REVIEW / SYNTHÈSE

Exploring and exploiting epigenetic variation in crops[1]

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Abstract: This review addresses the mechanisms by which epigenetic variation modulates plant gene regulation and phenotype. In particular we explore the scope for harnessing such processes within the context of crop genetic improvement. We focus on the role of DNA methylation as an epigenetic mark that contributes to epiallelic diversity and modulation of gene regulation. We outline the prevalence and distribution of epigenetic marks in relation to eukaryote developmental processes, and in particular identify where this may be relevant to crop traits both in terms of specific developmental stages and in relation to physiological responses to environmental change. Recent whole genome surveys have identified specific characteristics of the distribution of DNA methylation within plant genomes. Together with greater understanding of the mode of action of different maintenance and de novo methyltransferases, this provides an opportunity to modulate DNA methylation status at specific loci as an intervention strategy in crop genetic improvement. We discuss alternative approaches that may be suitable for harnessing such induced epiallelic variation. Most of the discussion is associated with *Brassica* crops, which demonstrate considerable morphological plasticity, segmental chromosomal duplication, and polyploidy.

Key words: epigenetics, crop plants, DNA methylation, breeding, *Brassica*.

Résumé : Cette synthèse porte sur les mécanismes via lesquels la variation épigénétique module la régulation génique et le phénotype. En particulier, les auteurs explorent le potentiel d'exploitation de ces processus dans le contexte de l'amélioration génétique. Les auteurs portent leur attention sur la méthylation en tant que marqueur épigénétique qui contribue à la diversité épi-allélique et à la modulation de l'expression génique. Les auteurs décrivent la fréquence et la distribution des marques épigénétiques en relation avec les processus du développement chez les eucaryotes et, en particulier, identifient pourquoi cela pourrait s'avérer pertinent chez les espèces cultivées tant à des stades pré*cis* du développement qu'en relation avec les réponses physiologiques aux changements environnementaux. Des études récentes à l'échelle du génome entier ont permis d'identifier des caractéristiques spécifiques de la distribution des sites de méthylation de l'ADN au sein des génomes des plantes. Combiné avec une meilleure compréhension du mode d'action des diverses méthyltransférases responsables soit de la méthylation initiale ou de son maintien, cela offre une opportunité de moduler la méthylation à des locus spécifiques pour des fins d'amélioration génétique. Les auteurs discutent d'approches alternatives qui pourraient permettre de tirer profit d'une telle variation épi-allélique induite. La plus grande partie de la discussion porte sur les *Brassica* cultivées, lesquelles présentent une plasticité morphologique considérable, de la duplication de segments chromosomiques et la polyploïdie.

Mots-clés : épigénétique, plantes cultivées, méthylation de l'ADN, amélioration génétique, *Brassica*.

[Traduit par la Rédaction]

Introduction

Over the past century genetic improvement of crops has underpinned massive increases in yield and food production. This has been achieved primarily through concerted breeding programs based upon crossing and recurrent selection of elite lines, within a framework of an increased understanding of Mendelian and quantitative genetics. It is noteworthy that many of these gains were made in a period of relative climate stability. However, the world is now entering an era of increased climate change and unpredictability, coinciding with a growing human population and increased per capita wealth with associated inefficient heterotrophic calorific intake. Together with reduced availability of productive agricultural land, current concerns over food security suggest a

Received 2 March 2010. Accepted 24 June 2010. Published on the NRC Research Press Web site at genome.nrc.ca on 04 November 2010.

Corresponding Editor: G. Scoles.

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[1]This article is one of a selection of papers from the conference "Exploiting Genome-wide Association in Oilseed Brassicas: a model for genetic improvement of major OECD crops for sustainable farming".

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pressing need for a deeper understanding of crop genotype x environment (G × E) interaction. In particular, it is timely to assess the scope for harnessing mechanisms that plants may have evolved to adapt to rapidly changing environments.

Many crop plant genomes are relatively large with complex organization arising from segmental or whole chromosome polyploidy, where subsequent gene duplication and divergence may lead to subtle novel functionality (King 2002). The availability of molecular markers over the past 20 years has led to increased interest in genetic analysis and identification of underlying genes, with a strong practical focus on marker-assisted selection. However, for many crops progress has been limited by lack of resolution both at the level of genome and trait definition. Although recent advances in high throughput sequence technologies hold the promise of increasing the ability to identify and characterize genes underlying many agronomic traits, there remain major challenges for resolving the underlying basis of trait variation.

Conventional crop selection is based on phenotype, which is considered a sum of genotype and environment. Quantitative trait loci (QTL) associated with a wide range of traits have successfully been identified for many crops; however, in many cases the genetic penetrance is low and subject to large G × E interactions. QTL analysis has provided valuable information on the relative contribution of component traits to overall phenotypic variation and identified loci that can be subject to greater or fewer G×E interactions (Fig. 1). From many perspectives, it is clear that the future of large-scale genomic selection for crop improvement and identification of underlying genes requires more emphasis on trait resolution. One of the most valuable outcomes of studying "immortal" segregating populations used in QTL analysis is the ability to assign variance to different environmental factors within multisite and multioccasion trials.

Superimposed on the underlying DNA sequence of eukaryotes are a series of epigenetic marks that can provide considerable agility in terms of modulating gene expression, ontology, and response to the environment. In contrast to most animals, plants display substantial developmental plasticity, as in general they lack the locomotor apparatus to remove themselves from short-term adverse environmental conditions. Plasticity is also evident in the relative facility with which plants can reorganize their genomes during and following polyploidy events (Szadkowski et al. 2010), as well as the more immediate and transitory effects of heterosis or hybrid vigour. It can be argued that this capacity for genome plasticity contributes to the totipotency of plant cells (Verdeil et al. 2007), which have attributes of universal stem cells.

Epigenetic phenomena have been observed throughout eukaryotes. Although the term epigenetics has been used in different contexts (Jablonka and Lamb 2002), it is now generally accepted to refer to changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence (Meehan et al. 2005). Epigenetic variation is typically associated with covalent modifications to DNA and histones, known as epigenetic marks, that affect chromatin conformation and are stable over rounds of cell division, but do not involve changes in the primary nucleotide sequence. Such epigenetic marks often affect the control of gene expression at the level of chromatin organization, contributing to regulation of many aspects of development or response to the environment (for a review, see Lukens and Zhan 2007). Thus epigenetic regulation encompasses the normal ontogenetic process of cellular differentiation in morphogenesis whereby totipotent stem cells undergo mitotic division, giving rise to a range of pluripotent cell lineages that contribute to differentiated states. This is achieved by activation of different subsets of genes. In animals this tends to be a unidirectional path within differentiating somatic cell lines, whereas in plants there is scope for the process to be reversed. In animals the pattern of epigenetic marks is reset at meiosis (Yoder et al. 1997), whereas in plants changes may accumulate within somatic lineages that on occasion may be inherited through meiosis (Finnegan et al. 1998; Takeda and Paszkowski 2006; Henderson and Jacobsen 2007; Saze 2008).

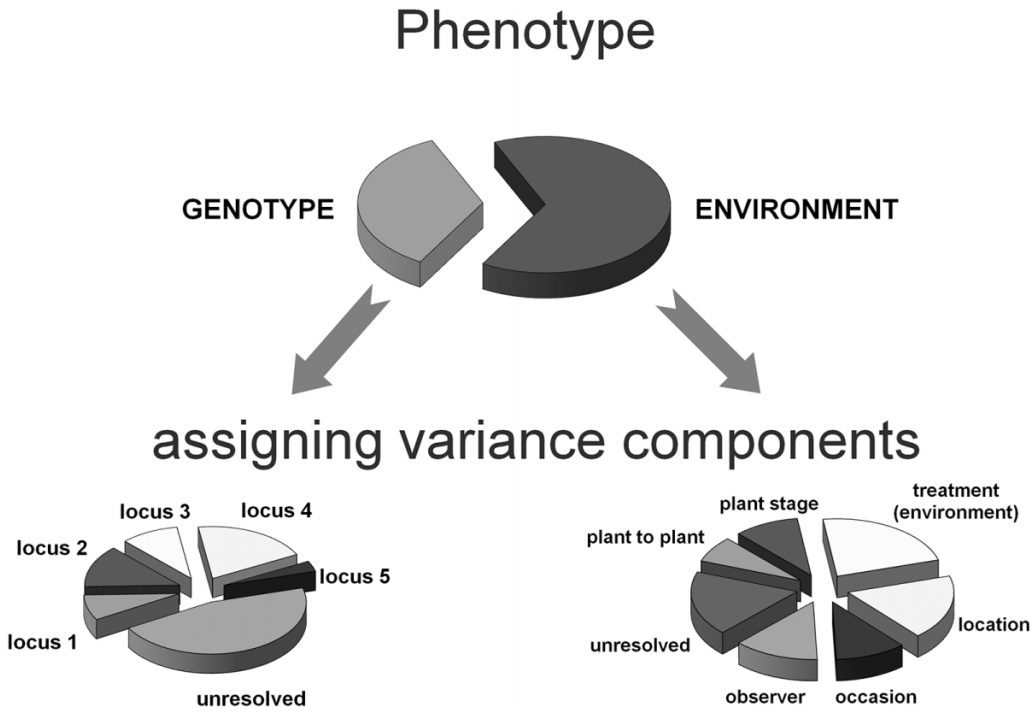
The aim of this review is to address the mechanisms by which epigenetic variation may modulate plant gene regulation and phenotype, and in particular to explore the scope for harnessing such processes within the context of crop genetic improvement. This is particularly pertinent in the context of climate change and the increasing need to develop crops with predictable response to local environmental conditions. The focus will be on the role of DNA methylation as an epigenetic mark that contributes to epiallelic diversity and modulation of gene regulation. Most of the discussion is associated with *Brassica* crops, which demonstrate considerable morphological plasticity, with different organs being selected under domestication for vegetable, oil, fodder, and condiment end use. Brassicas are also characterized by segmental chromosomal duplication and polyploidy, which in a heterozygote plant of an amphidiploid species such as *B. napus* (Canola, oilseed rape) has the potential to result in up to 12 allelic copies of genes that in the closely related and inbreeding *Arabidopsis* may only occur once. A number of recent studies in the genus *Brassica* have provided novel generic insight into the role of epigenetic variation in plant genome evolution, stability, and crop productivity. Finally, we explore the scope for intervening in the epigenetic status of crop plants as a potential tool in crop breeding.

Epigenetic variation and chromosome organization

Chromatin was first identified from cytological evidence and classified into two states. Heterochromatin is more compact and has generally been associated with silenced genes, although some transcription may take place (Gendrel et al. 2002). Euchromatin is less compact and is thought to be more transcriptionally active. Although boundaries are thought to exist between the two states, it is not fully understood if these are primarily physical or functional (Wei et al. 2005; Ishihara et al. 2006).

Nucleosomes are the primary unit of chromatin organization by which eukaryotic genomic DNA is packaged into nuclear chromosomes, in conjunction with core and linker histones (Tariq and Paszkowski 2004). Initiation of cellular differentiation is associated with changes in chromatin structure at both the level of higher order structures and of indi-

Fig. 1. The contribution of genotype and environmental factors to plant phenotype.



vidual genes (for a review, see de la Serna et al. 2006). More specifically, the distribution of nucleosomes in the context of coding and regulatory sequences directly affects the initiation and rate of gene transcription. Nucleosome distribution is determined by signals in the underlying DNA sequence as well as subtle changes in DNA methylation that affect interactions with core and linker histones. Chromatin remodelling resulting from changes in epigenetic marks is essential for activation of a number of genes, and failure to appropriately regulate chromatin structure may lead to developmental defects (de la Serna et al. 2006). Chromatin remodelling is usually achieved through enzyme-mediated movement of nucleosomes along the DNA, and typically involves ATP-dependent complexes that harness energy from ATP hydrolysis to disrupt or alter the local association of histones with DNA (Vignali et al. 2000). Histone acetyltransferases and histone deacetylase complexes can also regulate transcriptional activity by modulating acetylation of nucleosomal histones.

Epigenetic marks within genomic DNA sequences

Epigenetic marks primarily consist of methylation modification of DNA or acetylation, methylation, phosphorylation, or sumoylation of histones. It has recently become apparent, from studies using mouse embryonic stem cells and human cancer cells, that DNA methylation and histone modification pathways can be dependent on one another (Gilbert et al. 2007; Cedar and Bergman 2009). The extent and detailed patterns of epigenetic variation in plants has only recently started to be uncovered. Silencing in heterochromatin is associated with hypermethylation of DNA and specific covalent modifications of histones (Chang and Pikaard 2005; Mathieu et al. 2005; Vaillant and Paszkowski 2007; Zhang et al. 2007). These epigenetic marks interact with differential preferences for DNA binding that have consequences for the distribution of nucleosomes with respect to underlying DNA sequence and for the binding of other regulatory or structural proteins.

DNA methylation occurs primarily at the C^5 position of cytosines and is commonly called 5-methylcytosine (5mC). DNA methylation was initially found to protect genetic information in bacteria from being contaminated by invading DNAs. In addition, hypomethylation can result in derepression of transposable elements (Penterman et al. 2007), which then become active and may transpose into functional genes thus acting as a mutagen. DNA methylation has been implicated in timing of DNA replication, determination of chromatin structure, increased mutation frequency, and as a causal agent of some human diseases including cancer (for a review, see Finnegan et al. 1998; Bird 2002; Bender 2004).

Early studies of 5mC in wheat, tobacco, and other plant species revealed that approximately 80% of cytosines in CG dinucleotides are modified (Gruenbaum et al. 1981). However, compared with mammalian systems there are some distinct differences both in the prevalence and pattern of DNA methylation marks across the genome and also in terms of the role of DNA and chromatin changes in gene regulation. Plants possess greater genomic complexity in terms of potential DNA methylation targets. In metazoans, cytosine DNA methylation is solely within the context of CG dinucleotides, whereas in plants it may be associated with the symmetrical sequences CG and CHG and at a low frequency with nonsymmetrical sites such as CHH (Frommer et al. 1992; Finnegan et al. 1998; Tariq and Paszkowski 2004). However, DNA methylation at nonsymmetrical sites is probably not transmitted through the cycles of DNA replication.

The *Arabidopsis DNA METHYLTRANSFERASE 1* (*MET1*) gene is an orthologue of the mammalian DNA methyltransferase, *DNMT1* (Finnegan and Dennis 1993; Finnegan et al. 1996) and preferentially catalyses CG methylation (Finnegan et al. 1998; Brzeski and Jerzmanowski 2003; Cokus et al. 2008). Antisense *MET1* mutants in *Arabidopsis* display a broad spectrum of phenotypic abnormalities including decreased plant stature, smaller rounded leaves, decreased fertility, and reduced apical dominance (Finnegan et al. 1996), which suggest a crucial role for cytosine methylation for normal plant development.

The chromatin remodelling factor *DECREASE IN DNA METHYLATION 1* (*DDM1*) is required for maintaining methylation at both CG and CHG sites in plants. *DDM1* is a member of the SWI2/SNF2 protein family, and encodes a protein that binds to naked DNA to promote chromatin remodelling by inducing nucleosome repositioning (Brzeski and Jerzmanowski 2003), targeting both CpG and CpNpG methylation in nontissue-specific manner. *Arabidopsis ddm1* mutants have a reduced global 5mC level, 70% lower than wild type (Vongs et al. 1993; Kakutani et al. 1995).

The plant-specific DNA methyltransferase *CHROMOMETHYLASE 3*, characterized by the presence of a chromodomain embedded within the methyltransferase catalytic domain is responsible for catalysing non-CG methylation (Henikoff and Comai 1998; Bartee et al. 2001). In *Arabidopsis*, the *DOMAIN REARRANGED METHYLASE 1* and *DOMAIN REARRANGED METHYLASE 2* genes (*DRM1* and *DRM2*) encode methyltransferases that catalyse de novo DNA methylation both in symmetric and asymmetric contexts (Cao and Jacobsen 2002). Transcriptional analysis of *drm1* and *drm2* double homozygous mutants and downstream analyses of transgene *FWA* and endogenous *SUPERMAN* revealed the de novo catalytic activity of the *DRMs* in *Arabidopsis* (Cao and Jacobsen 2002).

Recent high resolution studies based on massively parallel sequencing have shown that the distribution of 5mC within the *Arabidopsis* genome is organized in a systematically different way from that found in mammals. In particular, a gradient of 5mCG is found across plant coding sequences (Cokus et al. 2008; Lister et al. 2008). Cokus et al. (2008) found genome-wide levels of 24% CG, 6.7% CHG, and 1.7% CHH methylation, whereas repeat-rich pericentromeric regions contained high levels of all three forms of methylation. Lister et al. (2008) also observed preferential CG and CHH methylation in euchromatic chromosome arms, whereas CHG methylation appeared most enriched in pericentromeric regions. A periodicity of 10 nucleotides corresponding to the length of one helical DNA turn was observed for nonsymmetric (CHH) methylation, with sequence-specific preferences associated with different methyltransferases.

Involvement of epigenetic marks in regulating plant and animal transcription

Chromatin structure plays a key role in modulating the regulation of eukaryote gene transcription. The enzymes involved in ATP-dependent chromatin remodelling have been extensively studied in mammalian differentiation pathways, and it has been revealed that these enzymes are cell-type- and gene-specific, thus making them much more precise in regulating cell differentiation (Tariq and Paszkowski 2004; de la Serna et al. 2006). In animals, epigenetic mechanisms contribute to orchestrating development from embryo onwards, with epigenetic marks inherited mitotically, and the overall pattern reset through meiosis (Yoder et al. 1997). Failure of epigenetic control in mammals has been associated with an increasing number of diseases, conditions, and ontogenetic processes, including cancers, psychiatric and autoimmune disorders, and ageing (Esteller 2003; Feinberg et al. 2006). In mammalian tissues, perturbations such as hypermethylation of tumour-suppresser genes can lead to cancer development, whereas hypomethylation of genomic DNA can lead to activation of potential oncogenes, which also results in cancer (for a review, see Bird 2002; Havliš and Trbušek 2002). Details of the close relationship between DNA methylation marks and chromatin structure are becoming more apparent, particularly through studies in *Arabidopsis*. From analysis of DNA methylation marks in *Arabidopsis* published by Lister et al. (2008), we had noted a tight relationship with exons and their boundaries as well as with the position of cross-linked mononucleosomes (unpublished data). This relationship has now been demonstrated by Chodavarapu et al. (2010) who found that nucleosome-bound DNA was more highly methylated than flanking DNA, and suggest that DNA methyltransferases are preferentially targeted to nucleosomal DNA.

In plants as in animals, DNA methylation has dual roles in defence against invasive DNA and transposable elements and in gene regulation. Epigenetic variation in plant genomes also orchestrates development both in terms of phase-change transitions and modulating the rate of development. One of the major differences between mammalian and plant DNA methylation is that in mammalian genomes, genomic methylation patterns are erased and reset early in embryogenesis (Yoder et al. 1997), whereas in plants methylation patterns are inherited through meiosis (Bender 2004). Reduced levels of DNA methylation in plants can result in a wide range of abnormalities including loss of apical dominance, reduced stature, altered leaf size and shape, reduced root length, homeotic transformation of floral organs and reduced fertility (King 1995; Finnegan et al. 1998).

Induction of hypomethylation (demethylation) generally results in a range of abnormal phenotypes. Hypomethylation may have *cis* and *trans* effects on gene regulation either acting to derepress a gene or where the gene affected is itself a repressor of another gene, the resultant phenotypic effect may appear to be downregulation of a target gene. In many cases the phenotypes arise from gain-of-function as a result of the removal of 5mC from repressed genes, transposons, and repeats. However a few phenotypes resulting from loss-of-function mutation have also been isolated.

As methylation patterns in plants are inherited through meiosis, it is important that the integrity of these patterns is maintained. A recent study of *Arabidopsis* has revealed that reactivation of transposable elements, mediated by the absence of the DDM1 protein, occurs in the vegetative nucleus during fertilization, but not in sperm cells (Slotkin et al. 2009). However, the vegetative nucleus does not contribute DNA to the zygote. The double fertilization of angiosperms involves transmission of two sperm cells from the pollen.

One fuses with two polar nuclei to form the primary vegetative endosperm nucleus and the other sperm with an oocyte to form the generative nucleus that develops into the zygotic embryo. Loss of DDM1 in the vegetative nucleus results in reactivation of transposable elements and 21nt siRNAs, which accumulate in the neighbouring sperm cells and target the silencing of retrotransposons. This may explain the increased rate of somaclonal variation in double haploid lines generated via microspore or anther culture, where embryos are solely derived via somatic regeneration from pollen sperm cells with no feedback from neighbouring vegetative nucleus. These findings could also suggest that the evolution of the vegetative nucleus as the sperm-companion may help to ensure that the integrity of epigenetic information from one generation to the next is protected in angiosperms (Slotkin et al. 2009).

Recent surveys of epigenetic variation in *Arabidopsis* using tiling microarrays have shown that at least a third of the expressed genes are methylated in parts of their coding regions, while only about 5% of genes are methylated within promoter regions (Zhang et al. 2006; Vaughan et al. 2007). However, the promoter-methylated genes do have a higher degree of tissue-specific expression (Zhang et al. 2006; Zilberman et al. 2006), and this suggests that such regions could be preferential sites for selection of subtle *cis*-regulation during, for example, fruit development. Methylation differences among ecotypes have been found to be common and heritable (Vaughan et al. 2007). The close correlation of methylation and transcription in *Arabidopsis* is further described by Zilberman et al. (2008), where the authors report that methylation affects the transcription of a large fraction of genes. In particular, they found that the histone variant H2A.Z is distributed preferentially in nucleosomes towards the 5′ of *Arabidopsis* genes and is excluded from sites of heavily methylated DNA within actively transcribed genes (Zilberman et al. 2008). H2A.Z has therefore been thought of as providing a mechanism to "protect" DNA from cytosine methylation in euchromatic regions.

The recent availability of methylation profiles from endosperm and embryo and aerial tissues (Gehring et al. 2009; Hsieh et al. 2009) demonstrates the control of seed development and imprinting by genome-wide demethylation of specific tissues. A preliminary methylation site amplified polymorphism (MSAP) analysis has been used to detect differences in the level- and site-specific methylation status during germination of *B. napus* (Lu et al. 2006), which also suggested that radical tissue was less methylated than cotyledon.

Epiallelic variation in plant development

An epiallele refers to variation in the levels of DNA methylation at a specific gene locus. Epialleles may confer novel heritable phenotypes affecting fitness across several generations (for a review, see Kalisz and Purugganan 2004). In recent years specific gene loci affecting key stages of plant development have been characterized and found to be regulated at the epiallelic level, primarily associated with specific changes in DNA methylation. Many of these genes are transcriptional regulators and directly involved in regulatory networks where this level of control mediates feedback with the environment and developmental phase transitions (Table 1).

In tomato, a spontaneously occurring dominant allele of the SBP-box (SQUAMOSA promoter binding protein-like) gene *LeSPL-CNR* at the colourless nonripening locus (*Cnr*) was found to be hypermethylated (Manning et al. 2006). SBP-box genes and their putative MADS-box promoter targets appear to regulate fruit tissue development in both dicots and monocots. This epimutant allele is responsible for the reduced *SPL* expression in *Cnr* fruits leading to inhibition of ripening. It is interesting to note that this dominant mutation arose spontaneously by somatic mutation within a crop variety and became genetically fixed through meiosis. More detailed analysis indicated considerable variation in the pattern of 5mC epigenetic marks within the promoter of *LeSPL-CNR* that contributes to modulation of the rate of pericarp ripening in different cultivars.

The *Arabidopsis bns* phenotype characterized by disrupted phyllotaxis, reduced apical dominance, and abnormal floral morphology was reproducibly isolated from a self-pollinated *ddm1* background (Saze and Kakutani 2007). The epimutation arose from de novo hypermethylation of *BONSAI* (*BNS*), a putative anaphase-promoting complex (APC) 13 gene. *BNS* methylation is dependent upon the presence of a long interspersed nuclear element (LINE) retrotransposon within the 3′ noncoding region.

Decreased DNA methylation can also alter flowering time (Horváth et al. 2003; Kondo et al. 2006). In *Arabidopsis*, the *FWA* gene is a positive regulator of flowering. This gene is maternally imprinted (Jullien et al. 2006) and is dependent on DNA methylation for its imprinted state (Soppe et al., 2000; Kinoshita et al., 2004).

The sporophytic self-incompatibility system of *Brassica* has been widely exploited in development of hybrids of diploid vegetable crops, and has also been used as a model system for studying this multiallelic system. The self-incompatibility locus *S* is characterized by a high level of allelic diversity, with a well described series of dominance relationships among *S* alleles. Tissue-specific monoallelic de novo DNA methylation at the *SP11* gene within the *Brassica rapa S* locus has been shown to contribute to these dominance relationships (Shiba et al. 2006).

Parent-of-origin imprinting mechanisms are characterized by epialleles, where only the maternal or the paternal copy of a gene is expressed in an individual. To date a small number of imprinted genes have been described in plants, where DNA methylation has been shown to confer the imprinted state. Seed growth in the Brassicaceae is controlled and co-ordinated by endosperm, integument, and embryo. Given the importance of seed development to harvest index and quality of oilseed crops such as Canola / oilseed rape (*B. napus*) and mustard oil (*B. juncea*), it is important to understand the role of epigenetic regulation in mediating variation in size and rate of development. In *Arabidopsis*, the maternal copy of *PHERES1* is silenced (Köhler et al. 2003). Although this silencing is not a result of methylation of the gene itself, a *cis* regulatory element needs to be silenced via DNA methylation for the paternal copy of *PHERES1* to be transcribed. In *Arabidopsis* the fertilization-independent genes *FIS2* and *MEA* are both silenced in developing seed via DNA methylation and in the paternal (en-

Table 1. Examples of epialleles in plants and their associated phenotypes.

				Methylation of status of alleles and associated phenotype		
Species	Crop trait	Gene	Gene class	Wild type	Epiallele	Reference
Arabidopsis thaliana	Seed development	♂*FWA*	Homeodomain-containing TF	Hypermethylated and positively regulates flowering	Hypomethylated allele confers late flowering, mediated by DEMETER	Soppe et al. (2000); Kinoshita et al. 2004
Zea mays	Seed development	♂*MEG1*	Cys-rich protein	Biallelic at later stages	Maternal parent-of-origin expression during early stages of endosperm; development	Gutiérrez-Marcos et al. 2004
A. thaliana	Seed development	♂*MEA*	Polycomb protein	Methylated	Mediated by DEMETER	Grossniklaus et al. 1998
A. thaliana	Seed development	♂*FIS2*	Transcription factor	Methylated	Mediated by DEMETER	Luo et al. 1999
A. thaliana	Seed development	*FIE*	Polycomb protein	—	—	Ohad et al. 1996
A. thaliana	Seed development	♀*PHERES1*	MADS TF	—	—	Köhler et al. 2003
A. thaliana	Seed development	♂*MPC*	Poly(A) binding protein	—	—	Tiwari et al. 2008
A. thaliana	Male appendages	*SUPERMAN*	Zinc-finger protein	Unmethylated. Normal flower development	Hypermethylated allele results in excessive staminoid organs	Jacobsen and Meyerowitz (1997)
A. thaliana		*PAI2*	Phosphoribosylanthranilate isomerase	Unmethylated. Does not fluoresce under UV light	Methylated allele leads to fluorescent shoots under UV light	Bender and Fink (1995)
A. thaliana	Plant architecture	*BNS*	Anaphase promoting complex	Methylated	Hypermethylation induced by *ddm1* disrupted phyllotaxis, reduced apical dominance and abnormal floral morphology	Saze and Kakutani (2007)
Solanum lycopersicon	Fruit (pericarp) ripening	*CNR*	MADS TF	Hypomethylated	Hypermethylation	Manning et al. (2006)
Zea mays	Grain colour	*PI*	DNA binding, similar to *MYB* oncogenes	Unmethylated allele *P-rr* confers uniform pigmentation of pericarp	Methylated alleles *P-pr-1* and *P-pr-2* give variegated pigmentation of pericarp	Das and Messing (1994)
Linaria vulgaris	—	*CYCLOIDEA*	Class II TCP transcriptional activator	Hypomethylated. Normal floral symmetry	Hypermethylated allele confers irregular floral symmetry	Cubas et al. (1999)
Arabidopsis	Vernalization	*FLC*	Nuclear localized	—	Modified histone derepresses	Bastow et al. (2004)
Brassica, *Arabidopsis*	Nucleolar dominance	*rDNA*	Ribosomal RNA	—	Nucleolar dominance	Preuss and Pikaard (2007)
Brassica	Self-incompatibility	*SP11*	Cys-rich protein	Promoter methylated	Demethylation leads to transcription	Shiba et al. (2006)
Oryza sativa	Stature	*D1*	—	—	Transcriptional initiation site differentially hypermethylated in metastable *Epi-d1*	Miura et al. (2009)

Note: ♂ = imprinted, silenced paternally in endosperm. ♀ = silenced maternally in endosperm.

dosperm) copy (Choi et al. 2002; Köhler and Makarevich 2006; Gehring et al. 2006).

In maize, the *maternally expressed gene 1* (*meg1*) displays a maternal parent-of-origin expression pattern during the early stages of endosperm development, but at later stages is expressed from both parental alleles. Moreover, there appears to be a correlation between hypomethylation of the maternal *meg1* alleles within the endosperm, and their transcription.

Physiological responses

The exposure of plants to biotic (Stokes et al. 2002) and abiotic stresses in natural environments can trigger epigenetic changes (for a review, see Lukens and Zhan 2007). Unlike variation in nucleotide sequence, it has been demonstrated that epigenetic polymorphisms can be modulated by environmental stimuli (Zhang et al. 2007), and so provide potential for novel heritable variation both desirable and undesirable.

Salt stress has been shown to be associated with semi-quantitative increases in AFLP polymorphism in rapeseed (Lu et al. 2007) as well as extensive changes in the pattern of different classes of MSAP bands. These latter changes include de novo methylation and demethylation events. Water deficit has been shown to increase DNA methylation levels at CCGG sites by 40% in peas (*Pisum sativum*) (Labra et al. 2002). Furthermore, Aina et al. (2004) have reported that heavy metal (Ni^{2+}, Cd^{2+}, and Cr^{6+}) stress led to hypomethylation in *Trifolium repense* and *Cannabis sativa*.

Increased temperature has been found to induce hypermethylation of the transposable element *Tam3* in *Antirrhinum majus* (Hashida et al. 2003), and cold stress can lead to genome-wide demethylation in maize roots, with the maize *ZmMI1* transposon-like sequence being transcribed only under cold stress (Steward et al. 2002). An early study by Burn et al. (1993) found that cold treatment of cultured *Nicotiana* cells resulted in 22% hypomethylation, similar to that achieved following a four day treatment with 100 µmol/L 5-azacytidine. They also demonstrated that 5-azacytidine treatment of *Arabidopsis* and *Thlaspi* induced nonvernalized plants to flower earlier than untreated controls.

The molecular responses to vernalization have since been well characterized in *Arabidopsis*, with three interconnected pathways interacting to affect the floral transition (for a review, see Alexandre and Hennig 2008). The most significant of these is the epigenetic silencing of *FLC* following cold treatment (Schmitz and Amasino 2007), where several regulators are involved in the pathway. This includes VIN3, a PHD-domain protein that is expressed at low temperatures, with dosage accumulation leading to transduction of chromatin modifications at the *FLC* locus. VRN2 has homology to Polycomb-group proteins and maintains *FLC* silencing following the vernalization response. Wood et al. (2006) have shown that a complex similar to metazoan Polycomb-group Repressive Complex 2 (PRC2), comprising VRN2, FIE, and CURLY LEAF/SWINGER and possibly other subunits associates with VIN3 at the *FLC* locus. Such complexes often contribute to establishing modifications to the core histone H3 (H3K27me3) at the target locus, and has been observed also at the *FLC* locus (for a review, see Alexandre and Hennig 2008). This in turn appears to recruit VRN1 and LHP1 as repressors, so maintaining a stable silenced state and continuance of flowering. The epigenetic behaviour associated with vernalization is underlined by the fact that such maintenance of repression requires cell division through mitosis, as reversion to the nonvernalized state can occur in nondividing tissues. Reduction of DNA methylation reduces transcription of *FLC* as well as *MAF-5*, probably by modifying expression of a *trans*-acting regulator (Finnegan et al. 2004).

A model for chromatin-level regulation of phosphate starvation responses has recently been proposed, based on evidence from *Arabidopsis* (Smith et al. 2010). This is based upon ARP6-dependent H2A.Z deposition that appears to modulate transcription of phosphate starvation response genes. In particular, ARP6 is required for accurate H2A.Z deposition at some phosphate starvation genes. H2A.Z is a histone variant that is preferentially localized towards the 5′ of genes in *Arabidopsis*, and has been shown to be excluded from sites of heavily methylated DNA within actively transcribed genes (Zilberman et al. 2008). It has been proposed that H2A.Z provides a mechanism to protect DNA from cytosine methylation in euchromatic regions. The nuclear actin-related protein ARP6, a conserved component of the SWR1 chromatin remodelling complex, regulates transcription by deposition of H2A.Z into chromatin. Thus it is reasonable to expect an interaction between DNA methylation and, amongst others, phosphate responses.

A direct relationship between nucleosomes containing the modified histone H2A.Z and the thermosensory response in *Arabidopsis* has recently been described by Kumar and Wigge (2010). They found that H2A.Z containing nucleosomes are essential for correct perception of ambient temperature, and that the presence of H2A.Z wraps DNA more tightly than in canonical H2A nucleosomes. Moreover, when H2A.Z deposition is prevented, plants display a constitutive warm temperature response. Again, there may also be a relationship between the tendancy of DNA methylation marks to exclude H2A.Z (Zilberman et al. 2008) and the observed temperature response.

Epigenetic variation in the context of crop development and adaptation

Epigenetic variation in crop plants may be detected at a phenotypic level by characteristic properties such as spontaneous modification of plant stature, fruit development and ripening, fertility, leaf shape, seed size, flowering time, and floral symmetry. Early studies indicated that loss of DNA methylation leads to aberrant chromosomal events, which can result in loss of genome integrity (Chen et al. 1998; for a review, see Bird 2002). Demethylation resulting from stress or environmental fluctuations may also lead to activation of transposons resulting in new mutants and somaclonal variation.

Of particular interest for the agronomic consequences of epigenetic variation is that planting distance in maize crop field may also influence DNA methylation (Tani et al. 2005). In an experiment with maize inbred lines and their offspring hybrids, Tani et al. (2005) observed increased DNA methylation in the inbred lines under high density

planting (18.5 plants/m^2), while hybrids were more stable at both high and low density planting (18.5 plants/m^2 and 0.513 plants/m^2). These changes in DNA methylation in response to environmental factors may be a programed response that could be advantageous for adaptation and fitness (Kalisz and Purugganan 2004; Doyle et al. 2008; Xu et al. 2009).

Anecdotal evidence from those who employ tissue culture techniques such as anther or microspore culture to generate homozygous "fixed" genetic lines has indicated that performance is often attenuated after one or more rounds of meiosis (selfing or otherwise). Doubled haploids are now widely used in crop breeding as a means of rapidly generating uniform genetic material, with lines used as parents for generation of F1 hybrids.

Somaclonal variation is a widely observed phenomenon (Phillips et al. 1994), and has often been associated with chromosomal rearrangements, including changes in chromosome numbers (polyploidy and aneuploidy), chromosome structure (translocations, deletions, insertions, and duplications), and mutations within the DNA sequence (Choi et al. 2000). It is becoming apparent that epigenetic changes can occur resulting in gene amplification or variation in DNA methylation. Somaclonal variation has been harnessed as a positive mechanism to induce additional genetic variability into germplasm, but can also be problematic when uniformity and stability of plant material is essential to plantations relying on micropropagated multiplication of clonal stocks. The number of mitotic cycles (somatic cell division) that have occurred appears to be associated with likelihood for errors to occur in the maintenance methylation via methyltransferases. Although much of the available information is anecdotal or correlative it should be noted that for crops vegetatively propagated on an industrial scale (e.g., in Rosaeceae, for fruit and flowers), the appearance of "sport mutants" is relatively common and is frequently identified as changes in fruit colour or homeotic changes. The level of cryptic mutation at this level has yet to be systematically quantified and related to mitotic generation time. Variation in levels of DNA methylation has been observed associated with somaclonal variation and different stages of development for a range of micropropagated trees (Valledor et al. 2007). For example, in date palm, variation in DNA methylation was detected in mother plants and off-shoots using methylation-sensitive amplified polymorphism, with most (81%) being present only in the off-shoots (Fang and Chao 2007).

Plasticity of *Brassica* genomes

The crop species within the genus *Brassica* are credited with having remarkably plastic genomes, which is attributable to gene duplication, intergenomic heterozygosity, and epigenetic phenomena (Lukens et al. 2006; Xu et al. 2009). Rapid alterations of gene expression and cytosine methylation have been detected in newly synthesized *B. napus* allopolyploids (Xu et al. 2009). It appears that this may result from the relatively large scale rearrangements at the very first meiosis of resynthesis (Szadkowski et al. 2010). The pattern of changes in DNA methylation following generation of resynthesized *B. napus* from the initial S_0 of interspecific hybrids of the diploid *Brassica* A and C genomes has been followed in some detail, through subsequent selfed generations to S_5 (Lukens et al. 2006; Gaeta et al. 2007). They found that changes in 5mCG were stably inherited and about 20 times more prevalent that those in 5mCHG, with more changes in the *Brassica* A genome than in the C genome. Interestingly, those in the A genome were predominantly associated with de novo methylation, while in the C genome they included both de novo and demethylation. Xu et al., (2009) made similar observations when they compared transcriptomic changes (4.1%) with changes in DNA methylation (6.8%) in synthetic *B. napus*.

In plants, nucleolar dominance involving chromatin-mediated silencing of rRNA genes is progressively established during post-embryonic development in tissues derived from shoot and root meristems. This plasticity may provide a means of tuning energy requirements for protein synthesis within the plant at different stages in development. Studies in *Brassica* and *Arabidopsis* have shown that an epigenetic-switch controls the number of active rRNA genes and involves methylation of the rRNA gene promoter and histone modification (Grummt and Pikaard 2003; Lawrence and Pikaard 2004; Preuss and Pikaard 2007). A consequence of the switch is the transition from a decondensed state associated with the modified histone H3K4me3 to a highly condensed state, where nucleolar organiser regions are associated with H3K9me2 and 5mC chromocentres (Pontes et al. 2007). Histone deacetylases HDA6 and HDT1 have been implicated in nucleolar dominance, as condensation is disrupted when these genes are downregulated (Pontes et al. 2007). An additional mechanism fine-tunes transcriptional initiation and elongation rates and so determines the total amount of rRNA generated per gene (Preuss and Pikaard 2007). Although it is still unclear what mechanisms specify and maintain patterns of 5mC in nucleolar organiser regions, gel blot analysis with limited locus and sequence-specific resolution has shown a positive, but imperfect correlation between coding and intergenic spacer DNA methylation of 45s rRNA (Woo and Richards 2008).

In *B. oleracea* MSAP analysis has shown an overall high mean rate of methylation ranging from 52% to 60% across different accessions (Salmon et al. 2008), with most MSAP-methylated fragments polymorphic between the populations and lines analyzed. The variation at DNA methylation level was not completely correlated with classification by morphotype or AFLP analysis. However, this level of variation is central to the potential for harnessing stable epigenetic variation for crop improvement.

In a key recent publication, Hauben et al. (2009) have demonstrated that quantitative traits of agronomic importance may be recursively selected through recurrent selfing in isogenic lines of Canola (*B. napus*), and that the stable phenotypic variation inherited is associated with epigenetic variation at the level of DNA methylation. By maintaining selection for respiration they were able to improve the trait of energy use efficiency, with gains of 5% yield increase beyond that achieved by heterosis. The changes in DNA methylation were detected by MFLP analysis, and where these were sequenced, all differentially methylated fragments were associated with coding sequences. In general, the se-

lected lines with improved low-respiration were hypomethylated. Moreover, in addition to epigenetic variation selected at the level of DNA methylation, they demonstrated stable inheritance of changes in histone modifications.

Induction of epiallelic variation to investigate and modulate crop phenotype

Mutations that reduce DNA methylation levels result in embryonic lethality in mammals and in various pleiotropic phenotypes in plants (Bird 2002). Although DNA methylation transcriptionally represses the expression of undesired sequences in the genome (e.g., transposons) and protects genomic DNA against invading DNAs, it can also cause some adverse effects in eukaryotic organisms, contributing to an increased substitution mutation rate from 5mC to T residues.

Although evidence is accumulating in relation to the potential of epialleles to generate novel phenotypes, to date little progress has been made in generating targeted epiallelic variation either to facilitate study of the relationship between DNA methylation and phenotype or to generate novel material as a basis for crop improvement. Different approaches can be taken to intervene in the methylation status of plant genomes. These include chemical treatment and mutational genetics that can be used to generate hypomethylated populations that are then suitable for analysis by reverse genetics screening and high throughput bisulphite sequencing.

Genome demethylation by chemical demethylating agents

Chemical demethylating agents such as 5-azacytidine (5-AzaC), provide a nontransgenic approach for inducing epiallelic variation. 5-AzaC is a potent inhibitor of DNA maintenance methyltransferases genes such as *MET1* and (or) *DMNT*, and therefore targets hypomethylation at symmetrical 5mCG sites in dividing cells. 5-AzaC has been shown to be effective in reverting the hypermethylation of tumour suppressor genes and suppressing cancer-specific cellular phenotypes (Christman 2002).

Hypomethylation with 5-AzaC has been used to generate a range of novel material in *B. oleracea* with altered phenotypes, including a range of morphological changes such as small plant stature, altered leaf development, abnormal proliferation of organs, deformed flower spikes, male sterility, absence of anthers, dwarf pistils, embryo abortion, poor seed set, altered carpel morphology, and altered flowering time (King 1995). Many of these phenotypes were very similar to those observed as somaclonal variation in tissue culture of *B. oleracea*.

Recently, a naturally occurring abnormal floral phenotype observed in potatoes was found to be associated with DNA methylation polymorphisms. Interestingly, the authors were able to reproduce the observed phenotype in 5-AzaC-induced hypomethylated plants and showed that, in both cases, the phenotype segregated with similar DNA methylation patterns and not with DNA sequence. This study and previous ones discussed above indicate that some naturally occurring morphotypes can be better explained by epigenetic variability (Marfil et al. 2009).

Downregulation of DNA methyltransferases and other regulators of DNA methylation

The *MET1* methyltransferase gene is highly conserved, and has been isolated from several other plant species including *B. rapa* (Fujimoto et al. 2006), *Oryza sativa*, *Nicotiana tabacum*, *Zea mays*, *P. sativum*, *Daucus carota*, *Vitis vinifera*, *Prunus persica*, *Solanum lycopersicum*, *Elaeis guineensis*, and *Populus trichocarpa*. In complex genomes where more than one functional copy has been identified, the different copies may have different tissue-specific functionality. For example, in *B. rapa* two copies have been identified, where one is transcribed both in vegetative and reproductive tissues and the other solely in pistils (Fujimoto et al. 2006). In rice two functional copies are both transcribed in callus, imbibed embryo, root, meristem, young panicle, anther, pistil, and endosperm, but vary with respect to their 5′ splicing patterns in different tissues (Yamauchi et al. 2008).

Repeated self-pollination of *ddm1* mutations in *Arabidopsis* leads to a spectrum of morphological abnormalities including short internodes, late flowering, reduction or increase in apical dominance, small leaf size, and reduced fertility (Kakutani et al. 1996), with some of these abnormal phenotypes segregating independently of the *ddm1*. In *B. rapa*, although no phenotypes were observed where *DDM1* had been downregulated by RNA interference (RNAi) mutants, hypomethylated states in transgenic plants were inherited by the next generation. In some cases these states were retained by plants where the RNAi construct had been removed through segregation (Fujimoto et al. 2008).

Harnessing epigenetic variation in crops: a blueprint for epibreeding

The availability of complete genome sequences for genera such as *Brassica*, which contribute to major oil and vegetable crops, is making it increasingly feasible to identify locus-specific copies of relevant genes. This should enable a detailed view of the distribution of epigenetic marks in paralogous pairs of genes in segmental duplicated chromosomal regions, of epiallelic variation at specific loci across the genepool, and in response to growth under different environmental conditions.

The ability to predict the likely consequences of locus- or region-specific patterns of DNA methylation in terms of rephasing of nucleosomes and other manifestations of chromatin remodelling should also greatly enhance the ability to combine whole transcriptome information with crop phenotype under different growth conditions.

Based on the chemical and genetic approaches outlined above, different strategies could be adopted to intervene in the epiallelic status of a plant. For crop improvement, an important consideration is the requirement for genomic stability. Thus it is vital to address the fact that downregulation of maintenance hypomethylation by methyltransferases such as MET1 is known to lead to progressive demethylation over generations, resulting in unpredictable phenotypes, lack of fertility, and loss of viability. Moreover, there are concerns that derepression or reactivation of transposable elements could result in additional undesirable insertion mutations at an unpredictable rate in subsequent generations.

However, there is potential to develop a strategy that involves transitory hypomethylation in a single generation, followed by rapid selection for the modified epiallele at a known target locus, with strong and rapid recurrent marker-assisted selection of the chromosomal segment into a wild-type background. Where 5-AzaC is used as the hypomethylating agent, then selection of the target epiallele could follow the approach adopted for Targeted Induced Local Lesions IN Genomes (TILLING) (Henikoff et al. 2004). This would involve making use of rapid screening for locus-specific changes in 5mC following bisulphite treatment of pooled DNA or individual plants. We have found that the high resolution melt technique is suitable for this purpose, where suitable care is taken in PCR primer design (Wojdacz and Dobrovic 2007). A similar selection approach may also be taken that makes use of constitutive or induced downregulation of *MET1* genes. Tissue-specific or transitory induced downregulation may be achieved either by transgenesis using RNAi with specific promoters or using virus induced gene silencing of methyltransferases.

Conclusion

There have been recent advances in understanding the distribution of epigenetic marks in plant genomes, together with well described examples of how epiallelic variation can contribute to whole genome and gene-specific regulation of development and interaction with the environment. This suggests that there is considerable scope for manipulation of crop phenotype in a predictable manner by intervening in the epiallelic status of breeding material, in particular at the level of DNA methylation marks. However, the strategies for pursuing such approaches are in their infancy, and considerably more work is required to gain a more comprehensive understanding of the mechanisms for inducing and stabilizing epigenetic variation in crops.

Acknowledgements

The authors are funded by the UK Biotechnology & Biological Sciences Research Council. An outline of this paper was originally presented by G.J. King at the OECD Conference "OECD-GenomeAssociation-Oz09" in Perth, Western Australia, November 2009, for which additional funding is gratefully acknowledged.

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