madlung *et al.* (2005), who showed activation of transposons in polyploid *Arabidopsis*. An additional possibility is that of altered gene expression arising from regulatory mismatch in hybrid or polyploid nuclei (Birchler *et al.*, 2005; Veitia, 2005). This mismatch may interact with altered cell volumes and stoichiometries of transcription factors and regulatory proteins in complex ways to generate widespread gene expression change, relative to that which existed in the parental genomes.

An additional lesson about epigenetic modification and polyploidy stems from work on cotton, where studies of duplicated (homoeologous) gene expression in synthetic and natural allotetraploids showed that the duplicated loci may be expressed at different ratios in different plant organs (Adams *et al.*, 2003, 2004). Most remarkably, some duplicate loci showed complete reciprocal silencing, *even among whorls of the same flower*. Because expression is constitutive in these organs (for the genes tested) in the parental diploids, the expression alterations observed are inferred to be mediated through one or more epigenetic processes that accompanied or shortly followed hybridization and/or polyploid formation.

An especially tantalizing observation from the work on *Gossypium* is that some of the biases in gene expression detected in natural allotetraploid cotton, the lineage of which is estimated to have originated 1–2 Myr ago (Wendel & Cronn, 2003), are reiterated in synthetic allotetraploids generated in the laboratory. This concordance suggests the evolutionarily provocative scenario that hybridization- and/or polyploidization-induced epigenetic modifications may remain stable over extraordinarily long periods of time. To the extent that they influence phenotype, and in consideration of the possibility that epigenetic control may be reversible, these observations suggest that epigenetic responses to organismal contact may generate enormous amounts of latent variation in gene expression,

which may be sustained and released for evaluation by natural selection over evolutionary timescales.

Why is gene expression so radically altered for so many genes following hybridization or polyploid formation? Does this pattern reflect the higher-order effect of merging ~30 000 genes from two divergent genomes into a common nucleus, resulting from the types of spatial effects described above, or is it the consequence of genome-wide regulatory mismatch and competition for rate-limiting transactivating factors, as envisioned in some recent thought-provoking papers (Birchler *et al.*, 2005; Veitia, 2005)? Alternatively, is the induction of specific silencing machinery more important in these responses? Apart from this mechanistic question, is adaptation involved in the initial response; that is, has evolution shaped genomes such that expression modulation reflects an adaptive response to dosage imbalance, permitting newly merged genomes to restore order in the face of regulatory confusion? Answers to these and related questions may soon be forthcoming, following experiments that are now enabled by advancing technology.

Morphological variation and epigenetic change

Epigenetically induced morphological variation has become a topic of interest not only in the plant world, but also to those concerned with human disease, as more and more human ailments are discovered to be the result of epigenetic and not genetic errors (Robertson & Wolffe, 2000; Huang *et al.*, 2003). Several model plant systems provide excellent examples of epigenetic effects on plant phenotype.

One of the premier examples concerns the genus *Brassica*, where an astonishing amount of morphological variability has been observed among presumptively genetically homogeneous allopolyploid lines of *Brassica rapa*, resynthesized from its two diploid progenitors, *Brassica napus* and *Brassica oleracea*. Following only six generations of divergent selection for flowering time, mean flowering time between lines selected for early and late-flowering differed by 12.5 d, even though all lines were generated from the same homozygous parents. In some lines, these changes were caused by homoeologous recombination near *FLOWERING LOCUS C3* (*FLC3*), with early flowering lines expressing twice as much *B. napus FLC3* (*BnFLC3*) and no *B. oleracea FLC3* (*BoFLC3*), but such recombination only accounted for ~30% of the observed variation (Pires *et al.*, 2004). Additionally, both copies of *BrFLC5* were silenced in all polyploids, yet expressed in all diploids. The mechanisms of interaction between the four *FLC* loci are not known, but clearly nonallelic variation resulting from polyploidy has induced enough variation to account for a 12.5-d difference in flowering time. Importantly, a trait such as flowering time is easily envisioned to be one that is readily visible to natural selection, as season length and time of seed set are important factors to plant survival (Schranz & Osborn, 2000; Pires *et al.*, 2004).

As flowering time may be epigenetically modulated, so might other aspects of plant phenology or morphology. A classic example of the latter concerns *Linaria vulgaris* (Cubas *et al.*, 1999, 2001), which normally has zygomorphic flowers but which exhibits a mutant form with actinomorphic flowers. Linnaeus commented on this variation during his lifetime, but it went without an explanation for more than two centuries, until molecular genetic tools permitted the identification of the *CYCLOIDEA* gene, which plays a role in control of floral symmetry. Even more interesting from the present perspective is that this morphology is controlled by a methylation polymorphism. The Lamiales seem particularly prone to this switch, with many well-documented cases of peloric mutants (actinomorphic flowers in a normally zygomorphic species) (Rudall & Bateman, 2003). The significance and relevance of these observations become greatly magnified when they are considered in light of the results discussed above for cotton, which raised the possibility both of global epigenetic responses and of epigenetic latency for perhaps millions of years.

Epigenetics and the preservation of duplicated genes and networks

The explosion in the amount of genomic data in recent years has been accompanied by a concomitant rise in awareness of the importance of genome duplication in evolution. This awareness, along with the realization that many duplicated genes quickly return to their former single-copy status, has led to a resurgence in theoretical and empirical work aimed at explaining differential loss and retention of duplicated genes. The basic idea is that, from the moment a gene becomes duplicated and hence potentially redundant (Ohno, 1970), there is a race against time, during which one copy or the other either acquires a new function or is obliterated by the normal mutational processes leading to pseudogene formation. Because evidence indicates that duplicated genes are retained far more often without novel function than would be expected based on mathematical models alone, a rather satisfying theory of subfunctionalization emerged (Force *et al.*, 1999; Lynch & Conery, 2000; Lynch & Force, 2000; Lynch *et al.*, 2001), which has now been supported by empirical evidence obtained in a study by Blanc & Wolfe (2004), wherein it was documented that there may be 'concerted divergence' of duplicated genes in pathways. Thus, many duplicated genes are retained following gene duplication because ancestral aggregate gene function (including temporal and spatial patterns of gene expression) has become partitioned between duplicated genes, each of which has suffered complementary mutations in the promoter regions such that both are essential for survival.

A key concept in the foregoing model is the race between subfunctionalization (and hence preservation) and pseudogenization (and hence loss). Only genes that are preserved become available for future acquisition of novel function. In this respect, 'epigenetic retention' of duplicated genes represents a powerful and sweeping mechanism (Rodin & Riggs, 2003)

that in principle could simultaneously affect hundreds to thousands of duplicated genes. That this possibility is more than just conjecture is evidenced by the results from polyploid cotton obtained by Adams *et al.* (2003, 2004), and by Rodin & Riggs (2003), who showed near-instantaneous partitioning of constitutive expression patterns in diploids accompanying their genomic merger into a derivative allopolyploid. The relevant point here is that, because of epigenetic silencing or biased expression of reciprocal homoeologs in different organs or tissues, the actual time to subfunctionalization for hundreds or thousands of duplicated loci may be near zero. To the extent that this scenario holds, it may help explain the unexpectedly high level of duplicate gene retention in plants, while simultaneously underscoring the significance of epigenetic mechanisms in plant evolution.

Epialleles and population genetics

Several recent studies have demonstrated that there exists within plant populations some level of standing variation in epigenetic marks such as cytosine methylation (Ashikawa, 2001; Knox & Ellis, 2001; Cervera *et al.*, 2002; Riddle & Richards, 2002; Liu & Wendel, 2003; Wang *et al.*, 2004b). At present, this is a relatively little-explored arena, so there exists no real understanding of the scope, pattern and scale of local, epigenetic variation. Assays for many kinds of epigenetic variation do not even exist; how does one quantify epigenetic phenomena for which there exists neither a molecular assay nor an appropriate theoretical framework? For some mechanisms, such as DNA methylation and histone modification, epigenetic assays at the population level are a realistic possibility, but for other relevant mechanisms, such as those involving shifts in chromosome territories and the restructuring of MARs, and NORs, not only do we lack assays but any results would be perplexing to interpret. Even more interesting is the realization that we have yet to develop a theoretical framework that might accommodate and test epigenetic observations (Kalisz & Purugganan, 2004). This need may be addressed in the next few years, as a consequence of what soon will be a wealth of accumulating empirical observations begging for theoretical underpinnings.

Perhaps more challenging are considerations of measuring indirect rather than direct epigenetic effects, even for phenomena

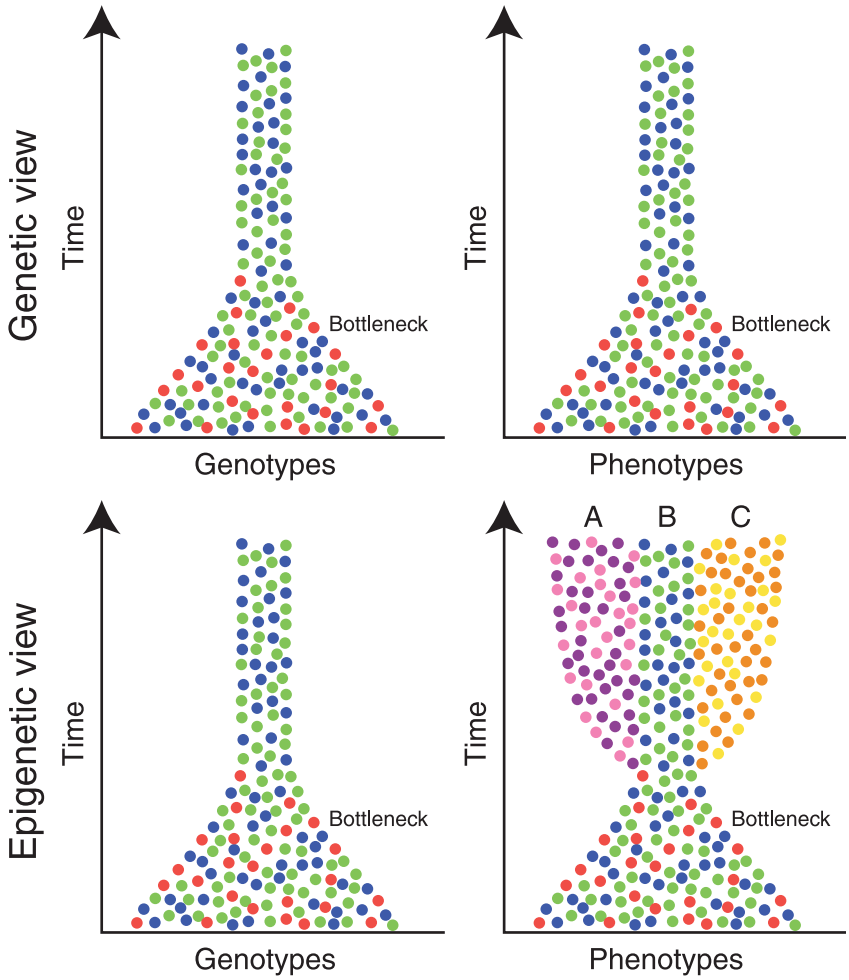

Fig. 1 Genetic and epigenetic views of bottlenecks. From a traditional genetic perspective, a bottleneck winnows genotypes and their corresponding phenotypes according to the requirements of drift and natural selection. As modeled in the upper panels, this is depicted as a loss of diversity at both the phenotypic and genotypic levels (loss of red dots; increased frequency of blue and green). An epigenetic perspective suggests that this winnowing process may be ameliorated at the phenotypic level by epigenetically mediated novel phenotypes. As illustrated here and described in the text, novel phenotypes (purple and yellow dots) may result from epigenetic modifications induced by genomic stresses, such as those caused by hybridization, polyploid formation, or extreme environmental selection or ecological change – processes inherently tied to genetic bottlenecks. Thus, in addition to the possibility modeled under the genetic view (B), new evolutionary opportunities (A and C) are envisioned.

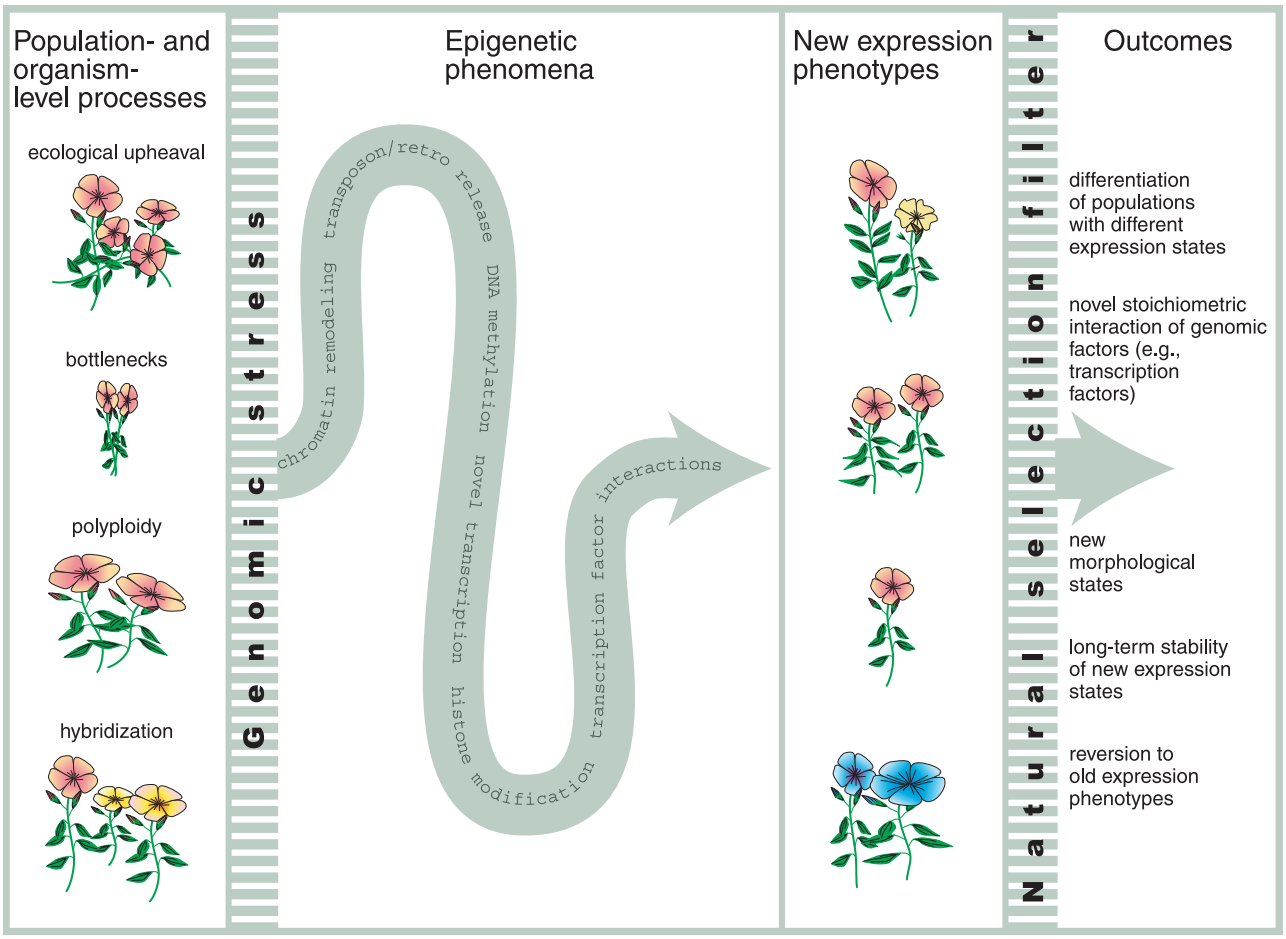

Fig. 2 Conceptualization of the interaction between epigenetics and evolutionary change. A number of population-level processes (left) cause genomic stress, leading to the induction of epigenetic phenomena (large arrow, center). These various phenomena operate in an ecological and evolutionary context to produce novel phenotypes (right center), ranging from molecular to morphological. These new phenotypes are subjected to the filter of natural selection – those surviving may then undergo longer-term evolutionary processes such as retention or loss of initially epigenetically fixed states.

for which assays of direct effects (such as cytosine methylation) may be practical. The interest here is in very real but difficult to measure alterations of spatial, temporal, and relative stoichiometries of mRNAs, translated proteins, and, ultimately, the phenotypic variations that these alterations may engender. How much natural variation is underwritten by epigenetic processes? How will we integrate new measures of epigenetic variation into our current equations and frameworks? This is likely to be a fertile arena for future investigation.

Epigenetics, populations, and evolutionary change

In this review, we have provided an introduction to epigenetic mechanisms and highlighted their potential relevance to the evolutionary biology of plants. It should be apparent from the foregoing section headings and the literature discussed that most of the empirical examples involve plant populations that are small, for example those involved in hybridization and/or polyploid evolution. It is tempting to speculate that both aspects of this observation are important; namely, that both 'genome disruption' or 'genomic shock' and evolutionary bottlenecks are features that promote the generation and fixation, respectively, of new variants in small populations. This idea is schematically illustrated in Fig. 1. Under a traditional genetic view, selection and/or drift acts on genotypes and alters allele frequencies within populations; following a bottleneck, it is expected that some portion of the pre-existing genetic variation will be selected, with an analogous effect on phenotypic variation (see, however, Goodnight, 1988; Cheverud *et al.*, 1999). The possibilities raised in this review present a new view. Specifically, an epigenetic perspective might explicitly consider that population bottlenecks are intimately associated with the processes that stimulate epigenetic instabilities. These bottlenecks may arise not only from hybridization and/or polyploidy, but also from extreme selection or environmental stress, which are

known to cause transposons to be released from suppression and thereby become activated. The full spectrum of genic and genomic alterations induced by these population-level phenomena is envisioned to generate novel genetic, epigenetic and phenotypic variation that may enable individuals to survive the bottleneck. Thus, the effects of the bottleneck involve far more than a mere winnowing of pre-existing variation, as in the traditional genetic view. Instead, in the epigenetic view, the bottleneck itself provides the stimulus for evolutionary novelty mediated by epigenetic responses, as well as the population genetic context in which novel variation might rapidly achieve fixation. This is schematically illustrated in Fig. 2, where population- and organism-level processes are shown to lead to genomic stress, thereby stimulating epigenetic repatterning, which itself leads to novel phenotypes that are then subjected to the evolutionary filter of natural selection.

An interesting twist on this scenario is that many epigenetic modifications are potentially reversible. This suggests the potential for a population to revert to a former phenotypic state if the bottleneck or environmental stress is reduced.

As molecular biologists develop and refine our understanding of epigenetic mechanisms, evolutionary biologists will continue to probe the connections between these mechanisms and evolutionary change at the population level. As shown in this review, enough is known already about the suite of mechanisms and their effects on gene expression and phenotype to consider the possibility that epigenetics plays a key role in many evolutionary scenarios.

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