Heritable Epigenetic Variation and its Potential Applications for Crop Improvement

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ABSTRACT Phenotypic variation within organisms is driven primarily by genetic diversity. However, there is a growing appreciation that epigenetic variation, resulting from a multitude of diverse chemical modifications to the DNA and chromatin, can have profound effects on phenotype. Heritable epigenetic marks persist through meiosis and can be stably transmitted to the next generation, resulting in transgenerational epigenetic inheritance. Importantly, when epigenetic changes occur near coding genes, affecting their transcriptional state, heritable epigenetic variation can result in heritable phenotypic variation. Large-scale interrogation of epigenome inheritance in *Arabidopsis* has revealed that spontaneous variation in DNA methylation occurs at a rate that is orders of magnitude greater than genetic mutation, indicating the key importance of epigenetic variation during evolution. Thus, there is a potential for epigenetics to play a role in crop improvement, including regulation of transgene expression and creation of novel epialleles. Here, we review cases of naturally occurring and genetically induced epialleles, and discuss how the studies from two epigenetic populations are rapidly increasing our understanding of epigenetic diversity.

Keywords Epigenetics, DNA methylation, siRNA, RNA-directed DNA methylation, Transgenerational inheritance, Epiallele, Natural variation, epiRIL

INTRODUCTION

The term epigenetics is generally used to refer to the study of heritable change in gene expression that is independent of DNA sequence variation (Berger *et al.* 2009; Haig 2004). Such change can occur at the DNA level through DNA methylation of cytosine residues or at the level of chromatin by posttranslational modifications of histones that influence the accessibility of the DNA to transcription activation. Epigenetic changes can result in altered gene transcription, and are an important mechanism in regulating gene expression during development and in response to environmental stimulation. Such epigenetic information represents the transcriptional memory associated with cell fate decisions, developmental switches, or stress responses; memory that often needs to be erased and reset during reproduction. By contrast, transgenerational epigenetic information refers to epigenetic marks that can be stably

transmitted through meiosis and inherited in the subsequent generation. Variation in this heritable epigenetic information is of particular agronomic interest as it too, like genetic variation, can result in phenotypic variation in the natural population.

The full extent to which epigenetic variation contributes to phenotypic variation remains to be determined (Richards 2011). Heritable traits that are caused by epigenetic changes are referred to as epialleles or epimutations. However, as discussed below, epialleles can be caused by genetic variations that are difficult to detect without comprehensive genome structure analysis. For example, epigenetic variation can often be caused by DNA methylation changes induced by gene duplication or transposon insertions (i.e. an obligated epiallele, Fig. 1) (Richards 2006). By contrast, pure epialleles that originate independently of any genetic variation are relatively rare (see Table 1 for summary). DNA methylation is the only epigenetic mark for which the mechanism of

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maintenance and stable inheritance is well understood (Law and Jacobsen 2010). Consequently, most of the naturally occurring epialleles identified and studied are associated with DNA methylation changes.

DNA methylation

DNA methylation is the addition of a methyl group to the 5-carbon of cytosine. In eukaryotic cells, this process is carried out by a family of DNA methyltransferase enzymes, which transfers methyl groups from the methyl donor S-Adenosyl methionine (SAM) to the cytosine. The resulting 5-methyl cytosine (5mC) is often repressive and can lead to gene silencing (Cedar and Bergman 2012). The repressive

nature of 5mC is thought to inhibit the binding of DNA by transcription factors (Watt and Molloy 1988) or to recruit additional chromatin proteins to form heterochromatic state that is inaccessible for transcription (Cedar and Bergman 2012). DNA methylation regulates diverse biological processes including cell differentiation, X-chromosome inactivation, transposon and gene silencing, and genomic imprinting (Law and Jacobsen 2010; Reik 2007).

DNA methylation in plants occurs in the contexts of CG, CHG and CHH (H = A, C, or T) (Cokus *et al.* 2008). DNA methylation in the CG context is carried out by METHYLTRANSFERASE1 (MET1), which is a homologue of the mammalian DNA METHYLTRANSFERASE1

Table 1. Examples of epimutation in plant.

Target	Trigger	Epigenetic Effect	Species	Phenotype	References
<i>CmWIP1</i>	<i>hAT</i> insertion	Spreading of DNA methylation to <i>CmWIP1</i>	<i>C. melo</i>	Promote female flower	Martin <i>et al.</i> 2009
<i>FWA</i>	<i>SINE</i> insertion	<i>SINE</i> DNA methylation and silencing of <i>FWA</i>	<i>Arabidopsis</i> genus	Late flowering	Soppe <i>et al.</i> 2000
<i>BONSAI</i> (<i>BSN</i>)	<i>ddm1</i>	Spreading of DNA methylation from <i>LINE</i> to <i>BSN</i> gene	<i>A. thaliana</i>	Stunted growth	Saze and Kakutani 2007
<i>booster1</i>	<i>MITE</i> repeats at upstream enhancer	Paramutagenic	<i>Z. mays</i>	Pigmentation	Stam <i>et al.</i> 2002
<i>PAI</i> genes	<i>PAI1-PAI4</i> inverted repeat	DNA methylation and silencing of all <i>API</i> genes	<i>A. thaliana</i>	Heavy methylation of all <i>API</i> genes	Bender and Fink 1995
<i>FOLT1</i>	<i>FOLT2</i> complex duplication	DNA methylation and silencing of <i>FOLT1</i>	<i>A. thaliana</i>	Reduced fertility	Durand <i>et al.</i> 2012
<i>SUPERMAN</i> (<i>SUP</i>)	Mutagenesis screens	DNA methylation and silencing of <i>SUP</i>	<i>A. thaliana</i>	Floral organ defects	Jacobsen and Meyerowitz 1997
<i>Lcyc</i>	Naturally occurring	DNA methylation and silencing of <i>Lcyc</i>	<i>L. vulgaris</i>	Change in floral symmetry	Cubas <i>et al.</i> 1999
<i>CNR</i>	Naturally occurring	DNA methylation and silencing of <i>CNR</i>	<i>S. lycopersicum</i>	Fruit ripening defect	Manning <i>et al.</i> 2006
<i>OsSPL14</i>	Naturally occurring	<i>OsSPL14</i> Promoter hypomethylation	<i>O. sativa</i>	Panicle branching and higher grain yield	Miura <i>et al.</i> 2010
<i>DWARF1</i> (<i>DI</i>)	Naturally occurring	DNA methylation and silencing of <i>DI</i>	<i>O. sativa</i>	<i>Dwarf</i>	Miura <i>et al.</i> 2009
<i>OsFIE1</i>	Naturally occurring	<i>OsFIE1</i> Promoter hypomethylation and ectopic <i>OsFIE1</i> expression	<i>O. sativa</i>	<i>Dwarf</i>	Zhang <i>et al.</i> 2012b

(DNMT1) (Vongs *et al.* 1993). Arabidopsis *met1* mutant has very little CG methylation and displays defects in morphology and fertility (Law and Jacobsen 2010; Zemach *et al.* 2010b). CHG methylation in Arabidopsis is maintained by the plant-specific DNA methyltransferase CMT3 whose chromodomain recognizes and binds to H3K9me2 marks (Bartee *et al.* 2001; Johnson *et al.* 2007; Lindroth *et al.* 2001). Both CG and CHG sites are symmetrical in that similar sequences exist on the complementary DNA strand. Upon DNA replication, hemi-methylated CG and CHG sites are recognized, and methylation on the newly synthesized strand is restored by MET1 and CMT3, respectively.

By contrast, methylation of the CHH sites has to be established and maintained by the siRNA directed DNA methylation (RdDM) pathway (Henderson and Jacobsen 2007; Matzke *et al.* 2009; Wassenegger *et al.* 1994). Lack of siRNAs leads to progressive loss of methylation on CHH sites. The production of siRNAs involves three core components: DNA-dependent RNA-Polymerase IV (Pol IV), RNA-dependent RNA Polymerase 2 (RDR2) and endoribonuclease DICER-LIKE 3 (DCL3). Pol IV preferentially targets transposons and repetitive elements to produce single-stranded RNA. The RNA was replicated by RDR2 to form double-stranded RNA, which is then processed by DCL3 into 24-nucleotide siRNAs. During *de novo* methylation, plant-specific RNA Polymerase V (PolV) and associated factors produce nascent transcripts from target loci that are recognized by siRNA-containing AGO4 complexes and recruit DRMs *de novo* methyltransferases to target loci for DNA methylation (Haag Pikaard 2011).

DNA methylation is an evolutionarily ancient process that is associated with gene silencing in eukaryotes. It has long been proposed to function as a genomic defense mechanism against invading transposable elements (TEs) (Law and Jacobsen 2010). DNA methylation is generally observed in heterochromatic regions like centromeres and pericentromeres, which consist mainly of TEs and repeats, and is the major epigenetic silencing mechanism to ensure their immobility (Law and Jacobsen 2010; Li *et al.* 2008; Wang *et al.* 2009; Zhang *et al.* 2006; Zilberman *et al.* 2007). DNA methylation is also seen in TEs and repeats in

euchromatic regions, including coding sequences (Cokus *et al.* 2008; Zilberman *et al.* 2007). DNA methylation is crucial for proper development. Defects in DNA methylation lead to embryonic lethality in mammals and developmental defects in plants. DNA hypermethylation in promoter or near transcription start sites can result in transcriptional silencing, and promoter demethylation and concomitant transcriptional activation has been demonstrated for many imprinted genes in plants (Gehring *et al.* 2006; Gehring *et al.* 2009; Hsieh *et al.* 2009; Hsieh *et al.* 2011; Waters *et al.* 2013; Waters *et al.* 2011; Zemach *et al.* 2010a).

Transgene silencing

Transgenic plants are routinely generated for crop improvement or for research purposes. The expression level of transgenes varies greatly among independent transgenic lines (Holtorf *et al.* 1995; Jones *et al.* 1985; Peach and Velten 1991). This variation is caused by many factors, including insertion location, copy number, and orientation of the inserted transgene. Desirable transgenic lines can be evaluated and selected from multiple transgenic lines. However, the transgene may become silenced in following generations, posing a more serious challenge in applying genetic engineering to crop breeding.

Transgene silencing is a typical epigenetic phenomenon that attracts broad attention. Although many questions remain, much has been learned about the molecular mechanisms of transgene silencing. Transgenes may be silenced at transcriptional level (transcriptional gene silencing, TGS), or at posttranscriptional level (posttranscriptional gene silencing, PTGS). A classic example of PTGS is sense-cosuppression observed in petunia (Napoli *et al.* 1990; Que *et al.* 1997). In transgenic petunia plants overexpressing a rate-limiting enzyme for anthocyanin production, the transgene and endogenous gene are simultaneously silenced, converting uniformly pigmented flowers to completely white or patterned flowers. The underlying mechanism is that the transgene mRNA is processed into abundant trans-acting siRNAs (tasiRNAs), which target and cleave the transcripts of both transgene and endogenous gene (De Paoli *et al.* 2009). Transgene silencing through PTGS is often less stable, because the process is reset at certain developmental stages in each

generation. By contrast, when a transgene is transcriptionally silenced, the silent state is often stably inherited across generations. This is because DNA methylation in the promoter or downstream regulatory sequences is a common cause for TGS, and as discussed above, symmetric CG methylation is faithfully maintained across generations (Dalakouras *et al.* 2012; Mathieu *et al.* 2007). In addition, during gametogenesis and early seed development, mechanisms exist to reinforce DNA methylation through RdDM on the gamete genomes (Hsieh *et al.* 2009; Mirouze *et al.* 2009; Slotkin *et al.* 2009).

Epimutations induced by genetic variation

Many of the known epimutations are induced by TE insertions or genome structural variation such as gene duplication. For example, insertion of a *hAT* transposon near the transcription factor gene *CmWIP1* induces DNA hypermethylation and transcription silencing of the *CmWIP1* gene in melon (Fig. 1 (A); Martin *et al.* 2009). Expression of *CmWIP1* leads to carpel abortion and promotes development of male flowers while silencing of *CmWIP1* gene suppresses anther development and produces female flowers. Thus the methylation state of *CmWIP1* gene determines the unisexual nature of melon flower. Consistent with the nature of epimutations, silent *CmWIP1* locus occasionally reverts to active state with reduced DNA methylation and reverted floral phenotype.

The Arabidopsis *FLOWERING WAGENINGEN* (*FWA*) gene is only expressed in endosperm from the maternal genome, but is transcriptionally silenced in embryo and in all other developmental stages (Kinoshita *et al.* 2004). In wild-type plants, *FWA* is silenced due to DNA hypermethylation of the *SHORT INTERSPERSED ELEMENTS* (*SINEs*) in the 5' regulatory region, and plants flower early. In *fwa* epimutant, *SINEs* DNA methylation is lost and *FWA* is de-repressed, causing significant delay in flowering time (Fig. 1 (B); Kinoshita *et al.* 2007; Soppe *et al.* 2000). A sequence survey revealed that *SINE* repeats structure in the *FWA* promoter is highly diverse among Arabidopsis accessions, indicating each repeat insertion derived independently (Fujimoto *et al.* 2008).

Another well-studied epiallele, *bonsai* (*bsn*), was isolated as a *ddm1* induced epimutation. DDM1 encodes a

SWI/SNF-like ATP-dependent chromatin remodeler, and is required for maintenance of DNA methylation in Arabidopsis (Jeddeloh *et al.* 1999; Lippman 2004). Loss of DNA methylation in *ddm1* background de-repressed a non-LTR retroelement located downstream of the *BSN* coding sequence, resulting in hypermethylation and silencing of *BSN* gene (Fig. 1(C); Saze and Kakutani 2007). Although *bonsai* is not a natural epimutant, it was reproducibly induced during self-pollination of the *ddm1* mutant. Its discovery suggests the existence of cryptic epigenetic variation in plant. The molecular phenotype, increase in *BONSAI* methylation or *ibm*, can be phenocopied by mutations in a *jmjC* domain containing gene *IBM1* which encodes a H3K9me2 demethylase that functions to block non-CG methylation in genic sequences (Saze *et al.* 2008).

Paramutation involves communication between homologous loci *in trans*, and results in altered DNA methylation and heritable chromatin change. One of the best-studied paramutation systems is the maize *booster1* (*b1*) gene. The paramutable *B-I* allele is active and causes intense pigmentation, whereas the paramutagenic *B'* is weakly transcribed with little pigmentation. In hybrids between *B-I* and *B'*, the transcription and chromatin state of *B-I* allele is converted to that of the *B'* allele, causing a dramatic decrease in pigment production. The change of *B-I* to *B'* is heritable and the newly converted *B'* becomes paramutagenic. A tandem repeat of seven *MITEs* in an enhancer region located 100-kb upstream of the *b1* coding sequence has been shown to be required for paramutation. The *MITE* repeats are hypomethylated in the paramutable *B-I* allele and hypermethylated in the paramutagenic *B'* allele (Fig. 1 (D); Haring *et al.* 2010; Stam *et al.* 2002). Genetic and molecular studies indicate that components of the RdDM pathway are required for the maintenance of paramutagenic states in maize (Alleman *et al.* 2006; Erhard *et al.* 2009; Hale *et al.* 2009; Hollick *et al.* 2005; Stonaker *et al.* 2009). Interestingly, transgenes carrying *b1* enhancer repeats can ectopically convert *B-I* into paramutagenic *B'* state, providing insights into possible applications for crop improvement (Belele *et al.* 2013).

In addition to TEs and repeats induced epigenetic variation, gene duplication and rearrangement can also

induce epigenetic changes. In *Arabidopsis Columbia* (Col) ecotype, the *PHOSPHORIBOSYL ANTHRANILATE ISOMERASE (PAI)* gene family of the tryptophan biosynthesis pathway contains three unlinked genes, *PAI1*, *PAI2*, and *PAI3*. But in Wassilewskija (Ws) ecotype an additional copy, *PAI4*, is present in a tail-to-tail orientation with *PAI1*, and all four loci carry heavy DNA methylation.

Mutant plants with the *PAI1-PAI4* locus deleted have reduced DNA methylation at remaining *PAI2* and *PAI3* loci, indicating their methylation is caused by *PAI1-PAI4* (Fig. 1(E); Bender and Fink 1995). Further experiments confirmed that the *PAI1-PAI4* inverted repeats induce DNA methylation of all *PAI* genes via RdDM (Bender 2004).

Fig. 1. Mechanisms of epimutation induced by genetic variation.

- (A) Insertion of the *hAT* transposon upstream of the *CmWIP1* gene led to DNA hypermethylation and silencing of *CmWIP1* gene, resulting in inhibition of male sexual organs development in melon. Reversion by loss of DNA methylation restores bi-sexual flower development.
- (B) In wild-type *Arabidopsis*, *SINE* elements near *FWA* promoter are methylated and *FWA* is silenced, resulting in early flowering. In the *fwa* epiallele, *SINE* elements are hypomethylated and *FWA* is de-repressed, resulting in late flowering.
- (C) In wild-type *Arabidopsis*, *LINE* elements next to *BONSAI (BSN)* gene are methylated. In *ddm1* mutants, *LINE* DNA methylation spreads toward *BSN* coding sequences, causing DNA hypermethylation and silencing of *BSN*.
- (D) The paramutagenic *B'* allele contains seven methylated *MITE* repeats in an enhancer region 100-kb upstream of the *booster1 (b1)* gene whereas in the paramutable *B-I* allele, the *MITE* repeats are hypomethylated. When both *B'* and *B-I* are present, the active *B-I* allele is converted to the inactive *B'* state in a stable and heritable fashion.
- (E) The *Arabidopsis Col-0* ecotype has 3 unlinked, active *PAI1*, *PAI2*, *PAI3* genes. In WS ecotype, an extra copy, *PAI4*, is present in a tail-to-tail orientation with *PAI1*. This *PAI1-PAI4* inverted repeat induces heavy DNA methylation in all four WS *PAI* genes.
- (F) In Sha accession, *FOLT2* is a complex duplicated locus with multiple truncated copies that produces siRNAs and silence *FOLT1* by RdDM. By contrast, Col-0 only has an active *FOLT1* copy and lacks *FOLT2*. This copy number induced epigenetic variation was not revealed until close examination of sterile RIL progenies that inherited only the silenced *FOLT1* from Sha, and chromosome segments from Col-0 that lacks *FOLT2*.

Another example of epigenetic variation induced by gene duplication is involved in genetic incompatibility between Col-0 and Shahdara (Sha) ecotypes. Col-0 has only one copy of the *FOLATE TRANSPORTER* gene, *FOLT1*, on chromosome 5 while Sha has an additional copy, *FOLT2*, on chromosome 4. The *FOLT2* is a complex locus with multiple truncated copies that produces siRNAs and silence *FOLT1*, but not *FOLT2*, in Sha. In a recombinant inbred line (RIL) population generated from a cross between Sha and Col-0, the silent *FOLT1* is stable in the absence of silencing inducer *FOLT2*. The RILs that inherited the silenced *FOLT1* copies from Sha, and chromosome 5 segments from Col-0 that lacks *FOLT2*, have reduced fertility due to lack of *FOLT* activity (Fig. 1 (F); Durand *et al.* 2012).

Epimutations that are not accompanied by genetic changes

One of the first plant epiallele was *clark kent* (*clk*) in Arabidopsis, which exhibits DNA hypermethylation at the *SUPERMAN* locus. The *clk* epiallele has phenotypes similar to loss-of-function mutants of *SUPERMAN*, with increased numbers of stamens and carpels. The unstable *clk* epiallele can revert back to normal phenotypes, and is accompanied by a reduction in DNA methylation at the *SUPERMAN* locus (Jacobsen and Meyerowitz 1997). Another example was a naturally occurring mutant of *Linaria vulgaris*, *peloric*, which changed the symmetry of the flower from bilateral to radial. The mutant flower is caused by promoter hypermethylation and silencing of *Lcyc*, a *CYCLOIDEA* gene homolog known to regulate floral asymmetry in *Antirrhinum*. The resulting epimutant has been stably maintained for hundreds of years. Consistent with the nature of an epiallele, mutant flowers occasionally reverted back to wild-type flowers, which are accompanied by loss of hypermethylation in *Lcyc* gene (Cubas *et al.* 1999).

The tomato fruit-ripening mutant *colorless non-ripening*, *cnr*, is a well characterized, naturally occurring epiallele of *CNR*, which encodes an SBP-box transcription factor. In this mutant, a segment of *CNR* promoter is hypermethylated, resulting in silencing of *CNR* expression and inhibition of normal ripening (Manning *et al.* 2006). Using chromatin

immunoprecipitation approach, the MADS-box transcription factor *RIPENING INHIBITOR* (*RIN*), an essential ripening regulator, was shown to interact with the promoters of genes important for the ripening processes, including *CNR* (Martel *et al.* 2011). Interestingly, the binding of *RIN* to the *CNR* promoter is greatly reduced in *cnr* mutant (Martel *et al.* 2011), supporting the model that DNA hypermethylation inhibits transcription by preventing binding of transcription factors.

Several epialleles affecting development or agronomic traits have been discovered in rice. The quantitative trait locus *WEALTHY FARMERS PANICLE* (*WFP*) encodes *OsSPL14*, was shown to promote panicle branching and higher grain yield in rice. The rice strain ST-12 that carries *OsSPL14^{WFP}* trait has much higher expression of *OsSPL14* in young panicles compared to Nipponbare. While no sequence variation was detected around *OsSPL14* gene in these two strains, several cytosines in the promoter were found to have high DNA methylation in Nipponbare, correlating with its lower expression level (Miura *et al.* 2010). The rice *epi-d1* allele showed DNA hypermethylation and epigenetic silencing of the *DWARF1* (*D1*) gene. The silenced state is associated with repressive histone and DNA methylation marks. The *epi-d1* epiallele is not stable and its dwarf phenotype correlates with DNA methylation and down-regulation of *D1* expression (Miura *et al.* 2009). The epimutation of *OsFIE1*, *epi-df*, is caused by DNA hypomethylation in the promoter, and ectopic expression, of *OsFIE1* gene. Ectopic expression of *OsFIE1* gene is also accompanied by reduced H3K9me2 and increased H3K4me3 levels, and resulted in alternation of H3K27me3 levels in hundreds of genes and a heritable dwarf phenotype (Zhang *et al.* 2012b).

Environment induced epigenetic variation

Plants, being sessile, must respond to environmental stresses through physiological changes. It is well documented that chromatin modifications play a role in plants' response to environmental stimuli. More recently, a growing number of reports have demonstrated potential change in DNA methylation can be induced by various biotic and abiotic stresses (Lang-Mladek *et al.* 2010; Pecinka *et al.* 2010; Tittel-Elmer *et al.* 2010). In order for the environmental

stimuli to induce stable epigenetic change, the perceived stimuli must be propagated and perpetuated through cell divisions. Several studies looked at the stability of epigenetic states under various stress conditions and found that silencing of reporter transgenes, as well as endogenous loci, is released upon various abiotic stresses such as high salinity, draught, heat, or UV radiation (Lang-Mladek *et al.* 2010; Pecinka *et al.* 2010; Tittel-Elmer *et al.* 2010). However, such stress-induced transcriptional activation is mostly transient, and silencing is rapidly restored upon returning to optimal growth conditions. Other studies suggest a more widespread and potentially stable DNA methylation changes among different environmental conditions. Lira-Medeiros *et al.* (2010) applied methylation sensitive amplification polymorphism (MSAP) method to study genetic and epigenetic (CpG methylation) variation in two mangrove tree populations grown in different water stress environments. Trees grown adjacent to river are larger, taller, and have higher CpG methylation compared to trees grown in nearby high salinity ground. Interestingly, cytosine methylation polymorphism is greater than genetic variation, in both populations. Colaneri and Jones (2013) simulated drought condition and examined DNA methylation and transcription profile changes in *Arabidopsis* seedlings. Among the ~1,000 genes with expression level changes, 316 showed altered DNA methylation level near their transcriptional start sites. Jin *et al.* (2013) found substantial changes in DNA methylation in the promoter regions of the *ETHYLENE RESPONSIVE FACTOR 6 (ERF6)*, *SUPPRESSION OF RVS 161 DELTA 4 (SUR4)* and *3-KETOACYL-COA SYNTHASE 13 (KCS13)*, which regulate cotton fiber growth, over the course of one year. Together these observations suggest a dynamic DNA methylation plasticity in response to growth environments.

Biotic stress is widely studied environmental effects that have also been implicated in changing DNA methylation and creating heritable epigenetic variation. Using the apomictic dandelions that reproduce through unfertilized seeds, Verhoeven *et al.* (2010) examined epigenetic alterations induced by various biotic stresses in the absence of genetic variation. The authors used MSAP to profile DNA methylation difference among dandelion populations that were treated with chemicals to induce herbivore and

pathogen defenses, and found considerable methylation variation throughout the genome. Furthermore, many DNA methylation changes can be faithfully transmitted to the subsequent generation, suggesting that stress-induced methylation changes are common and can be heritable. Luna *et al.* (2012) showed that *Arabidopsis* exposed to pathogens induces systemic acquired resistance (SAR) and produces progeny that are primed for defense response, indicating a transgenerational inheritance of SAR response. Supporting the observation, genome-wide DNA methylation and transcription profiling identified widespread DNA methylation, and associated gene expression changes in response to biotic stress (Downen *et al.* 2012).

Stress-induced epigenetic variation can also arise from activation and mobilization of transposons. The genomes of higher plants consist mainly of TEs and repeats. For example, up to 85% of the maize genome is composed of TEs, particularly the retroelements (Schnable *et al.* 2009). Thus keeping majority of the genome epigenetically silenced is a daunting task and requires a suite of robust and highly regulated silencing mechanisms. Thus it is not surprising to find that even in global DNA hypomethylation mutant *met1* or *ddm1* backgrounds, many TEs are de-repressed but rarely mobilized (Tsukahara *et al.* 2009). As discussed earlier that heat stress destabilizes transcriptional gene silencing, the *Arabidopsis* retroelement *ONSEN* was transcriptionally activated in response to heat stress, resulting in synthesis and accumulation of extrachromosomal *ONSEN* DNA copies. Significantly, a high frequency of new *ONSEN* insertions was observed in the progeny of stressed siRNA pathway mutants, but not in stressed wild-type progeny. Furthermore, *ONSEN* insertions confer heat responsiveness to nearby genes. Thus, stress-induced epigenetic changes and mobilization of TEs can confer new regulatory circuitry to nearby genes, generating novel phenotypic traits (Ito *et al.* 2011). These observations resemble the Sicilian blood orange that arose from an insertion of a *copia*-like retrotransposon adjacent to a gene encoding RUBY, a MYB transcription activator of anthocyanin production. The retrotransposon provides a regulatory network that confers cold dependency of *RUBY* expression (Butelli *et al.* 2012).

Unlocking the epigenome for crop improvement

Throughout the life cycle, organisms are constantly exposed to chemicals, nutritional deficiency, or subject to influence of maternal behavior shown to cause altered gene expression that can persist and sometimes be transmitted to the next generation (Anway *et al.* 2005; Cooney *et al.* 2002; Dolinoy *et al.* 2006; Kaminen-Ahola *et al.* 2010; Vandegehuchte *et al.* 2010; Weaver *et al.* 2004). In honeybees, larvae fed with royal jelly develop into queens while larvae fed with regular food become worker bees. Experimental down-regulation of a honeybee DNA methyltransferase *Dnmt3* can result in increased number of queens from the larvae not fed with royal jelly, indicating that nutritional input stored as epigenetic information can be used for developmental decision-making in insect (Kucharski *et al.* 2008). Similarly, the variable yellow *agouti* (A^{vy}) mouse whose coat color variation correlates with epigenetic marks established early in development, has been used to study the impacts of nutritional and environmental influences on the epigenome of the fetus. Animal fed with methyl-rich diet such as folic acid has higher DNA methylation level at the A^{vy} locus, and ethanol exposure led to transcriptional silencing and hypermethylation at the A^{vy} locus (Dolinoy 2008; Dolinoy *et al.* 2006; Kaminen-Ahola *et al.* 2010; Waterland and Jirtle 2003; Waterland *et al.* 2007). These observations indicate that modulating expression of epigenetic maintenance enzymes such as DNA methyltransferases or methyl donor SAM biosynthesis genes can have profound effects on the integrity of epigenome. Indeed, several recent chemical genomic screens have identified small molecular compounds that can suppress epigenetic silencing of transgene reporters. Intriguingly, folic acid metabolism and the methyl donor SAM biosynthesis pathway are frequent targets of these small molecule inhibitors (Dinh *et al.* 2013; Noutoshi *et al.* 2012; Zhang *et al.* 2012a).

Attempts to generate epigenetic diversity in crop species have been hindered by the lack of global DNA methylation mutants such as *met1* and *ddm1*. Instead, chemical inhibition of epigenetic maintenance or regulation provides an attractive alternative. Akimoto *et al.* (2007) subjected rice seeds to methylation inhibitor 5-azadeoxycytidine treatments during germination. The surviving progenies were propagated

in the field for over 10 years, and some of the altered phenotypic characters were stably inherited, including a line that showed acquired resistance to a bacterial pathogen *Xanthomonas oryzae* due to promoter DNA hypomethylation and induced expression of a disease resistant gene *Xa21G*.

By contrast, rapid progress has been made in unveiling phenotypic diversity and plasticity masked in wild type *Arabidopsis* by DNA methylation. In particular, there is a great deal of interest and excitement in the comprehensive analyses of epigenetic diversity using recombinant inbred populations generated from parental strains that differed only in DNA methylation. These so called “epi-recombinant inbred lines”, or epiRILs, were created by crossing DNA methylation mutants (i.e., *met1* and *ddm1*) to wild-type plants. The resulting inbred lines carry homozygous, epigenetically diverge genomic segments inherited from wild-type and hypomethylated parents. The epiRILs displayed variable and heritable morphological or developmental traits, including flowering time, plant height, biomass, TE mobilization, salt and pathogen responses (Johannes *et al.* 2009; Reinders *et al.* 2009). Examining the DNA methylation profiles of individual epiRILs confirmed that segments of parent-specific epi-alleles are visible and can be maintained within the epiRILs for at least eight generations (Reinders *et al.* 2009). Together these studies provided conceptual and technological breakthrough to our understanding, and potential application, of epigenetic variation and inheritance. They also provide the first insight into the extent of phenotypic variation generated by random combination of parent-specific epigenomes. It is thus likely that heritability of epigenetic variation can contribute to a significant degree in complex phenotypic traits. Supporting the theory, when a larger panel of epiRILs was tested under ecologically realistic conditions, they were found to display heritable phenotypic diversity and exhibit heritability patterns resembling those of the natural accessions in most of the traits examined (Roux *et al.* 2011). In another independent study, epiRILs were tested for responses to biotic stress induced by jasmonic acid and salicylic acid, and found to carry significant heritable variations in growth rates and in responses to defense hormones (Latzel *et al.* 2012). Furthermore, when biomass, response to drought, and nutrient ability were considered as traits of ecological

importance, epiRILs further confirm the notion that variations in DNA methylation can cause substantial heritable variations of these ecologically important traits, suggesting possible evolutionary selection on epigenetic variation (Latzel *et al.* 2013; Zhang *et al.* 2013).

Frequency of spontaneous epigenetic variation

The meiotically heritable epialleles discussed above, such as *peloric* and *cnr*, are thought to occur spontaneously in their respective populations. However, it is not possible to know how frequent such spontaneous heritable epialleles could arise in the natural population.

Using model plant *Arabidopsis*, two recent studies provided an intriguing answer to the question. Both studies used whole genome methylation sequencing technique to examine spontaneously occurring variation in DNA methylation at base-pair resolution by comparing plants propagated by single-seed descent for 30 generations. Significantly, both studies found that the rate of spontaneous variation in DNA methylation was at least four orders of magnitude greater than spontaneous genetic mutations (Schmitz *et al.* 2011; Becker *et al.* 2011). On average, about 10% of all methylated cytosines have increased or decreased methylation in at least one of the progenies examined (Becker *et al.* 2011). Interestingly, the distribution of these differentially methylated cytosines is not random, and overlaps significantly with loci that are affected by *met1* or RdDM mutations (Schmitz *et al.* 2011), suggesting incomplete reinforcement of methylation states is a major factor in generating spontaneous epialleles.

CONCLUSION

Genetic diversity has long been considered as the main foundation for phenotypic variation. As a result, the design of breeding programs has traditionally focused on genetic variation. The lessons learned from epiRIL and related epigenetic studies strongly indicates a great deal of epigenetic variation is associated with phenotypic diversity. These studies also suggest previously observed phenotypic diversity in genetic based studies might have a stronger epigenetic attribution, and warrant further re-evaluation. However,

epiRIL based study in major crop species is still not possible without global DNA methylation mutants such as *met1* and *ddm1* in *Arabidopsis*. Inhibiting DNA methylation or other histone modifications by small molecule inhibitors can provide feasible alternatives as exemplified by the rice DNA methylation inhibition study (Akimoto *et al.* 2007).

It is conceivable that in future design of breeding programs, epigenetic variation will increasingly be taken into consideration. Particularly with the advance of genomic technologies and reducing cost for high throughput DNA sequencing, epigenetic marker-assisted breeding strategies can be applied to select for agronomical desirable epigenetic quantitative traits. Crop improvement via locus-specific epigenetic manipulation has become increasingly feasible with continuing improvement of TALE- or CRISPR-based genome editing technologies (Chen Gao 2013; Maeder *et al.* 2013; Mendenhall *et al.* 2013).

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