Evolutionary Significance of Epigenetic Variation 16

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Contents

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16.1 Introduction

Several chapters in this volume demonstrate how epigenetic work at the molecular level over the last few decades has revolutionized our understanding of genome function and developmental biology. However, epigenetic processes not only further our understanding of variation and regulation at the genomic and cellular levels, they also challenge our understanding of heritable phenotypic variation at the level of whole organisms and even the process of evolution by natural selection (Jablonka and Lamb 1989, 1995; Danchin et al. 2011). Although many of the epigenetic mechanisms involved in differential gene expression are reset each generation, some epigenetic marks are faithfully transmitted across generations (Jablonka and Raz 2009; Verhoeven et al. 2010a). In addition, we now know that natural variation exists not only at the DNA sequence level but also the epigenetic level (e.g., Vaughn et al. 2007; Herrera and Bazaga 2010). This may be particularly common in plants, and several studies suggest that epigenetic variation alone can cause significant heritable variation in phenotypic traits (e.g., Cubas et al. 1999; Johannes et al. 2009; Scoville et al. 2011). Because of these observations, there is currently increasing interest in understanding the role of epigenetic processes in ecology and evolution (e.g., Richards 2006, 2011; Bossdorf et al. 2008; Johannes et al. 2008; Richards et al. 2010a).

In spite of the speculation about the potential evolutionary implications of epigenetic processes, most previous work has involved agricultural crops and model species such as *Arabidopsis thaliana*, frequently under artificial conditions, and we therefore still know little about the importance of epigenetic processes in natural populations (Richards 2008, 2011; Richards et al. 2010b). With this in mind, we review some of the salient examples of known heritable phenotypic effects of epigenetic mechanisms as well as how epigenetic variation can be created. We address the important issue of disentangling genetic and epigenetic components of heritable phenotypic variation with particular emphasis on how some epigenetic effects may be determined by genotype, while

J.F. Wendel et al. (eds.), *Plant Genome Diversity Volume 1*,
DOI 10.1007/978-3-7091-1130-7_16,

others act independently of genotype. We discuss what little we know about how these patterns are manifest in natural populations and what we need to do to discover how these mechanisms work in the real world. Finally, we conclude with some ideas about how these concepts may enhance our understanding of evolutionary processes.

16.2 Phenotypic Effects of Epigenetic Variation

The logical first step to understanding the importance of epigenetic effects is characterizing the phenotypic response to epigenetic variation. There are few known simple and obvious phenotypic effects that result from changes in epigenetic marks at single genes. However, through manipulation of methylation levels and isolation of methylation mutants, researchers have made substantial progress in demonstrating important phenotypic effects that result from changes at only the epigenetic level.

16.2.1 Single Gene to Phenotype Epigenetic Effects

One of the most celebrated studies of single-gene epigenetic effects on the phenotype and epigenetic inheritance is an elegant example of the change from bilateral to radial floral symmetry in *Linaria vulgaris* (Fig. 16.1). Cubas et al. (1999) found that the radial symmetry phenotype was associated with methylation changes at the gene *Lcyc* which controls dorsoventral asymmetry. In the variety of *Linaria* with radial flowers, *Lcyc* is extensively methylated and transcriptionally silent. The epigenetic modification is heritable and co-segregates with the phenotype. Occasionally, the phenotype reverts during somatic development, which correlates with demethylation of *Lcyc*, restoration of gene expression and the development of normal bilateral flowers.

Although the *Linaria* example is satisfying, there are not many other simple examples of the phenotypic effects of epigenetic alteration of one or few genes, especially changes that are stably inherited for generations (see further discussion in Paszkowski and Grossniklaus 2011). There are several examples of epigenetic effects that are not heritable in plant developmental biology: for instance, studies have shown that epigenetic silencing (caused by cold treatment) of the floral repressor Flowering Locus C (FLC, Sung and Amasino 2004; Shindo et al. 2006), followed by increased methylation of histone 3 lysine 9 (H3K9) and histone 3 lysine 27 (H3K27), triggers flowering in many *A. thaliana* accessions that overwinter as a rosette. However, this response is necessarily non-heritable, so that the next generation of seedlings will also overwinter before flowering.

Fig. 16.1 A clear example of phenotypic effects of natural epigenetic variation, and epigenetic inheritance. Cubas et al. (1999) showed that the naturally occurring change from normal bilateral (*right*) to radial symmetry (*left*) of *Linaria vulgaris* was associated with methylation and silencing of the gene *Lcyc* (Photos from Palevitz 1999)

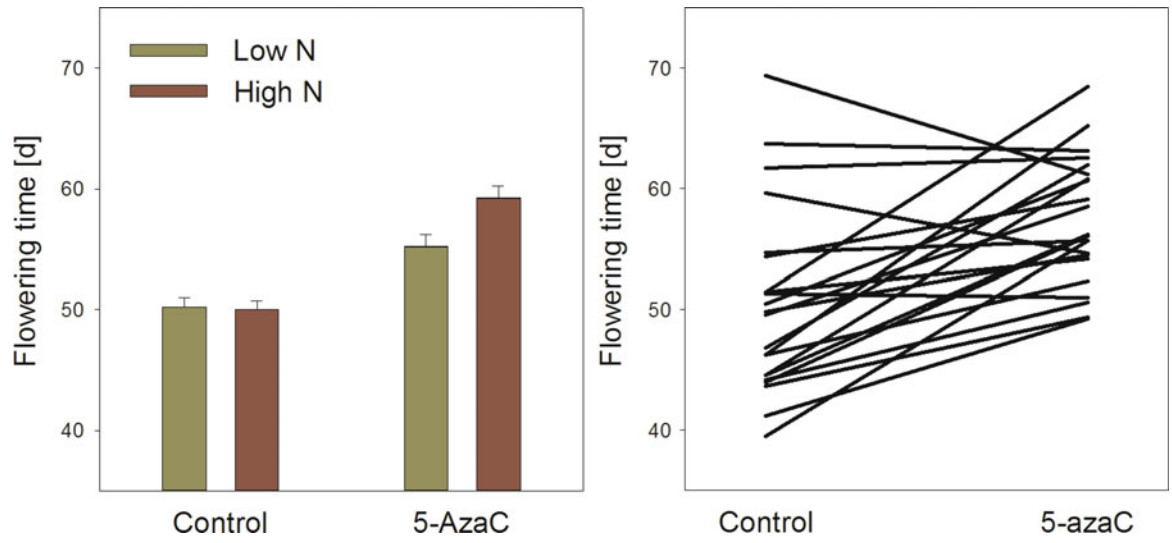

Fig. 16.2 Experimental demethylation through 5-azacytidine (5-azaC) significantly alters the phenotype of *A. thaliana*. *Left panel*: the phenotypic plasticity to nutrient addition of control vs. 5-azaC-treated plants. *Right panel*: variation in flowering time responses to 5-azaC among 22 different *A. thaliana* ecotypes (Modified from Bossdorf et al. 2010)

16.2.2 Experimental Manipulations of DNA Methylation

One approach to understanding the phenotypic effects of DNA methylation has been to manipulate genome wide levels of methylation through the application of the demethylating agent 5-azacytidine (5-azaC), a chemical that is incorporated into DNA during replication and thereby inhibits the enzyme methyltransferase, causing partial demethylation of the DNA (e.g., Burn et al. 1993; Fieldes and Amyot 1999a; Tatra et al. 2000). This creates different epigenetic variants of the same genotypes and therefore allows researchers to demonstrate phenotypic effects of epigenetic changes. Several studies have demonstrated that the effects of demethylation on ecologically important traits can be significant. For example, Burn et al. (1993) showed that treatment of *A. thaliana* and *Thlaspi arvense* with 5-azaC significantly altered plant flowering time, while Fieldes and colleagues (Fieldes 1994; Fieldes and Amyot 1999b; Fieldes et al. 2005) found that 5-azaC affected the growth, fitness and phenology of *Linum usitatissimum*. More recently, Bossdorf et al. (2010) found that experimental alteration of DNA methylation not only altered the growth, fitness and phenology of *A. thaliana*, but also the phenotypic plasticity of these traits in response to nutrient addition (Fig. 16.2). Moreover, there were significant differences among the 22 studied *A. thaliana* genotypes in the degree to which trait means and plasticities were affected by 5-azaC. Demethylation also altered overall patterns of among-line variability, which indicates that epigenetic changes can not only affect the short-term environmental responses (phenotypic plasticity) of plants, but also the evolutionary potential of important traits and their plasticities (Bossdorf et al. 2010).

16.2.3 Methylation Mutants

Another approach to isolating the phenotypic effects of methylation has been the isolation of mutants that have non-functional or reduced function in the methylation machinery. Using southern blot analysis, Vongs et al. (1993) isolated three hypomethylation mutants from approximately 2,000 ethylmethanesulfonate (EMS) mutagenized plants from the Columbia genotype (Col-0) of *A. thaliana*. The three lines were referred to as *ddm* mutants in reference to their *d*ecrease in *D*NA *m*ethylation. A large portion of the change in methylation was found in the repeat regions of the genome, and two of the mutants (*ddm1-1* and *ddm 1-2*) had methylation levels reduced to only 25–30% of wild type, while the third was reduced to 83% of wild type. The genes involved in these mutations were later characterized as chromatin remodeling proteins (Jeddeloh et al. 1999). However, the original screening of the homozygous mutants did not show any obvious difference in phenotype from wild type (Vongs et al. 1993). Backcrosses to wild type demonstrated that hypomethylation was gradually lost through segregation, but hypomethylated fragments were slow to be re-methylated.

Screening of another 5,000 EMS mutagenized plants revealed two more mutants with decreased methylation,

this time in the METHYLTRANSFERASE1 (MET1) gene (Kankel et al. 2003). These were initially referred to as *ddm2-1* and *ddm2-2*, but were renamed *met1-1* and *met1-2* when the mutations were identified in the MET1 gene. In this case, methylation levels were reduced by 50% in *met1-2* and plants displayed normal development and morphology. In contrast, *met1-1* showed delayed flowering time, which was associated specifically with the demethylation of the floral repressor FWA and creation of an FWA epiallele. As with the *ddm1* mutants, hypomethylated segments of the genome from *met1* mutant could be inherited in wild type backcrosses.

16.2.4 Epigenetic Recombinant Inbred Lines (epiRILs)

Although the first set of *A. thaliana ddm1* mutants did not initially demonstrate any obvious phenotypic effects of reduced methylation, epigenetic recombinant inbred lines (epiRILs) developed from backcrosses of these and the *met1* mutants to Col-0 wild type have proven to be powerful tools for detecting effects of variation in DNA methylation on quantitative traits (Johannes et al. 2009; Reinders et al. 2009). Johannes et al. (2009) created 505 epiRILs by crossing the *ddm1-2* to wild type Col-0 and backcrossing a single F1 female with Col-0. From this first backcross, 509 offspring with the *DDM1*/*DDM1* wild type genotype were chosen to initiate lines of single seed descent for six self-fertilizing generations. Despite the fact that these lines are nearly isogenic, the authors found increased variance and significant among-line variation in plant height and flowering time across the 505 lines that survived the inbreeding process, and they ascribed the phenotypic variation to the differences in DNA methylation patterns. Subsequent work with these epiRILs has confirmed that there is consistent and significant heritable variation in many other ecologically important traits among these lines, e.g. fitness traits (Roux et al. 2011) and drought responses (Zhang and Bossdorf, unpublished data; Fig. 16.3).

In a similar recombination study, Reinders et al. (2009) created 68 epiRILs by crossing the *met1-3* to wild type Col-0 and backcrossing a single F1 female with Col-0. From this first backcross, F2 offspring with *MET1*/*MET1* wild type genotype were selfed, which provided for 100 F3 lines to initiate lines of single seed descent for four additional generations. At F7, seed from nine random plants were bulked to provide the F8 used for phenotyping and methylation analysis. As in the Johannes et al. (2009) epiRIL study, this population of epiRILs showed significant heritable among-line variation in growth and response to stress. In addition, the population was characterized by a bimodal distribution of flowering time, which the authors related to

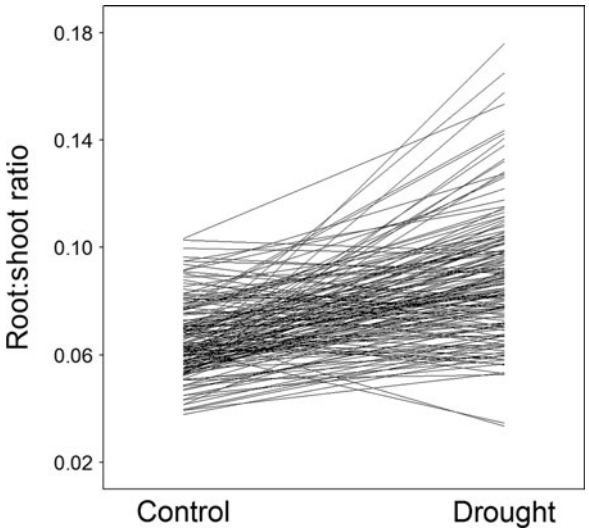

Fig. 16.3 Variation in drought responses of 160 epiRILs (Zhang and Bossdorf, unpublished data)

the fact that the floral repressor FWA is methylated in wild type and demethylated in *met1-3*. The FWA locus in 11 late flowering lines each had a "*met*-like epiallele".

Both epiRIL studies elegantly demonstrate the potentially strong effects of epigenetic changes on plant phenotypes. However, they also show that the stability of phenotypes based on epi-alleles can be difficult to predict and is locus-dependent (Reinders and Paszkowski 2009). Both studies show only partial inheritance of methylation changes, and partial remethylation, especially of transposable element (TE) loci whose active remethylation is ensured by an RNA-dependent DNA methylation machinery that recognizes TE-derived transcripts and subsequently silences the TE locus (Teixeira et al. 2009). Johannes et al. (2009) tested the stability of 11 loci (including FWA) that were methylated in wild type and hypomethylated in *ddm1-2* as well as three loci that were not methylated in either parental line. They examined the methylation of these 14 loci across 22 epiRILs representing the two ends of the flowering time spectrum (Fig. 16.4). The three loci that were unmethylated in both parents were stably inherited as unmethylated in the offspring. Five of the eleven differentially methylated sequences segregated close to the expected Mendelian fashion; for the other six loci (including FWA) however, there was almost complete reversion to the methylated state. This indicates that there are mechanisms to correct for hypomethylation in the long term, and that these mechanisms are able to methylate de novo in later generations, without using methylation inherited from either parent (Reinders and Paszkowski 2009; Teixeira et al. 2009). Potentially, such mechanisms could contribute to population level variation and ultimately evolution.

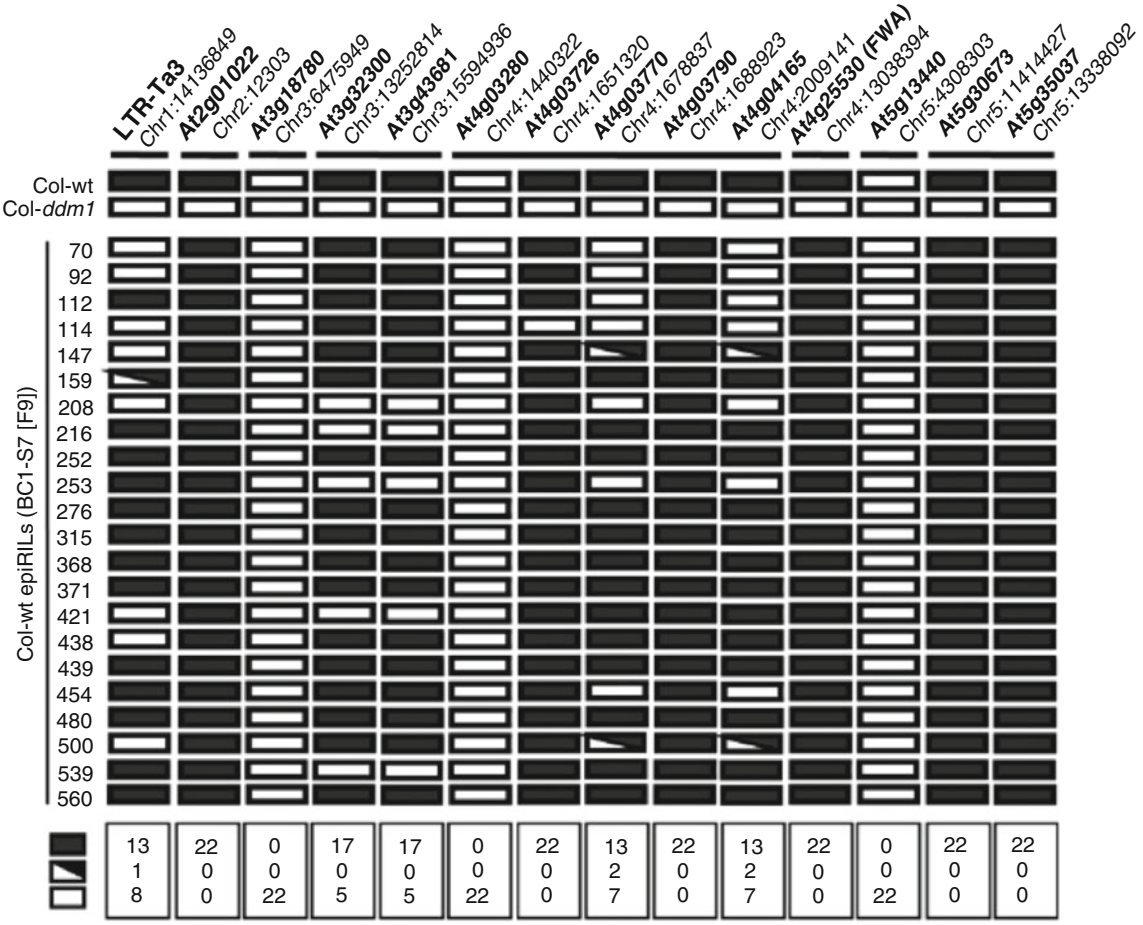

Fig. 16.4 Segregation of methylation patterns of 14 loci across 22 Col-0 × *ddm1-2 A. thaliana* epiRILs. Name and position of loci are listed across the *top* with *horizontal bars* indicating closely linked sequences. *Black rectangles* represent high (*wild type*) methylation. *White rectangles* represent *ddm1*-induced hypomethylation. *Partial rectangles* represent intermediate levels of methylation. Segregation is summarized across the 22 epiRILs for each sequence at the *bottom* (Reprinted from Johannes et al. 2009)

16.3 Creation of Heritable Epigenetic Variation

Having established that epigenetic variation can have significant phenotypic effects, the next question is how heritable epigenetic variation is created. From what is known, there seem to be three main mechanisms: (1) natural epimutations, (2) environmental induction, and (3) genomic events such as hybridization and polyploidization.

16.3.1 Epimutations

One source of natural epialleles (that are not triggered by defective enzymes in the DNA methylation machinery, as in the case of the epiRILs) are natural epimutations created by imperfect maintenance of DNA methylation patterns and other epigenetic marks through mitosis and meiosis (Genereux et al. 2005). For instance, methylation polymorphisms may develop by a failure of maintenance methyltransferase enzymes to copy methylation patterns to daugther strands during DNA replication (Vaughn et al. 2007).

While the source of most epialleles is largely unknown, the studies with the *A. thaliana* epiRILs discussed earlier indicate increases in epigenetic variation even after the function of the methylation machinery has been restored (i.e. in *DDM1*/*DDM1* or *MET1*/*MET1* homozygous epiRILs). In addition to suppressed DNA demethylation activities, this increase in variation is manifest through the redistribution across the genome of other silencing marks like H3K9 and H3K27 histone methylation (Lippman et al. 2004; Reinders et al. 2009), which could dramatically alter phenotypes.

Existing empirical evidence suggests that epimutations may be more frequent but also more labile than mutations of DNA sequence. For instance, in a study of apomictic dandelions, Verhoeven et al. (2010a) showed that even in a constant environment, some DNA methylation differences

developed between individual plants (7.5% of the polymorphic DNA methylation loci) and that most of these changes were inherited across generations. Moreover, it has been shown that in some sequence contexts, DNA methylation errors are quickly and actively repaired, e.g. through RNA-directed DNA re-methylation (Teixeira et al. 2009), whereas in other DNA contexts no active restoration occurs and DNA methylation changes may turn into stable polymorphisms.

16.3.2 Environmental Induction

Another mechanism by which epigenetic variation can be created is environmental induction. Exposure to environmental stress triggers epigenetic changes, which regulate the transcriptomic stress response (Chinnusamy and Zhu 2009), and some of these stress-induced changes may persist even after the stress is relieved, resulting in a stress 'memory' that can be stable throughout the lifetime of an organism or even across generations. For instance, humans that were prenatally exposed to famine during the Dutch Hunger Winter (1944–1945) still show modified patterns of DNA methylation at *IGF2* after six decades (Heijmans et al. 2008). When gestating female rats were transiently exposed to endocrine disruptors, their male offspring showed reduced spermatogenic activity even four generations later, and this phenotypic effect was correlated to altered DNA methylation in the germ line (Anway et al. 2005).

Environmental induction of epigenetic variation that is stably inherited across generations seems to be particularly important in plant biology. One explanation for this might be that in plants, unlike in mammals, the germ line is separated from somatic tissue at a much later developmental stage (Jablonka and Lamb 1989; Wessler 1996). As a result, there might be more opportunities for plants to transmit epigenetic modifications to offspring that were acquired in somatic tissue during the plant's life, including environmentally-induced epigenetic modifications. Several studies have used multi-generation experiments to show that parental exposure to biotic or abiotic stresses resulted in modified DNA methylation in unexposed offspring in tobacco (Boyko et al. 2007), dandelion (Verhoeven et al. 2010a) and *A. thaliana* (Boyko et al. 2010). For example, Verhoeven et al. (2010a) used methylation sensitive amplified fragment length polymorphism (MS-AFLP) to show that plants with identical genotypes exposed to different stresses had more changes in methylation sensitive markers compared to control (Fig. 16.5). In particular, chemical induction of herbivore and pathogen defenses triggered considerable methylation variation throughout the genome. Although some of these methylation differences reverted back to the original in the next generation of plants grown in a common environment, Verhoeven et al. (2010a) provide some of the first evidence that the majority of the stress-induced changes in methylation are inherited in the next generation. However, the authors did not present any information on the potential phenotypic effects of these methylation changes, and cannot rule out the possibility that the stress treatment induced changes at the DNA sequence level (e.g. through mutation or the activity of transposable elements). Further studies are required of these plants with stress-induced changes to see if the changes may be adaptive.

In *A. thaliana*, parental plants exposed to abiotic stresses produced offspring that not only showed modified transcriptomes and genomic DNA methylation, but sometimes also altered responses to stress exposure. For instance, parental exposure to salt stress resulted in offspring with a greater salt tolerance, and this transgenerational response

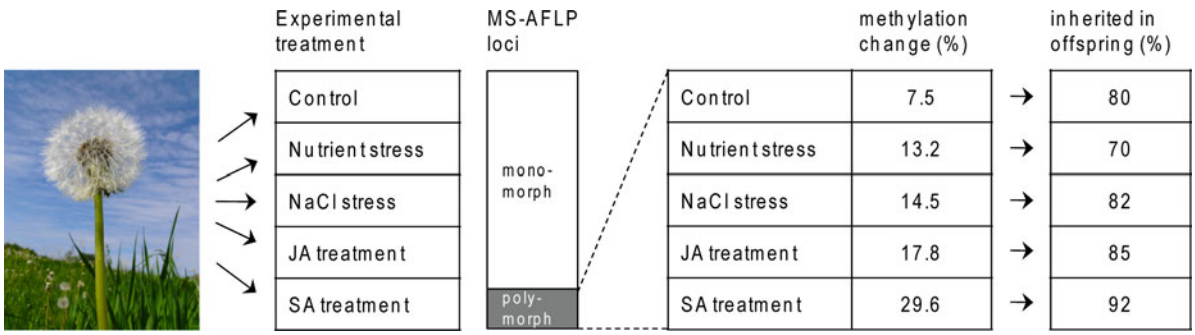

Fig. 16.5 Induction of DNA methylation changes by ecological stresses and their heritability in asexual dandelions (*Taraxacum officinale*). A single apomictic dandelion genotype was exposed to different experimental environments. From a total of 359 MS-AFLP marker loci, 20 showed polymorphism within the experiment. Although these 'susceptible' loci showed some background level of methylation change also in the control group, the rate of methylation change that was observed within the subset of susceptible loci increased significantly due to stress treatment, particularly due to treatment with jasmonic (JA) or salicylic (SA) acid. JA and SA are plant hormones involved in herbivore and pathogen defenses. Most of the induced methylation changes were inherited in apomictic offspring that were not exposed to stress but raised in a common control environment (Verhoeven et al. 2010a)

appeared to depend on DNA methylation because the enhanced salt tolerance in the offspring disappeared when plants were treated with the demethylating agent 5-azaC (Boyko et al. 2010). This study suggests that one function of stress-induced epigenetic changes could be to prime offspring for enhanced stress resistance, presumably by heritably modulating the expression of stress-related genes. However, such a function implies that epigenetic changes are gene-specific, and we know that at least in some cases stress-induced epigenetic changes can also be untargeted or random. In dandelion, for instance, replicated individuals showed only limited consistency in their stress-induced DNA methylation changes (at anonymous MS-AFLP marker loci, Verhoeven et al. 2010a).

16.3.3 Polyploidization and Hybridization

Novel epigenetic variation can also be generated through genomic events such as hybridization and polyploidization (Liu and Wendel 2003; Adams and Wendel 2005; Grant-Downton and Dickinson 2006). The merging and doubling of genomes creates significant challenges for complex organisms, and epigenetic modifications may play an important role in reconciling the regulatory incompatibilities that are created through such genomic events (Chen 2007), for instance through epigenetic regulation or silencing of duplicated genes and re-activation of epigenetically silenced transposons (Liu and Wendel 2003; Wang et al. 2004; Lukens et al. 2006; Chen 2007; Paun et al. 2007; Hegarty and Hiscock 2008). Full genome duplication means complete functional redundancy of all genes, allowing for duplicates to take on new function or to be completely silenced and preserved for possible future function, thereby providing a sort of latent evolutionary potential (Adams et al. 2003; Rodin and Riggs 2003; Rapp and Wendel 2005; Ainouche et al. 2009; Slotkin et al. 2012, this volume). Although the epigenetic consequences of genome doubling have been documented, for instance in *A. thaliana* (Mittelsten Scheid et al. 1996, 2003), *Gossypium hirsutum* (Keyte et al. 2006) and dandelions (Verhoeven et al. 2010b), the hybridization of different genomes appears to have even more dramatic effects on epigenetic variation (Salmon et al. 2005; Parisod et al. 2009; Chelaifa et al. 2010).

A well-studied example of plant hybridization and polyploidization, and its epigenetic consequences, are the recent studies on *Spartina* (salt marsh grass) by Ainouche and coworkers. Within the last 150 years, natural hybridization between *S. alterniflora* and *S. maritima* produced the hybrid *S.* ×*townsendii*, which through polyploidization produced the highly invasive and phenotypically plastic allopolyploid *S. anglica* (Salmon et al. 2005; Ainouche et al. 2009). The *Spartina* system provides a rare opportunity to contrast genetic and epigenetic effects of hybridization and polyploidization. Ainouche and coworkers investigated genetic and epigenetic differences, as well as transposable element activity and gene expression differences between hybrids, allopolyploids and parental species (Salmon et al. 2005; Parisod et al. 2009; Chelaifa et al. 2010). They found that both hybridization and polyploidization were associated with substantial DNA methylation changes, particularly around transposon loci. However, these changes were more dramatic during the hybridization event, and therefore, if epigenetic changes contributed to the invasive success of *S. anglica*, then the preceeding hybridization event may have been an important step in the evolution of this invader.

16.4 Relationships Between Genetic and Epigenetic Variation

One of the main challenges in understanding the importance of epigenetics in evolution is the relationship between genetic and epigenetic effects, and the degree to which phenotypic variation can be explained by epigenetic effects independent of genetic effects. Richards (2006) speculated that while some epigenetic effects will be entirely determined by genotype, others may be only "facilitated" by specific genotypes, or may be completely independent. Disentangling these different possibilities in reality, however, is complicated not only because we know little about these interactions, but also because so far the genetics of most complex traits is not well understood.

The network of genes involved in the flowering time pathway in *A. thaliana* is one of the best-studied regulatory pathways that control a complex life history trait in plants (Mouradov et al. 2002; Simpson and Dean 2002), and is therefore a useful starting point for unraveling genetic-epigenetic relationships. This is particularly true because flowering at the appropriate time requires the integration of many environmental signals to initiate a developmental switch in plants. The flowering time response requires the coordination of hundreds of genes organized into four main pathways responsive to specific environmental stimuli: the autonomous, gibberellin, photoperiod, and vernalization pathways (Mouradov et al. 2002; Simpson and Dean 2002). Within these pathways, mutant and transgenic studies have revealed genes that respond directly to specific environmental factors (e.g., PHYA detects far red light, PHYB, D and E detect red light; Schmitt et al. 1999; Simpson and Dean 2002), whereas quantitative genetic studies have shown dramatic phenotypic effects of natural polymorphisms in some of these genes (Caicedo et al. 2004; Olsen et al. 2004; Stinchcombe et al. 2004; Korves et al. 2007). The two best-known genes involved in the vernalization pathway are FRIGIDA (FRI) and the floral

repressor FLC (Johanson et al. 2000; Weinig et al. 2002; Olsen et al. 2004). FRI represses flowering by activating the expression of FLC. Candidate gene association studies, while not definitive, have suggested that common allelic variation at FRI and FLC are associated with flowering time diversity in natural field conditions (Caicedo et al. 2004; Stinchcombe et al. 2004). In particular, an active FRI-FLC pathway results in late flowering, whereas an inactive FRI-FLC pathway results in early flowering.

While these studies of the flowering time response in *A. thaliana* have been generally focused on candidate genes and DNA sequence based mechanisms, recent studies indicate that epigenetic processes play an important role in this regulatory network, in particular in the incorporation of environmental cues into genomic responses.

16.4.1 Epigenotype Dependent on or Inseparable from Genotype

The vernalization pathway in *A. thaliana* controls epigenetic silencing of the floral repressor FLC, and is an important component of the regulation of flowering time (Sung and Amasino 2004). Moreover, Shindo et al. (2006) recently explored how this epigenetic component of FLC regulation differed among different *A. thaliana* genotypes, and they found that there was significant variation among genotypes in the critical length of cold treatment that was required for FLC repression (when exposed to shorter cold treatments, FLC would become reactivated and flowering time would be repressed). Molecular comparisons between a genotype that rapidly responded to vernalization (Edi-0) to one that required much longer vernalization (Lov-1) suggested that the critical length of vernalization period required was related to (1) different levels of initial histone trimethylation at H3K27 in the FLC promoter and (2) different rates of acquiring the silencing marks at H3K27.

While in this case epigenetic variation is clearly associated with phenotypic variation in an important plant trait, different genotypes are involved, so the epigenetic differences could be a consequence of genetic instead of epigenetic differences. In fact, Shindo et al. (2006) hypothesize that sequence polymorphisms in the FLC gene itself could be partially responsible for the observed differences in accumulation of trimethylation at H3K27.

Although the epigenetic patterns of FLC seem to be genotype specific, the mechanism of inheritance for this epigenetic effect is unknown. Is the epigenetic pattern re-established de novo in each generation, or after its initial establishment does it persist regardless of genotype? To discriminate between these possibilities, it would be informative to do controlled crosses between genotypes with either quick or slow vernalization responses to see how the trimethylation of H3K27 segregates. Another approach to understanding the epigenetic programming of FLC would be to explore the patterns in the mutant lines. Crosses with the *ddm1* mutant show that in general expression profiles look like that of *ddm1* (Lippman et al. 2004), but no reports have specifically diagnosed the epigenetic status of FLC in these crosses. Targeted studies of the epigenetic silencing of FLC in the epi-RILs may be helpful in understanding the relative contribution of genetic and epigenetic regulation of this ecologically important gene.

16.4.2 Epigenotype Can Act Independently of Genotype

In the case of silencing of FLC through accumulation of trimethylation of H3K27, variation in the epigenetic response is associated with different genotypes. But are there also cases where epigenetic effects are independent of genetic effects? Evidence that this is possible comes from studies of epimutations such as the *Linaria* study of Cubas et al. (1999; Fig. 16.1), because in the absence of genetic variation, phenotypic effects can be unambiguously ascribed to the epigenetic alterations. However, even in such studies one cannot rule out the possibility that certain genotypes are more prone to epigenetic changes and that there may be some interdependence of genotype and epigenotype (i.e., "facilitated" epigenetic effects, sensu Richards 2006).

Essentially, all of the approaches described in Sect. 16.2 that have been used to examine phenotypic effects of epigenetic variation, i.e. natural or artificial epimutations, or epigenetic recombinant inbred lines, have successfully demonstrated that there *can* be epigenetic effects independent of genotype, but many of these methods were artificial. The question is still open as to the extent that independent epigenetic effects exist and play a role in natural populations (see Sect. 16.5 below).

16.4.3 Genotypic Variation Created by Epigenetic Effects: Transposable Elements and Heat Shock Proteins

In addition to the possibility of "facilitated" epigenetic effects (Richards 2006), there are other ways for genetic and epigenetic mechanisms to interact. One important phenomenon in this context is the possibility of genetic changes caused by transposons. Most insertions of transposable elements (TEs) are to be likely neutral or deleterious (reviewed in Brookfield 2005; Slotkin et al. 2012, this volume), but some may be adaptive in providing novel regulatory function or by promoting adaptive alternative splicing

or transcription factor binding sites (Kidwell and Lisch 2000; Feschotte 2008; Slotkin et al. 2012, this volume).

Transposable elements are concentrated in the heterochromatin of plants where they typically are epigenetically silenced by histone and DNA sequence methylation (Lippman et al. 2004; Slotkin and Martienssen 2007; Vaughn et al. 2007). Lippman et al. (2004) showed that in the *A. thaliana* Col-0 wild type, both DNA and H3K9 methylation are significantly correlated with the location of transposable elements and repeats. In *A. thaliana ddm1* mutant lines, both DNA and H3K9 methylation are reduced, specifically in the genomic areas where transposable elements are located, and several classes of transposons, which are not active in the wild type, have a high frequency of transposition (Kakutani 2002; Lippman et al. 2004; Tsukahara et al. 2009).

When transposable elements reinsert in the genome, they not only change DNA sequence, but they can also affect gene expression (Lippman et al. 2004; Feschotte 2008) and have dramatic effects on phenotype through the production of epialleles (Wessler 1996; Slotkin and Martienssen 2007; Tsukahara et al. 2009; Slotkin et al. 2012, this volume). Many studies have speculated that environmental stress could result in phenotypic variation that results from changes in epigenetic silencing of transposons, which could ultimately cause transposition and change in the genetic make-up of an individual (Kidwell and Lisch 2000; Rapp and Wendel 2005; Slotkin and Martienssen 2007; Jablonka and Raz 2009; Mirouze and Paszkowski 2011). While this type of transposon activity in response to environmental stress has not yet been demonstrated in plants (Colot V 2010, personal communication, see Slotkin and Martienssen 2007 for examples in *Drosophila* and *Schizosaccaromyces pombe*), increased transposon activity in response to *genomic* stress, including hybridization and polyploidization, is well-documented (McClintock 1984; Wessler 1996; Liu and Wendel 2003; Rapp and Wendel 2005; Mirouze and Paszkowski 2011). In a recent example, Parisod et al. (2009) used methyl-sensitive transposon display (MSTD) to investigate if increased transposon activity resulted from hybridization of *Spartina* species. They found that hybridization resulted in major methylation changes, especially near TE insertions in the maternal *S. alterniflora* genome, but in the three families of transposable elements that they examined, they did not find evidence of transposition. In general, the adaptive significance of such (possibly epigenetically induced) transposable element activity remains unclear.

One emerging story about the epigenetic silencing of TEs involves the activity of heat shock proteins (Hsp). Hsp90 is a required component of many regulatory complexes and as such Hsp90 activity affects many different pathways. Studies have found that the Hsp90 is a *phenotypic capacitor* and that its normal activity buffers phenotypes against environmental variation by suppressing the expression of genetic variation in several developmental pathways (Rutherford and Lindquist 1998; Queitsch et al. 2002). When Hsp90 is altered, a variety of developmental abnormalities develop which can become fixed even when Hsp90 activity is restored. In addition, the response of a given genotype appears to be dependent on the nature of the previously silent genetic variation (Queitsch et al. 2002).

The concept of phenotypic capacitors contributing to phenotypic robustness (also known as canalization) dates back to Waddington's experiments with *Drosophila* over 50 years ago (Waddington 1942, 1953). Waddington found that after 12 generations of selecting a phenotype elicited by exposure to 40 C for 4 h in *Drosophila*, some offspring would elicit the phenotype without the 40 C treatment. He later described this phenomenon as genetic assimilation. Without any understanding of the mechanistic basis for the inheritance of the trait, Waddington argued that the coding of the trait had become assimilated by the genotype (Waddington 1953). It is only recently that this buffering has been linked to an epigenetic effect (Sollars et al. 2003; Specchia et al. 2010). In a mutant screen of *Drosophila,* Sollars et al. (2003) found that Hsp90 is involved in chromatin regulation. The authors argue that instead of just relying on cryptic genetic variation that is revealed when Hsp90 is reduced as the sole source of heritable phenotypic variation, adaptive differences may arise epigenetically and allow for a more rapid response to selection. This source of phenotypic variation may be less stable than strictly DNA sequence based variation, and it is most likely that a combination of genetic and epigenetic mechanisms interact to produce adaptation.

In addition to affecting expression through alteration of chromatin structure, recent studies have connected the activity of Hsp90 with the activity of Piwi-interacting RNA. These small RNAs are involved in the silencing of TEs and mutations in Hsp90 show increased transcription for all TEs tested (Specchia et al. 2010). Similar to previous studies of Hsp90 where the response depends on the genetic background, Specchia et al. (2010) found that different genotypes may induce different transposon insertions. Combined, these studies suggest that Hsp90 activity (and perhaps other phenotypic capacitors) can contribute to phenotypic variation through both restructuring of chromatin or the indirect activation of TEs. In both cases, gene expression may be altered and the effects can be inherited. We know of no studies that have tested these effects in an ecological context, but the possible effects for evolutionary biology are intriguing.

16.5 Patterns of Natural Epigenetic Variation

Epigenetic inheritance in natural contexts is currently little explored, although some of the best-known heritable epialleles occur naturally in wild populations (e.g., *Linaria vulgaris*, Fig. 16.1). It could well be that many epialleles have more subtle effects and are not as easily discovered, but they may nevertheless significantly contribute to quantitative variation in ecologically relevant traits and therefore, ultimately, to adaptation.

To date, analyses of natural epigenetic variation have either used high-resolution genetic information to understand variation in specific traits (usually on model species without explicit links to populations or environments), or using low-resolution genetic information such as MS-AFLPs to address population-level questions. We expect progress to be made from more intimate merging of these two approaches, which should allow linking of epigenetic polymorphisms with known functional effects on gene expression and/or traits to performance and fitness under ecologically relevant conditions and in natural populations. Still, some important insights already have been obtained from analyses of natural epigenetic variation.

16.5.1 High Levels of Heritable Epigenetic Variation Exist Within and Between Natural Populations

Early studies of natural variation in DNA methylation in *A. thaliana* revealed that methylation patterns are similar between plants from the same inbred accession (demonstrating faithful inheritance of DNA methylation marks) but different between plants from different accessions (Cervera et al. 2002). Subsequent genomic analyses confirmed that there are high levels of DNA methylation polymorphism between accessions. The extent of polymorphism differs considerably between genomic regions, and polymorphisms are particularly abundant within genes (Vaughn et al. 2007). This probably has to do with the mechanisms that generate and maintain DNA methylation. For instance, the methylation of transposable elements is often guided by small RNAs and can be actively restored de novo, which leads to high levels of TE methylation but limited methylation polymorphism among different accessions. In contrast, many *A. thaliana* genes have DNA methylation that is heritable but fairly unstable and that apparently is not restored de novo after loss of methylation marks. Over time, this seems to lead to high levels of genic methylation polymorphism between different accessions that don't seem to reflect changes in gene expression or isolation by distance patterns (Vaughn et al. 2007).

High levels of DNA methylation variation within and between populations are also detected in several other plant species including cotton (Keyte et al. 2006), wild barley (Li et al. 2008), mangrove trees (Lira-Medeiros et al. 2010) and Mediterranean violets (Herrera and Bazaga 2010). Several such studies in non-model species have used MS-AFLP analysis to quantify DNA methylation polymorphisms at anonymous marker loci throughout the genome. In these studies, population genetic measures of diversity within or among populations were often significantly higher for methylation-sensitive markers than for normal (methylation-insensitive) genetic markers, i.e. there was often both greater diversity within populations and greater population differentiation at the epigenetic level than at the genetic level.

16.5.2 Natural Epigenetic Variation May Be Partly Autonomous

In principle, only epigenetic variation that is not under complete control of DNA sequence variation has the potential to explain phenotypic variation beyond that already explained by DNA sequence variation. It is clear that heritable, natural epigenetic variation is sometimes not autonomous, but under genetic control (e.g. FLC as discussed above). This genetic control can not only be direct, but also indirect through small RNAs, as among-accession differences in DNA methylation can be controlled by differences in accession-specific small interfering RNAs (Zhai et al. 2008), which presumably reflect genetic differences in siRNA-generating loci (such as TEs and repetitive sequences). Nevertheless, in the natural population studies described above, correlations between genetic variation and DNA methylation variation were often surprisingly weak. In *A. thaliana*, for instance, a matrix of pairwise similarities between individuals based on methylation polymorphisms was uncorrelated to a similarity matrix based on genetic polymorphisms at genomewide AFLP markers (Cervera et al. 2002), and patterns of gene-level methylation between *A. thaliana* accessions did not reflect genetic relatedness of the accessions (Vaughn et al. 2007).

Lack of correlation between genetic and epigenetic variation suggests that epigenetic variation may not be under strict genetic control. However, lack of correlation does not necessarily prove independence. For instance, in MS-AFLP versus AFLP comparisons, it is possible that MS-AFLP polymorphisms are under trans-acting genetic control (by loci that are not themselves part of the AFLP dataset). The data from natural populations are certainly consistent with partial autonomy of DNA methylation variation, but a more conservative conclusion that can currently be drawn is that many DNA methylation polymorphisms in natural populations are not under cis-acting genetic control (Cervera et al. 2002).

16.5.3 Epigenetic Variation Can Be Correlated to Adaptive Population Differentiation

If heritable epigenetic variation plays a role in adaptation, then local differences in ecological habitat characteristics may select for different epialleles in different populations. Just as with selection on genetic polymorphism, this will result in population-level associations between heritable epigenetic polymorphisms and ecological habitat characteristics. So far, such associations are unexplored for epialleles with well-characterized functional effects on gene expression or traits. However, some interesting population epigenetic observations have recently been made using methylation-sensitive markers on natural populations of *Dactylorhiza* orchid species (Paun et al. 2010), the mangrove *Laguncularia racemosa* (Lira-Medeiros et al. 2010) and the Mediterranean violet *Viola cazorlensis* (Herrera and Bazaga 2010, 2011). Each of these studies found correlations between epigenetic diversity and different habitats, but all three studies were performed on field collected material and did not control for environmentally induced epigenetic effects so the potential for these epigenetic effects to be involved in adaptation is unclear (Richards et al. 2010b).

For example, the *Viola cazorlensis* populations are genetically differentiated, and individual AFLP marker loci can be identified that show higher-than-expected population differentiation compared to genomic background levels of differentiation. Such 'outlier' loci are usually attributed to divergent natural selection between populations, which maintains population-specific alleles at genes closely linked to these loci under divergent selection, whereas the rest of the genome is more homogenized between populations. It turns out that these outlier genetic polymorphisms in *V. cazorlensis* are statistically correlated both to flower morphology and also to DNA methylation variation in plants collected from their natural habitat (Herrera and Bazaga 2008, 2010). Thus, adaptive genetic divergence may be associated with epigenetic differentiation between the populations. However, since Herrera and Bazaga (2010) were unable to grow the plants in common garden, many of these epigenetic differences could also be environmentally induced. Alternatively, this epigenetic differentiation could be a downstream consequence of selectively maintained genetic variation. In another study, Herrera and Bazaga (2011) found both DNA sequence and methylation polymorphisms in *V. cazorlensis* were correlated specifically with herbivory damage. Structural equation models suggested that genotype contributed directly to herbivory damage and epigenotype, but could not discriminate the relationship between epigenotype and herbivory damage. The two best models equally predict a consequential and causal role between epigenetic variation and herbivory suggesting that there could be a combination of effects that are induced by herbivory and affect the likelihood of herbivory. Another possibility is that random epigenetic mutations arise and build up rapidly within isolated populations, potentially resulting in (neutral) epigenetic differences between populations that correlate with genetic differentiation of the populations.

In summary, the use of anonymous MS-AFLP markers, which has dominated the work in more ecologically-oriented epigenetics research so far, has provided promising first insights from natural populations, in the wild and in non-model species. The high levels of natural epigenetic variation, its limited correlation with genetic variation and its association with adaptive population differentiation are all consistent with (but not conclusive evidence for) a role for epigenetic inheritance in adaptation and evolution. It is possible to investigate whether patterns of natural epigenetic variation in the field associate with phenotypes or environmental factors. However, such questions are currently unexplored and require common environment manipulations because of the environmentally labile nature of epigenetic effects (Richards et al. 2010b).

Ultimately, however, marker-level data provide only limited information. To gain a deeper understanding of the causes and consequences of the observed natural epigenetic variation, the next step should be to merge ecological approaches (linking epigenetic variation to fitness, ecological environments and natural populations), molecular approaches (gene-level information on sequence, epigenetic and activity status), and common garden experiments. Scoville et al. (2011) have made progress in combining these approaches by identifying a target gene that may be epigenetically modified and contribute to epigenetic inheritance of trichome density in *Mimulus guttatus*. The genetic basis of trichome production has been extensively studied in the model plants *A. thaliana* and *Antirrhinum majus,* and Scoville et al. were able to select homologs in *M. guttatus* of genes known to be involved in trichome development. They examined the relationship between expression of candidate genes and inheritance of damage-induced trichome production in high and low trichome parental lines and four recombinant inbred lines (RILs) exposed to damage and control conditions. Their findings indicate that down-regulation of *MgMYBML8* is correlated with the inheritance of increased trichome density in one of the parents and three of the four RILs. However, their study does not explore epigenetic mechanisms that may be involved in regulating this expression, which merits further work (Richards and Wendel 2011). It is this challenge of grafting detailed epigenomic tools onto an ecological genetics approach that will eventually provide a deeper understanding of natural epigenetic variation.

16.6 Impact of Epigenetics on Our Understanding of Evolutionary Concepts

The evolutionary relevance of epigenetic inheritance has been much discussed in recent years (Richards 2006; Bossdorf et al. 2008; Johannes et al. 2008; Jablonka and Raz 2009; Richards et al. 2010a, b). Mechanisms that generate heritable variation are a driving force behind all evolution. The heritable modulation of gene activity through epigenetic inheritance could represent a variation-generating mechanism, in addition to mutation (that creates novel gene polymorphisms) and the joint processes of recombination and segregation (that create novel gene combinations). Because heritable epigenetic variation can be induced by environmental conditions, it has been argued that epigenetic inheritance can be a mechanism for 'soft inheritance', where an environmentally-induced phenotype is transmitted to offspring generations (Richards 2006). This resembles Lamarckism and the inheritance of acquired characters, a concept that was dismissed long ago in the modern evolutionary synthesis but that some researchers argue deserves re-evaluation (Jablonka and Lamb 1989, 1995; Gissis and Jablonka 2011).

16.6.1 Selection on Heritable Epigenetic Variants

In a strict sense, the evolutionary implications of epigenetic inheritance are due to phenotypic effects of epiallelic variants that are (at least partially) independent of DNA sequence variants and that are exposed to natural selection, thereby affecting population responses to selection in ways that cannot be explained by DNA sequence variation alone. Independence or partial independence of epigenetic variation from genetic variation causes partial de-coupling of phenotypic change and genotypic change. As discussed above, this can arise, for instance, due to imperfect maintenance of heritable epigenetic marks, or by environment-induced epigenetic modifications that are subsequently stably transmitted to offspring generations. In addition, epiallelic variants arise more frequently and are more inducible and reversible than DNA sequence mutations. These features can affect micro-evolution in several ways, as described below.

16.6.1.1 Epigenetic Inheritance Facilitates Exploration of the Adaptive Landscape

A dynamic and reversible epigenetic code can add adaptive flexibility to the more stable and hard-wired genetic code. In principle, through random epigenetic variation a single genotype can heritably vary in different phenotypic directions, permitting the exploration of novel niches without abandoning the old one. Pal and Miklos (1999) modeled the consequences of epigenetic inheritance for adaptation, and found that the ability to generate heritable epigenetic variation can speed up the process of reaching a fitness peak in the adaptive landscape. It may also facilitate peak shifts, or the transition from one fit genotypic state to another fit genotypic state despite reduced fitness of intermediate genotypic states. This is because under randomly generated epigenetic variation, some individuals of a reduced-fitness intermediate *genotype* may still possess a high-fitness and heritable *phenotype* due to the added epigenetic contribution to the phenotype, thus effectively flattening the fitness landscape. After approaching a novel fitness peak, processes of canalization and genetic assimilation may take over and genetically stabilize the novel phenotype (Pal and Miklos 1999; see also Sect. 16.6.2.2).

High epigenetic mutation rates could particularly enhance the adaptive possibilities of asexual or low-diversity taxa, or in rapidly changing environments, when the rate of genetic change can be a limiting factor for generating novel variation (Jablonka and Lamb 1989). A concrete example of when epigenetic effects may be a particularly important source of phenotypic variation is in the expansion of invasive species. Understanding local adaptation in plant invasions has been challenging given the likelihood of reduced genetic variation following a population bottleneck, which is assumed to severely constrain the evolutionary potential of a given population or species. Although many invasive species benefit from alternative sources of increased DNA sequence variation through multiple introductions (Durka et al. 2005; Lavergne and Molofsky 2007; Rosenthal et al. 2008; Gammon and Kesseli 2009) or hybridization (Daehler and Strong 1997; Pysek et al. 2003; Bímová et al. 2004; Mandák et al. 2004; Bailey et al. 2009), several invasives appear to do well even with low levels of sequence based variation (Hollingsworth and Bailey 2000; Dlugosch and Parker 2008a, b; Richards et al. 2008; Loomis and Fishman 2009). Despite the observation of decreased sequence based variance in these studies, only one reported a substantial decline in phenotypic variance (Simberloff et al. 2000). Rapp and Wendel (2005) argue that even with reduced genetic variation, epigenetic effects could expand the phenotypic possibilities that may result from epigenetic modifications induced by genomic stresses, such as those caused by extreme environmental selection or ecological change which are often experienced by populations that go through a genetic bottleneck. This possibility is particularly relevant given that past hybridization or polyploidization may have produced latent evolutionary potential through the preservation of epigenetically silenced duplicate genes

that are poised to take on new function and contribute to phenotypic variation (Adams et al. 2003; Rodin and Riggs 2003; Rapp and Wendel 2005; Ainouche et al. 2009).

The chance sampling of genotypes involved in the invasion process, combined with non-DNA sequence based sources of phenotypic variation, can lead to divergence in phenotypes of these populations even in the absence of abundant sequence based variation and differential selection (Rapp and Wendel 2005; Keller and Taylor 2008; Prentis et al. 2008). For example, Richards et al. (2008) found that *Fallopia* populations have invaded a diversity of habitats on Long Island, NY and have persistent phenotypic variation with almost no sequence variation (Figure 16.6a, b). However, using MS-AFLP, Richards and colleagues showed that these populations harbor five times as many polymorphic epigenetic loci as DNA sequence loci (Fig. 16.6c, d; Richards, Schrey and Pigliucci unpublished).

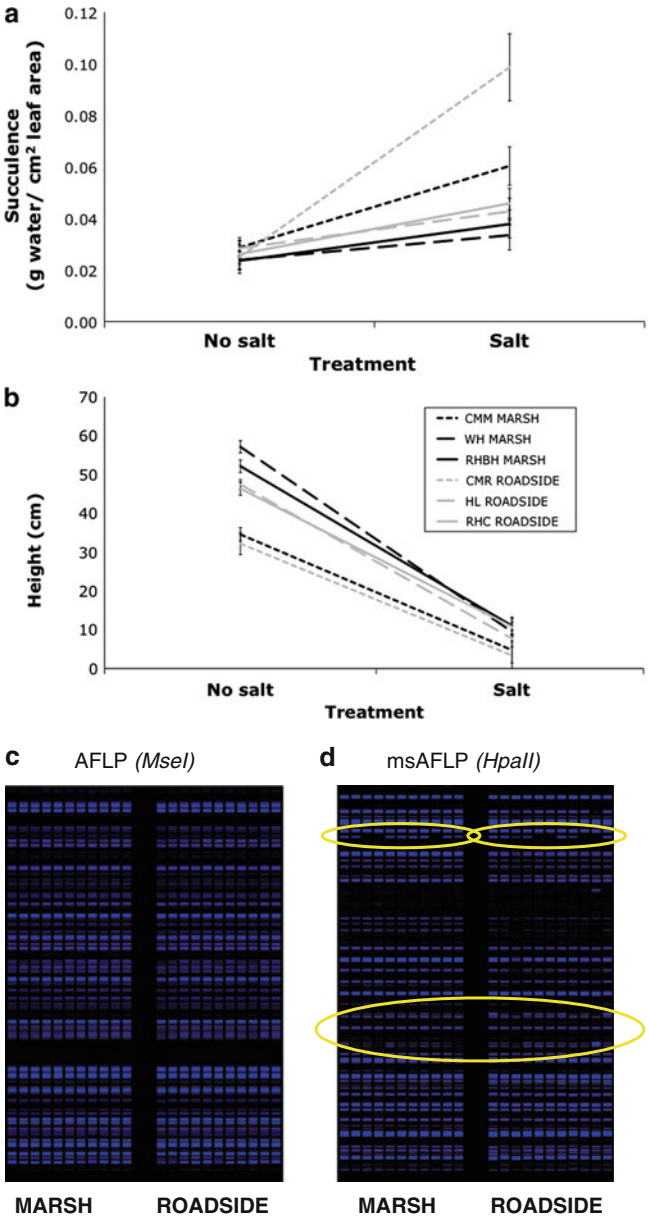

Fig. 16.6 *Fallopia japonica* and *F.* ×*bohemica* invading Long Island habitats. Shown are reaction norms (means ± 1 SE) for (**a**) succulence and (**b**) height of *Fallopia spp.* in response to two salt treatments for three road side and three salt marsh populations (From Richards et al. 2008). Also shown are (**c**) AFLP markers indicating no polymorphism across one of the roadside and one of the marsh populations, and (**d**) polymorphism within and among the same to populations for MS-AFLP (Epigenetic loci; Richards, Schrey and Pigliucci unpublished)

16.6.1.2 Epigenetic Inheritance Adds a Trans-generational Component to Phenotypic Plasticity

While undirected modifications will contribute to random epigenetic variation and can result in increased offspring trait variances, directed (targeted) modifications that occur in the same way in different individuals can result in shifted offspring trait means. In the latter case, epigenetic inheritance can be an underlying mechanism for trans-generational phenotypic plasticity (or 'maternal effects'; Richards et al. 2010a; Richards 2011; Scoville et al. 2011). Such trans-generational effects are commonly reported in the ecological literature, and may persist for more than one offspring generation. Particularly in cases where trans-generational effects involve highly specific stress responses (such as increased leaf trichome density in offspring of herbivory-exposed parental plants; Holeski 2007; Scoville et al. 2011) or persist for multiple generations (Whittle et al. 2009), authors have speculated that epigenetic inheritance could be an underlying mechanism. Thus, in contrast to genetic mutations, beneficial heritable modifications can be triggered simultaneously in multiple individuals in the population, thereby speeding up the population response to a changed environment (Jablonka and Raz 2009). The microevolutionary consequences of environment-induced parental effects can be diverse (Fox and Mousseau 1998) and they can impact both the rate and direction of evolutionary change in response to selection (Kirkpatrick and Lande 1989). By 'preparing' offspring for specific environmental conditions, parents can increase their offspring performance and fitness, as demonstrated both in the lab (Agrawal et al. 1999) and under natural field conditions (Galloway and Etterson 2007).

16.6.2 Selection on Genetic Variants Mediated by Epigenetic Change

In addition to selective effects of heritable epigenetic variants, epigenetic inheritance mechanisms can add to the evolutionary potential that is based on selective effects of genetic variants.

16.6.2.1 Epigenetic Control Over Transposable Elements Affects Rates of Genetic Change

Release of epigenetic TE silencing, which can occur in response to stressful conditions, can trigger sudden bursts of novel genetic variation due to transposition and associated processes. This adds raw material for natural selection during times of stress, when novel variation can be advantageous (McClintock 1984; Wessler 1996; Rapp and Wendel 2005), but also allows for novel genome function especially by way of regulatory genes and added transcription factor binding sites (Feschotte 2008). It is important to note that novel epigenetic variation (not just novel genetic variation) can be generated in this way as well, as release and subsequent re-silencing of TEs has the potential to generate novel epiallelic variation at functional genes that are physically close to TE insertion sites (Slotkin and Martienssen 2007; Paszkowski and Grossniklaus 2011). Several authors have pointed out potential macro-evolutionary consequences of stress-induced release of TE activity and silencing. Long periods of more or less stable TE silencing that are interrupted with moments of unleashed TE activity could account for a 'punctuated equilibrium' model of evolutionary change, when bursts of TE-induced genetic variation permit brief periods of rapid evolutionary change. Sudden TE-induced genome restructuring can displace a population far from its adaptive peak in the fitness landscape, facilitating the transition to other fitness peaks (Zeh et al. 2009). TE-induced genome restructuring might also result in genetic incompatibilities between subpopulations, leading to rapid reproductive isolation and speciation (Rebollo et al. 2010).

16.6.2.2 Epigenetic Change Can Initiate Heritable Change That Guides Subsequent Selection

An epigenetic code that is more dynamic and flexible than the genetic code can play an important role in initiating evolutionary change that is subsequently taken over by the more stable genetic code. Epigenetic modifications could 'hold' a potentially advantageous phenotype for multiple generations, allowing time for more stable genetic variants to stabilize the phenotype. This sequence of events has been proposed as a mechanism for genetic assimilation, the process by which an initially plastic phenotype that is stimulus-induced becomes heritably fixed and stimulus-independent (Waddington 1953). More generally, West-Eberhard (2005) argued that evolutionary change is commonly initiated by developmental plasticity (for instance in response to environmental stimuli) and is subsequently followed by selection of genetic variants that stabilize or otherwise accommodate the changed phenotype. According to this view, genes may be followers rather than initiators of evolutionary change. Rapid accumulation of epigenetic modifications can also be a first step in some macro-evolutionary processes. For instance, different populations may rapidly build up epigenetic differences, either due to epigenetic drift or environment-specific induction of epigenetic modifications, and such epigenetic differences can be a first steps towards reproductive isolation (Jablonka and Lamb 1998) because epigenetic incompatibilities can be responsible for compromised fitness of hybrids (Vrana et al. 2000, in Jablonka and Raz 2009). Once the process of reproductive isolation has started, it facilitates the evolution of genetic incompatibilities and speciation.

16.7 The Future of Ecological and Evolutionary Epigenetics

While molecular studies of epigenetic phenomena over the last few decades have revolutionized our understanding of genome function and developmental biology, these processes also challenge our understanding of heritable phenotypic variation at the level of whole organisms and even the process of evolution by natural selection (Jablonka and Lamb 1989; 1995; Danchin et al. 2011). In this chapter, we have discussed how epigenetic variation is associated with variation in phenotypic traits, that natural variation exists at the epigenetic level, that epigenetic variation can be induced by environmental stress and that some epigenetic marks are faithfully transmitted across generations. However, since previous work emphasizes agricultural crops and model species, frequently under artificial conditions, we still know little about the importance of epigenetic processes in natural populations (Richards 2008; Richards et al. 2010b). Fortunately, several researchers have begun to explore questions related to ecology and evolution in natural populations. The future of ecological and evolutionary epigenetics holds many more studies like those of Verhoeven et al. (2010a, b), Hererra and Bazaga (2010, 2011) and Scoville et al. (2011) which begin to address the question of epigenetic responses to the environment and ultimately epigenetic contributions to adaptation. This task will continue to be challenging because of the labile nature of epigenetic effects, the complicated relationship between epigenetic effects and DNA sequence variation and the tools required to tie functionality to epigenetic changes.

Because epigenetic variation is to some extent environmentally labile and reversible and many of the developmental processes that underlie response to different environments involve epigenetic changes, patterns of epigenetic differentiation among individuals that are measured in different environments will most likely include a reversible component (e.g., for FLC) that results in phenotypic

plasticity and a non-reversible or relatively stable component due to heritable epigenetic differentiation (e.g., *Lcyc* in *Linaria vulgaris*). As we have discussed recently (Richards et al. 2010b), analyses of epigenetic variation are similar to analyses of phenotypic variation, and common garden experiments are necessary to firmly establish inheritance of epigenetic effects and separate between plastic and heritable components of variation. As in classic analyses of phenotypic variation, demonstrating adaptation requires assessing response to reciprocal transplant studies in the field or studies in a controlled environment. In the case of epigenetic effects, a common environment approach will be even more critical to rule out the possibility that any association of epialleles is not merely a transient and environmentally induced association.

Another issue that will be important to consider in future studies is the level of independence of epigenetic variation. We are particularly interested in whether epigenetic variation that is unrelated to DNA sequence variation has the potential to explain phenotypic variation beyond that already explained by DNA sequence. Verhoeven et al. (2010a) have provided a first glimpse into the inheritance of induced methylation patterns for a single genotype in response to multiple environmental stresses. Expanding this approach to include (1) multiple genotypes and (2) associated phenotypes will be critical information to evaluate the importance of induced and heritable epigenetic changes.

Providing the association between epigenetic changes and phenotypic changes will be particularly challenging. A major limitation to our current understanding of epigenetics in natural systems is that marker-level data provide only limited information, and cannot typically be associated with phenotype. To gain a deeper understanding of the causes and consequences of the observed natural epigenetic variation, we must find a way to explore the phenotypic effects of specific epigenetic activity in natural settings. Scoville et al. (2011) have made some progress in this respect by targeting known genes that underlie inheritance of environmentally induced trichome formation in *M. guttatus*. The increasing application of next generation sequencing combined with epigenetic specific approaches (i.e. bi-sulfite sequence conversion or tiling microarrays) could be incorporated into ecological experimental design and should help in the process (Richards and Wendel 2011).

While there are few ecological epigenetics studies making steps in the right direction, we can continue in this quest by learning from the extensive studies in the field of ecological genetics, which has long been interested in deciphering organismal response to the environment and how natural selection leads to adaptation. Applying the new tools and understanding of epigenetics and genome function in general to a robust ecological design will be powerful for assessing the importance of these effects in the real world.

Acknowledgements The authors would like to thank Jonathan Wendel for the invitation to write this chapter as well as JF Wendel and X Zhang for feedback on previous versions of the manuscript. This work was supported by University of South Florida (CR), the Netherlands Organisation for Scientific Research (KJFV), and the Swiss National Science Foundation (OB).

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