CellPress
PARTNER JOURNAL

Molecular Plant
Review Article

Crop Epigenomics: Identifying, Unlocking, and Harnessing Cryptic Variation in Crop Genomes

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http://dx.doi.org/10.1016/j.molp.2015.01.021

ABSTRACT

DNA methylation is a key chromatin modification in plant genomes that is meiotically and mitotically heritable, and at times is associated with gene expression and morphological variation. Benefiting from the increased availability of high-quality reference genome assemblies and methods to profile single-base resolution DNA methylation states, DNA methylomes for many crop species are available. These efforts are making it possible to begin answering crucial questions, including understanding the role of DNA methylation in developmental processes, its role in crop species evolution, and whether DNA methylation is dynamically altered and heritable in response to changes in the environment. These genome-wide maps provide evidence for the existence of silent epialleles in plant genomes which, once identified, can be targeted for reactivation leading to phenotypic variation.

Key words: epigenomics, DNA methylation, whole-genome bisulfite sequencing (WGBS), crops, comparative genomics

Ji L., Neumann D.A., and Schmitz R.J. (2015). Crop Epigenomics: Identifying, Unlocking, and Harnessing Cryptic Variation in Crop Genomes. Mol. Plant. **8**, 860–870.

INTRODUCTION

Genome-wide studies have undergone an unprecedented advancement following the increased availability of high-quality reference genome assemblies. The first plant reference genome was generated for *Arabidopsis thaliana* in 2000 (Arabidopsis Genome, 2000); since then, more than 40 high-quality plant reference genomes have been published (Goodstein et al., 2012). Utilizing these publicly available reference genomes, the first genome-wide maps of DNA methylation were produced for a number of plant species revealing that all studied plants use DNA methylation (Lane et al., 2014), but not necessarily in exactly the same way (Gent et al., 2013; Takuno and Gaut, 2013; West et al., 2014). DNA methylation is one of the most extensively studied chromatin modifications in plants (Niederhuth and Schmitz, 2014). It can be meiotically and mitotically heritable (Calarco et al., 2012) and is one mechanism that provides a link between morphological variation and the DNA sequence. This base modification is involved in a number of plant processes, as changes in DNA methylation states are often associated with alteration to gene expression and resulting phenotypes (Eichten et al., 2014; Niederhuth and Schmitz, 2014).

Methodologies to explore the patterns of DNA methylation in genomes have rapidly advanced during the last 10 years. Initially, methylation-sensitive restriction digestion methods were used to profile genome-wide distributions of DNA methylation (Schmitz and Zhang, 2011). Taking advantage of the sensitivity of certain restriction enzymes' ability to digest methylated DNA, methods were developed to visualize differential methylation after methylation-sensitive restriction digestions with electrophoresis, microarray hybridizations, and other detection methods (Schumacher et al., 2006; Laird, 2010). However, this general approach is limited by the sites that each enzyme digests and the distribution of each enzyme's target motif, and cannot determine DNA methylation states within each fragment, thus giving a relatively low-resolution method for the detection of DNA methylation within the genome. Following this method was the development of immunoprecipitation-based assays (Laird, 2010). 5-Methylcytosine (5mC) specific antibodies are used to immunoprecipitate methylated cytosines (Cross et al., 1994) and then are coupled with gene-chip or high-throughput sequencing (MeDIP-chip or MeDIP-seq), which improves the resolution at which DNA methylation can be detected to dozens of base pairs (Zhang et al., 2006). However, this approach is restricted by each antibody's specificity and is heavily

Published by the Molecular Plant Shanghai Editorial Office in association with Cell Press, an imprint of Elsevier Inc., on behalf of CSPB and IPPE, SIBS, CAS.

dependent on the distribution of 5mC throughout the genome (Schmitz and Zhang, 2011). In 2008, the emergence of sodium bisulfite conversion combined with high-throughput sequencing (whole-genome bisulfite sequencing [WGBS]) led the field into the single-base resolution era, and significantly advanced our understanding of how genomes utilize DNA methylation (Cokus et al., 2008; Lister et al., 2008). The principle of this technique is to use a sodium bisulfite reaction to convert unmethylated cytosines to uracils, which are then converted to thymines during PCR amplification (Hayatsu et al., 1970; Shapiro et al., 1973, 1974; Hayatsu, 1976; Frommer et al., 1992; Clark et al., 1994, 2006; Ji et al., 2014). High-resolution sequencing of the converted DNA is then compared with a reference genome, allowing determination of the methylation status of every cytosine in the genome at a single-base resolution, as methylated cytosines are protected from conversion by this reaction and read as cytosines after sequencing (Cokus et al., 2008; Lister et al., 2008).

WGBS has led to a number of discoveries and novel fields of research in both plant and animal species. For example, human DNA methylation patterns differ between specific cell types and show methylation in two distinct contexts (Lister et al., 2009; Ziller et al., 2013). Methylation in a CG context occurs ubiquitously, whereas methylation in a CH (H = A, T, C) context occurs in brain and embryonic stem cells (Lister et al., 2009, 2013). In contrast to humans, DNA methylation in plants is separated into three distinct contexts: CG, CHG, and CHH. Each of these require different mechanisms for their establishment/maintenance and have different effects on their target DNA sequences (Law and Jacobsen, 2010). In *A. thaliana*, CG methylation is maintained by the DNA methyltransferase MET1 (Finnegan et al., 1996; Ronemus et al., 1996; Mathieu et al., 2007; Lister et al., 2008) whereas CHG and CHH is controlled by methylation of histone H3 lysine 9 (H3K9) and maintained by the methyltransferases CMT3 and CMT2, respectively (Lindroth et al., 2001; Zemach et al., 2013; Stroud et al., 2014). Regions of the genome methylated in all three contexts often lead to silencing in the targeted region and in some cases, neighboring regions (Law and Jacobsen, 2010). The RNA-directed DNA methylation pathway (RdDM) requires the activities of the DNA methyltransferase DRM1/DRM2 (Cao et al., 2000) and is one of the primary mechanisms for non-CG methylation (Law and Jacobsen, 2010). In *A. thaliana*, this pathway is guided by 24 nucleotide short interfering RNAs (siRNAs) derived from transcripts generated by the plant-specific RNA polymerase Pol IV (Huettel et al., 2007; Zhang et al., 2007; Pikaard et al., 2008). DNA methylation-induced silencing is mostly found in transposable elements (TEs), repeat sequences, and some genes (Cokus et al., 2008; Lister et al., 2008). In contrast, protein-coding genes methylated in only the CG context are referred to as ''gene-body methylated'' (Zhang et al., 2006; Zilberman et al., 2007; Takuno and Gaut, 2013). Although its functions remain unclear, many studies of plant genomes reveal that this type of methylation is enriched in exonic regions and depleted of siRNAs (Lister et al., 2008). Furthermore, these genes have moderately high expression levels and tend to be more stably expressed, being less responsive to environmental and developmental cues (Zhang et al., 2006; Zilberman et al., 2007; Coleman-Derr and Zilberman, 2012).

Although genetic and genomic studies of DNA methylation in *A. thaliana* have made scientific advances in the field, how these results transfer to crop species is unclear. Multiple studies in a range of crop species have reported the role of DNA methylation in controlling gene expression and resulting morphological variation (Manning et al., 2006; Eichten et al., 2011, 2012, 2013; Regulski et al., 2013; Schmitz et al., 2013a; Zhong et al., 2013; West et al., 2014), highlighting the importance of studying DNA methylation in these species. To date, methylomes of eight crop species have undergone genome-wide sequencing using WGBS (Table 1), including *Brassica napus* (oilseed rape) (Chalhoub et al., 2014), *Brassica oleracea* (Parkin et al., 2014), *Glycine max* (soybean) (Schmitz et al., 2013a; Song et al., 2013), *Oryza sativa* (rice) (Feng et al., 2010; Zemach et al., 2010a, 2010b; Chodavarapu et al., 2012; Li et al., 2012; Stroud et al., 2013; Takuno and Gaut, 2013; Wang et al., 2013; Hu et al., 2014), *Populus trichocarpa* (black cottonwood) (Feng et al., 2010; Liang et al., 2014), *Solanum lycopersicum* (tomato) (Zhong et al., 2013), *Sorghum bicolor* (sorghum) (Olson et al., 2013), and *Zea mays* (maize) (Eichten et al., 2013; Gent et al., 2013; Regulski et al., 2013; West et al., 2014; Zhang et al., 2014). If we extend the scope to include non-crop species, the total number increases to 14, including *Amborella trichopoda* (Amborella) (Amborella Genome, 2013), *Arabidopsis lyrata* (Seymour et al., 2014), *A. thaliana* (Cokus et al., 2008; Lister et al., 2008), *Brachypodium distachyon* (Brachypodium) (Takuno and Gaut, 2013), and *Capsella rubella* (Seymour et al., 2014).

In this review, we describe the general pattern and distribution of DNA methylation in published crop methylomes. Although all crop species studied utilize cytosine methylation, how this base modification is translated into control of gene expression, transposon regulation, and phenotypic variation is not well understood. In particular, we discuss reported roles of DNA methylation in crop developmental processes and its role in evolutionary mechanisms such as whole-genome duplications. We also investigate the possible role of DNA methylation in the response by plants to changes in the environment, and discuss the advantages of modifying DNA methylation states for the generation of novel variation useful to breeding programs.

DEVELOPMENTAL ROLES OF DNA METHYLATION IN CROP PLANTS

A comprehensive understanding of the mechanisms underlying the diverse growth and development of plants to a harvestable product is essential in generating better crop cultivation and breeding methodologies. Previous studies indicate that certain developmental processes are influenced by changes in DNA methylation. Two recent studies looked for evidence for variation in DNA methylation between organ and tissue types in soybean and sorghum, respectively (Zhang et al., 2011; Song et al., 2013). Song et al. (2013) produced single-base resolution methylomes for four organ types at 13× coverage in soybean. Although they found little difference between methylation status of organs, they did find support for rare hypomethylation events associated with higher gene expression levels of nearby genes. However, whether these were spontaneous in nature or were developmentally controlled differentially methylated regions

Species	Tissue	Reference
Amborella trichopoda	Leaves	Amborella Genome, 2013
Arabidopsis lyrata	Roots and shoots	Seymour et al., 2014
Brachypodium distachyon	Leaves and flower buds	Takuno and Gaut, 2013
Brassica napus	Leaves and roots	Chalhoub et al., 2014
Brassica oleracea	Leaves	Parkin et al., 2014
Capsella rubella	Roots and shoots	Seymour et al., 2014
Glycine max	Leaves	Schmitz et al., 2013a
	Roots, stems, leaves, and seed cotyledons	Song et al., 2013
Oryza sativa	Endosperm, embryos, shoots, and roots	Zemach et al., 2010a
	Leaves	Feng et al., 2010
	Leaves	Zemach et al., 2010b
	Leaves and flower buds	Takuno and Gaut, 2013
	Panicles	Li et al., 2012
	Leaves	Chodavarapu et al., 2012
	Embryos, endosperms, leaves, roots, and shoots	Wang et al., 2013
	Leaves and calli	Stroud et al., 2013
	Shoots	Hu et al., 2014
Populus trichocarpa	Leaves	Feng et al., 2010
	Leaves	Liang et al., 2014
Solanum lycopersicum	Leaves and fruits	Zhong et al., 2013
Sorghum bicolor	Roots	Olson et al., 2013
Zea mays	Unfertilized ears	Gent et al., 2013
	Shoots	Regulski et al., 2013
	Leaves	Eichten et al., 2013
	Shoots, embryos, and endosperm	Zhang et al., 2014
	Leaves	West et al., 2014

Table 1. List of DNA Methylomes and Epigenomic Studies Published throughout the Plant Kingdom and Their Associated References (*Arabidopsis thaliana* Is Not Listed).

(DMRs) is unclear. Sorghum tissue methylation profiles produced at lower resolution using methylation-sensitive amplified polymorphism revealed similar findings, with insignificant methylation changes across seven tissues except for the endosperm (Zhang et al., 2011). The endosperm possessed genome-wide hypomethylation, which concurs with studies on *A. thaliana*, rice, and maize endosperm (Hsieh et al., 2009; Zemach et al., 2010a; Waters et al., 2011).

The lack of variation found in the soybean and sorghum studies can most likely be explained by the complexity of the tissues studied, although it should be noted that changes in DNA methylation may not be required for many plant developmental processes. These studies searched for variation between tissue types, which possess a large diversity of cell types. For instance, the only significant intra-individual variation found between the two studies was for the endosperm, a tissue composed of just a few cell types. Future studies of genome-wide patterns of DNA methylation using a single cell or single cell type will likely have greater success in elucidating the role of DNA methylation in plant development (Figure 1). For example, studies in rice, tomato, and maize have already demonstrated increased ability to detect genome-wide changes to DNA methylation by using methods to reduce the number of cell types being profiled (Zemach et al., 2010a; Waters et al., 2011; Zhong et al., 2013).

In the rice endosperm, genome-wide demethylation was observed in non-CG contexts associated with short euchromatic TEs, but demethylation within CG contexts was localized to only 7% of loci (Zemach et al., 2010a), which is similar to previous findings in *A. thaliana* (Gehring et al., 2009; Hsieh et al., 2009; Ibarra et al., 2012). The observed loss in non-CG methylation in rice is proposed to reactivate genes normally silenced in the sporophyte. Further investigation revealed that 165 demethylated genes were preferentially expressed in the endosperm, including genes responsible for synthesizing starch proteins, which are confined to the endosperm, providing support for this tissue-specific gene activation process (Zemach et al., 2010a). In *A. thaliana*, the demethylation in endosperm is mediated by the DNA glycosylase *DME* (*DEMETER*) (Gehring et al., 2006), but an ortholog is not present in rice (Zemach et al., 2010a). Instead, in rice it appears that *ROS1a* (*REPRESSOR OF TRANSCRIPTIONAL GENE SILENCING1a*), a homolog of *DME*, likely carries out this function (Ono et al., 2012).

Evidence from a comprehensive epigenomic study in tomato provides another example of the potential function of DNA methylation to influence a plant developmental process. Many crop species have fleshy fruits, which is a differentiated floral tissue that evolved to aid seed dispersal in some flowering plants. Plants with this trait undergo a distinctive development stage known as fruit ripening following seed maturation, in which the fruit undergoes numerous physical changes to attract seed dispersal vectors (Howe and Smallwood, 1982). The plant hormone ethylene is involved in controlling the onset of fruit ripening, but it is only effective after fruit seeds first become viable, implying that other factors exist (Klee and Giovannoni, 2011). A genome-wide analysis of DNA methylation throughout a developmental time course in a carefully dissected tomato fruit pericarp tissue demonstrated that multiple ripening-related genes including the transcription factor *CNR* (*COLORLESS NON-RIPENING*), the pectinase polygalacturonase *PG2A* (*POLYGALACTURONASE 2A*), and the rate-limiting enzyme in fruit carotenoid synthesis *PSY1* (*PHYTOENE SYNTHASE*) possess hypermethylated 5′ regions prior to fruit ripening (Zhong et al., 2013). However, these regions become

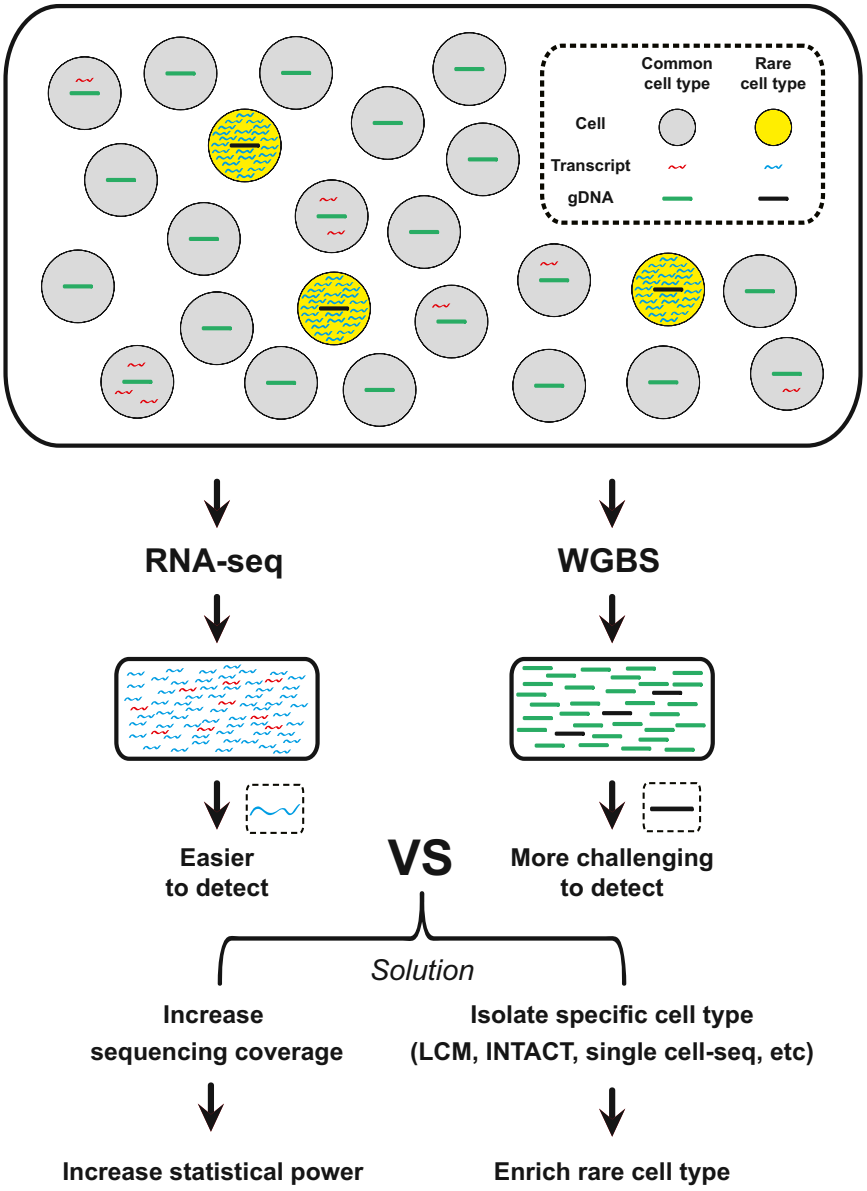

Figure 1. Schematic View of the Challenges Associated with Detecting Differentially Methylated Regions Versus Differentially Expressed Genes.

High-throughput sequencing provides unprecedented resolution of DNA methylomes and transcriptomes. However, a major challenge for studying single-base resolution changes in DNA methylation controlled by the environment or by development is cellular complexity associated with most experiments. Whereas multiple transcripts are produced from a single gene, there is only a single genomic region associated with each gene for each chromosome. The amplification of abundant transcripts from a single locus increases the ability to detect differentially expressed genes in complex cell populations compared with detection of differentially methylated regions. Methods to overcome these challenges include enriching specific cell types or sequencing epigenomes of single cells. Furthermore, increasing the sequencing depth increases statistical power to detect differentially methylated regions.

INTACT, isolation of nuclei tagged for specific cell types; LCM, laser capture microdissection; WGBS, whole-genome bisulfite sequencing (Deal and Henikoff, 2010, 2011).

demethylated and undergo active transcription during ripening stages (Figure 2). Furthermore, early introduction of the methyltransferase inhibitor 5-azacytidine caused premature demethylation in many of these genes and induced early fruit ripening (Zhong et al., 2013).

Using chromatin immunoprecipitation sequencing (ChIP-seq) of RIPENING INHIBITOR (RIN), a key ripening transcription factor, an association was identified with the promoters of numerous genes important for fruit ripening (Zhong et al., 2013). The function of RIN in fruit ripening is dependent on *CNR* (Martel et al., 2011). The *cnr* mutant used in these studies is an epiallele with a hypermethylated promoter that reduces *CNR* gene expression (Manning et al., 2006; Martel et al., 2011; Zhong et al., 2013). A genome-wide analysis of methylation patterns throughout fruit development demonstrated many RIN targeted genes fail to undergo demethylation in the *cnr* mutant, indicating that *CNR* is required for the changes in DNA methylation of RIN targeted loci and explaining the lack of transcriptional activity for these genes (Figure 2) (Zhong et al., 2013). This comprehensive study demonstrates the complexity, and necessity, of the dynamic methylation changes important to fruit ripening in tomato (Zhong et al., 2013). Collectively, studies from endosperm tissue and the study from tomato provides evidence for the role of methylation in certain developmental processes (Zemach et al., 2010a; Zhang et al., 2011; Song et al., 2013; Zhong et al., 2013) and also demonstrates the need for technologies and methodologies that enrich for specific cell types to increase the resolution and detection of methylation changes genome wide.

THE ROLE OF DNA METHYLATION IN THE EVOLUTION OF PLANT GENOMES

How DNA methylation patterns change over evolutionary time scales in plant genomes is a relatively uncharted field of research. Plant genomes are exceptionally plastic and can withstand major changes to total genomic DNA content as a result of whole-genome duplications and hybridization events in addition to spontaneous mutations, gene conversion, and structural rearrangements (Panaud et al., 2014). How DNA methylation patterns evolve in response to these changes is an important question. Current evidence for the roles of DNA methylation in the evolution of crop genomes are primarily limited to studies in rice, maize, and soybean (Eichten et al., 2013; Schmitz et al.,

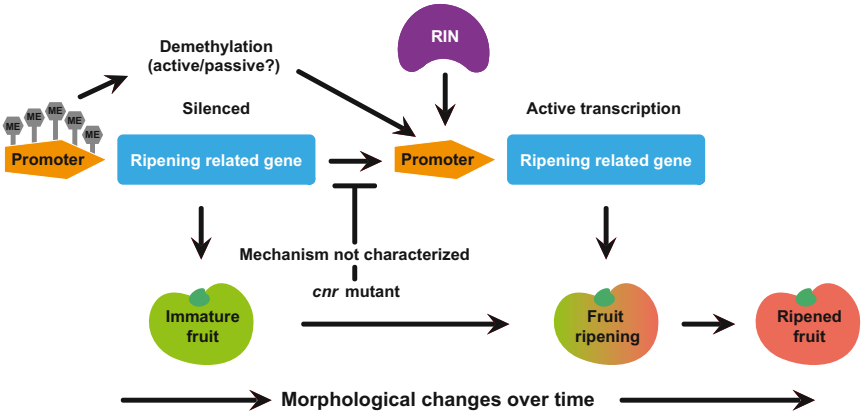

Figure 2. A Model Depicting the Involvement of DNA Methylation in Fruit Ripening.
In immature fruit, numerous genes associated with ripening have methylated promoters, which inhibit RIN targeting and subsequent transcriptional activation. In an uncharacterized manner, the promoter regions become demethylated during ripening stages and corresponding genes experience binding by RIN and active transcription, triggering fruit ripening. The *cnr* mutant inhibits the demethylation process and prevents fruit ripening.

2013a; Takuno and Gaut, 2013). Genome-wide evidence from these species supports that many DNA methylation variants are linked to underlying changes in the DNA sequence (Eichten et al., 2013; Schmitz et al., 2013a, 2013b). Spontaneous rates of methylation polymorphisms were recently estimated using *A. thaliana* mutation accumulation lines (Becker et al., 2011; Schmitz et al., 2011), which revealed that single methylation polymorphisms occur at a rate many orders of magnitude greater than the spontaneous mutation rate for these lines (Ossowski et al., 2010). In contrast, larger regions of differential methylation that are commonly associated with changes in gene expression occur at a rate similar to the mutation rate. Additional evidence for the occurrence of spontaneous epimutations is supported by the identification of DMRs in sections of the maize genome that are identical by descent (Eichten et al., 2013). It should be noted that there is currently no evidence for the role of single methylation polymorphisms in the control of gene expression, whereas there is abundant evidence for the control of gene expression by DMRs. With the application of WGBS to plant genomes, the identification of spontaneous epimutations will increase. Therefore, it is necessary to understand whether these novel methylation states contribute to the evolution or domestication of these species and how these new methylation states are inherited over generational time scales.

One approach used to address this question is mapping DNA methylomes of recombinant inbred lines (RILs) and their respective parental lines. Using this approach in soybean, the vast majority of DMRs that contain changes in CG, CHG, and CHH methylation were associated with the genotype they were associated with in the parental genomes (Schmitz et al., 2011). These results suggest that many of the methylation variants may be linked to local genetic variants, but could also be due to recently derived spontaneous epimutations that are stably inherited over the few generations required to generate this RIL population. A similar approach was used in maize RILs, which also showed a stable inheritance pattern of DNA methylation through generations and revealed that nearly half of DMRs were associated with genetic variation (Li et al., 2014a). In contrast, another study of maize RILs revealed evidence for paramutation-like effects, as methylation states could switch between genotypes (Regulski et al., 2013). The differences between the conclusions of these two studies is likely a result of how the data were analyzed, revealing another difficult challenge in this field. These studies show that the vast majority of DNA methylation states are stably inherited over short time scales in crop genomes, but leave a number of interesting questions about the role of these DMRs in phenotypic variation, their function over evolutionary time scales, and their potential to respond to new environments. Future studies of experimental plant populations will be necessary to clarify the role, if any, of spontaneous epimutations in affecting beneficial crop traits. To supplement these studies, the stability of epimutations across generations should be investigated, which will enhance previous studies on the generation of epimutations across generations in *A. thaliana* (Becker et al., 2011; Schmitz et al., 2011).

Although there is evidence for spontaneous changes to DNA methylation over short time scales (Becker et al., 2011; Schmitz et al., 2011), certain domains of DNA methylation, such as gene body methylation, are conserved over evolutionary time scales (Takuno and Gaut, 2013). One characteristic of all plant DNA methylomes that have been sequenced thus far is the occurrence of gene body methylation, which is proposed to be of evolutionary significance although its exact function remains to be determined (Takuno and Gaut, 2012). This type of methylation is positively correlated with moderately expressed genes (Zhang et al., 2006; Zilberman et al., 2007; Coleman-Derr and Zilberman, 2012), and is often found in longer genes that are slowly evolving. A comparative epigenomic study between rice and *Brachypodium* provided some insight into the evolutionary conservation of gene body methylation (Takuno and Gaut, 2013). By comparing reference genomes of these two species, these authors found 7826 collinear orthologs, and subsequent analysis confirmed a relatively high positive correlation of CG methylation levels between them (r = 0.76). Inclusion of data from maize orthologs also revealed strong positive correlations between all three species (Takuno and Gaut, 2013). This study suggested a conserved role of gene body methylation in plant evolution (Figure 3). This study builds upon earlier observations of the presence of gene body methylation across diverse eukaryotic genomes (Feng et al., 2010; Zemach et al., 2010b).

Results from sequencing DNA methylomes from insects, algae, plants, and animals confirmed that gene body methylation is not exclusive to plants (Feng et al., 2010; Zemach et al., 2010b). To explore the conservation of gene body methylation, a phylogenetic analysis of enzymes involved in DNA methylation

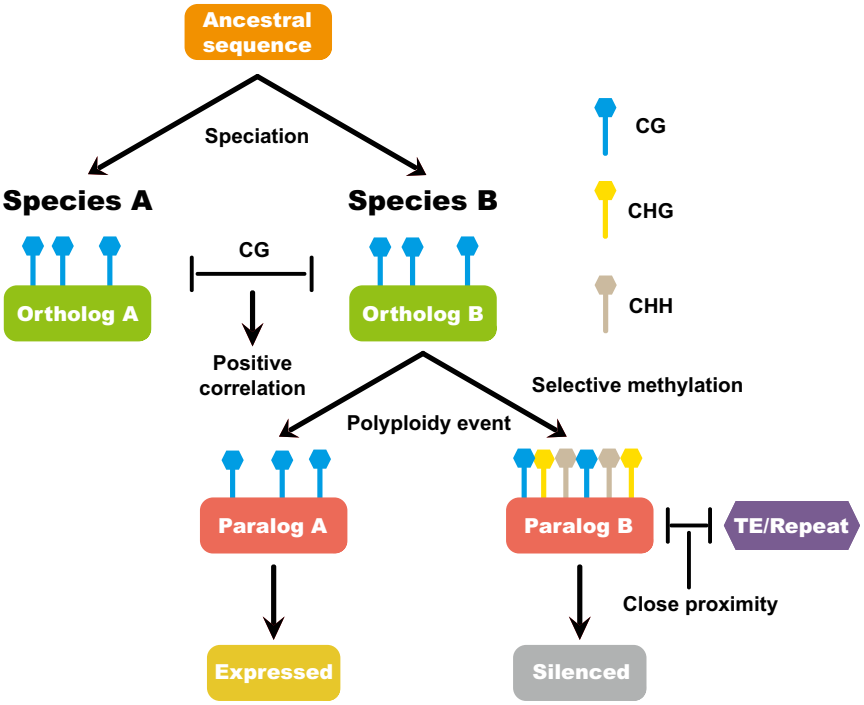

Figure 3. Schematic Model of DNA Methylation Dynamics in Orthologs and Paralogs.
Between different species, orthologs have a strong positive correlation of CG methylation levels characteristic of gene body methylation. Within a species, one paralog within a set is at times subject to silencing by DNA methylation. Silenced paralogs often have close proximity with transposable elements (TE) and repeat regions compared with their counterparts.

found that UHRF1 (ubiquitin-like with PHD and RING finger domains 1) and its homologs exist in all species with CG methylation, but is absent from species lacking CG methylation (Feng et al., 2010). It should be noted that although numerous genes in animals and plants contain CG methylation in the gene bodies, the gene body methylation found in plants that is associated with moderately expressed genes that are slowly evolving is not found in animals. Therefore, it is unlikely that the function and evolutionary consequence of gene body methylation between plants and animals is the same. Finally, the function of gene body methylation in plant genomes is also unclear, although it has been speculated that it may simply be a by-product of transcription or other nuclear processes (Takuno and Gaut, 2012).

It is currently unknown how much variation in DNA methylation occurs immediately after whole-genome duplications or upon hybridization and polyploidy events. What can be addressed is the conservation of methylation states between duplicated sequences over evolutionary time. One hypothesis is that upon duplication of genes, one paralog could be subjected to silencing by DNA methylation (Figure 3). Silencing a paralog is a useful means to cope with duplications from increased dosage generated from polyploidy events in plants, similarly to how dosage is controlled in X-chromosome inactivation in animals (Wutz, 2011). A previous study in *A. thaliana* reported conservation of CG methylation states between paralogs (503 pairs), but whether conservation of non-CG methylation states exists was not studied (Widman et al., 2009). Several crop species genomes contain many more paralogs than *A. thaliana* and may be more ideal experimental systems for studying these effects. The soybean genome contains almost 10,000 sets of paralogs within its genome as a result of recent whole-genome duplications (Schmitz et al., 2013a). Pairs of paralogs in soybean were recently analyzed for differential DNA methylation, revealing that at least ~5% of pairs are differentially targeted by DNA methylation that is characteristic of gene silencing (Schmitz et al., 2013a). To investigate potential reasons behind these observations, the distances of the methylated paralogs with nearby TEs were calculated. The results revealed that ~9.3% of differentially methylated paralogs overlapped with RdDM-targeted TEs. Furthermore, these numbers will only increase, as regions targeted by RdDM outside of the coding sequence were not considered because of the difficulty in associating regulatory elements to genes. These studies provide evidence of how DNA methylation changes in response to polyploidy events. More comprehensive studies are needed at distinct stages in the evolution of polyploids to help better understand this mechanism and determine whether this is a universal mechanism common in all plants and other kingdoms that undergo duplications. Finally, in parallel with the studies of DNA methylation at gene duplication events in plants, a recent selective DNA methylation phenomenon was reported in humans, indicating that this process may be a general feature of genomes that use cytosine DNA methylation (Keller and Yi, 2014).

ENVIRONMENTAL INFLUENCES ON GENOME-WIDE PATTERNS OF DNA METHYLATION

Environmental cues are known to induce changes to DNA methylation (Dowen et al., 2012) and present a potential source of novel variation. Experiments investigating non-natural environmental factors in the form of tissue culture plant regeneration showed consistent changes in genome-wide methylation that is heritable over multiple generations (Figure 4) (Stroud et al., 2013; Vining et al., 2013; Stelpflug et al., 2014). These studies are of particular importance because of the critical role tissue culture holds in the crop breeding industry. At present, little evidence exists to suggest that natural environmental factors can cause dynamic changes in DNA methylation that are heritable over generations.

Three reports recently studied epigenomic changes in different crop species during plant regeneration through tissue culture (Stroud et al., 2013; Vining et al., 2013; Stelpflug et al., 2014). The most comprehensive of these studies was in rice (Stroud et al.,

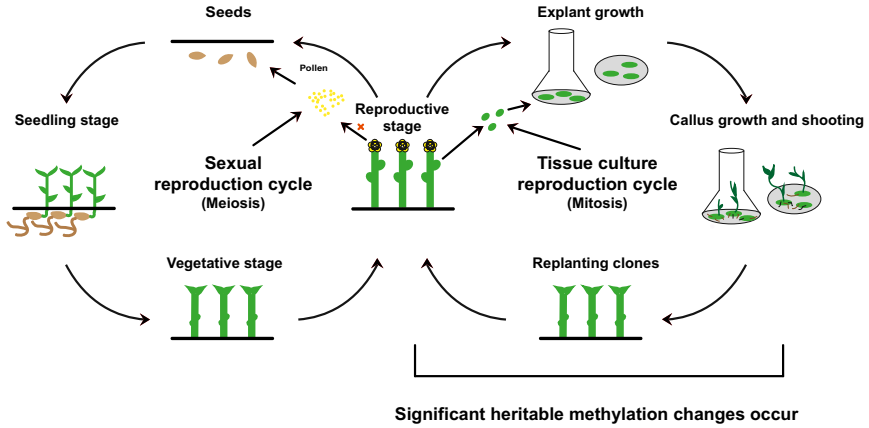

Figure 4. Models of DNA Methylation Changes in Natural Plant Sexual Reproduction and Tissue Culture Reproduction Cycles.

Plants regenerated as a result of tissue culture display significant variation in DNA methylation. Mostly hypomethylated regions are observed, which are often heritable in subsequent generations. Clonally propagated plants and plants that are continually regenerated by tissue culture do not undergo normal sexual reproduction, which is an important check point to ensure that deleterious sequences such as transposons are maintained in a silenced state. The red "X" indicates cross/selfing pollination.

2013), which generated single-base resolution methylomes of different rice accessions that had undergone routine transgenic protocols commonly used to generate novel phenotypes for agricultural purposes. Comparison of these methylomes with wild-type inbred lines identified novel, heritable methylome changes, showing tissue culture as the primary cause of the observed methylome variation (Stroud et al., 2013). Lines generated through tissue culture regeneration experienced global hypomethylation changes. To mimic breeding procedures, the lines also underwent transgenic transformation during the same period. To demonstrate that the observed hypomethylation changes were a result of the tissue culture process and not transgene introduction, T2 plants homozygous for the transgene were compared with T2 plants for which the transgene was segregated away. In addition, these results were compared with data from wild-type plants that solely underwent tissue culture regeneration in the absence of transformation. In all lines that underwent tissue culture regeneration, similar hypomethylation patterns emerged. Furthermore, the heritable stability of these changes in following generations was demonstrated, as T4 and T6 generation plants descending from the same regenerant showed 84% of CG methylation loss seen in T2 plants (Stroud et al., 2013).

The methylation changes observed in the rice study were similar to those found in separate studies in black cottonwood and maize (Vining et al., 2013; Stelpflug et al., 2014). In black cottonwood, genome-wide changes to DNA methylation were identified at different time points throughout the tissue culture process, showing that hypomethylation changes occur through a gradual process (Vining et al., 2013). In addition, a study in maize further confirmed the heritability of tissue culture induced DNA methylation changes, and compared and contrasted the abundance and stability of hypo- versus hypermethylation changes (Stelpflug et al., 2014). In the 479 DMRs found in this study using MeDIP-chip, 67% were hypomethylated, with 75% of these hypomethylated DMRs seen in multiple tissue culture regenerants; alternatively, only 47% of hypermethylated DMRs were seen in multiple tissue culture regenerants. Furthermore, 60% of hypomethylated DMRs were observed in R_1 offspring, whereas only 7% of hypermethylated DMRs were stably inherited in offspring. This study also found a small, but significant, overlap of tissue culture generated DMRs with natural DMR variation, suggesting that stress encountered in tissue culture may overlap with natural genomic stress, and therefore may predict loci susceptible to methylation variation (Stelpflug et al., 2014). These three studies demonstrate that tissue culture regeneration can generate heritable, genome-wide methylation changes, primarily in a hypomethylated manner (Figure 4). These results likely reveal the challenges cells encounter in maintaining DNA methylation states in conditions that promote active and rapid cell divisions. Lastly, expression profiles generated in these studies also demonstrated that hypomethylation changes in promoter regions lead to increased expression rates for some of these genes, possibly demonstrating a cause of the observed phenotypic somaclonal variation seen in breeding programs (Stroud et al., 2013; Vining et al., 2013; Stelpflug et al., 2014).

The effect of natural environmental factors on DNA methylomes remains to be investigated in great detail. However, a recent single-base resolution methylome study in poplar showed an increase in total cytosine methylation following water-stressed conditions (Liang et al., 2014). Absent from this study was heritability of these changes in subsequent generations or in the absence of the environmental stimulus. Further studies analyzing the transgenerational inheritance of altered DNA methylation states are necessary to understand whether plant genomes use this base modification to prepare for future environments. Further efforts to investigate the dynamics of DNA methylation in natural plant populations may be the key to answering this question. However, it should be noted that a recent study analyzing the dynamics of DNA methylation in *A. thaliana* wild isolates that are diverged ~100–200 years from a common ancestor found no evidence for widespread effects of the environment to the DNA methylome (Hagmann et al., 2015).

CONCLUSIONS AND FUTURE PROSPECTS

Epigenomics is an emerging field that is expanding our abilities to explain observed phenotypic variation through the identification of multiple cellular products such as RNAs, protein–DNA interactions, chromatin modifications, and chromatin accessibility (Lane et al., 2014). DNA methylation is one of the most intensely investigated areas in this field as numerous phenotypes and cellular mechanisms have been explained as a result of changes in DNA methylation. Lately, studies focused on DNA methylation have been greatly enhanced by the improvement in methylation detection methods brought on by cheaper,

high-throughput sequencing technologies; in particular, the invention of the WGBS method has greatly advanced studies of DNA methylation (Cokus et al., 2008; Lister et al., 2008).

Studies in *A. thaliana* have paved the way for many major mechanisms to explain how DNA methylation functions in plant genomes. However, it is clear that although all plant genomes studied utilize cytosine methylation, they do not necessarily use it in the same way. For example, in maize the use of CHH methylation is limited to DNA TEs upstream of genes, termed CHH islands (Gent et al., 2013), and is largely absent in appreciable quantities in genes or throughout major heterochromatin domains such as the pericentromere (Gent et al., 2013; Regulski et al., 2013; West et al., 2014). Instead, H3K9me2 (dimethylated H3K9) and CHG methylation have a more pronounced role in silencing transposons inserted primarily into introns and in silencing large heterochromatin domains (West et al., 2014). Clearly, as new DNA methylomes become available for crop species additional features will be discovered, revealing novel pathways and functions of DNA methylation in plant genomes.

At present a large obstacle facing the field is reference genomes, which are the foundation for in-depth analyses. Although many plant genomes have been released, the quality of the draft assemblies and annotations can hamper downstream genomic analyses. In addition to this barrier, it is important to note other characteristics unique to methylome studies presenting obstacles that will need to be overcome. For example, in contrast to RNA-seq (RNA sequencing), which can provide informative results for highly expressed genes from diverse cell types, studies of the same population of cells can impede genome-wide studies of DNA methylation (Figure 1). For each rare cell there exists only one diploid genome, whereas in some cases there exist numerous copies of transcripts, especially for highly expressed genes, which provide greater statistical power to find genes that are differentially expressed. This leads to a risk of missing rare methylation patterns unique to a few cells when a large number are collected and pooled together. One way to overcome this obstacle is higher coverage in sequencing. However, financial constraints inhibit widespread application of this particular solution. Another method would be to improve resolution at the DNA collection stage. Studies using tissue types with limited cell types, such as fruit pericarp tissue and rice/*A. thaliana* endosperm (Zemach et al., 2010a; Zhong et al., 2013), have been able to find methylation differences between tissue types, in contrast to studies using large cell type mixing such as leaf or floral tissue (Gehring et al., 2009; Schmitz et al., 2013a). Progress has already been made toward improving cell type-specific methylation data. Earlier this year a protocol was developed to sequence the methylome from a single embryonic mouse stem cell (Smallwood et al., 2014), demonstrating that low-input and single-cell methylome profiles are possible. Successful isolation of DNA from a single cell or single cell types is a bottleneck for most experiments and will need to be overcome to advance plant epigenomic studies.

Another unique and challenging aspect to methylome studies is the identification of DMRs. At present, the majority of DMR finding methods starts from identifying a differentially methylated site (DMS) and then using either physical or statistical methods such as a hidden Markov model to combine multiple DMSs into a single DMR (Robinson et al., 2014; Seymour et al., 2014). Certainly many efforts have been made to advance this field, and a variety of different statistics-based programs have been released (Robinson et al., 2014). However, this diversity also hampers the comparative analysis of different studies and, in some cases, increases the difficulty to link DMRs with gene expression profiles and phenotypes. Other challenges in discovering DMRs include low coverage data and how multiple samples with replicates are handled. Given how young this field is, future efforts to develop novel DMR finding strategies are required to provide a community standard approach that is robust enough to cope with all of the challenges presented in this review.

Most epigenomic studies thus far have focused on characterizing chromatin features of the genome in a single tissue. Identification of regions and genes in the genome that are transcriptionally silenced by DNA methylation is a major first step toward the goal of generating phenotypic variation as a result of the expression of cryptic alleles. As demonstrated in *A. thaliana* epiRILs (epigenetic RILs), there exist significant hidden variations present in the genome that can be uncovered by perturbing DNA methylation patterns genome wide (Johannes et al., 2009; Reinders et al., 2009; Roux et al., 2011). Importantly, many of these induced methylation variants are stable over time scales necessary to breed novel traits, so much so that quantitative trait loci for some phenotypes have been identified using DMRs as genetic markers (Teixeira et al., 2009; Cortijo et al., 2014). Whether similar epigenomic populations can be created in crop genomes and leveraged to introduce identified epimutations into current breeding programs remains unknown. One of the major challenges to these populations will be whether mutations in DNA methylation machinery are tolerated, as was recently observed in rice and maize (Hu et al., 2014; Li et al., 2014b), due to the abundance of transposon sequences in crop genomes compared with the trimmed down genome of *A. thaliana*. An alternative strategy is to use more surgical approaches to directly engineer the epigenome, as has been described in *A. thaliana* (Johnson et al., 2014). Regardless, a combination of technical and biological innovation will be necessary to realize the potential of epigenomic variants in crop genomes.

FUNDING

This study was supported by research grants to R.J.S. from the National Science Foundation (IOS-1339194) and the National Institutes of Health (R00GM100000).

ACKNOWLEDGMENTS

We would like to thank the editors for the opportunity to write this review, and thank Chad Niederhuth, Adam Bewick, and Xiaoxiao Sun for their helpful comments and discussions. No conflict of interest declared.

Received: December 1, 2014
Revised: January 20, 2015
Accepted: January 22, 2015
Published: January 29, 2015

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