Quantitative Epigenetics: A New Avenue for Crop Improvement

Vijay Gahlaut [1],*, Gaurav Zinta [1], Vandana Jaiswal [1,2],* and Sanjay Kumar [1,2],*

[1]   Biotechnology Division, CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, 176061, India; gzinta@gmail.com

[2]   Academy of Scientific and Innovative Research (AcSIR), CSIR-IHBT, Palampur, Himachal Pradesh, 176061, India

*Correspondence: vijaygahlaut@ihbt.res.in (V.G.), vandana@ihbt.res.in (V.J.), sanjaykumar@ihbt.res.in (S.K.)

Abstract: Plant breeding conventionally depends on genetic variability available in a species to improve a particular trait in the crop. However, epigenetic diversity may provide an additional tier of variation. The recent advent of epigenome technologies has elucidated the role of epigenetic variation in shaping phenotype. Further, the development of epigenetic recombinant inbred lines (epi-RILs) in the model species such as Arabidopsis has enabled accurate genetic analysis of epigenetic variation. Subsequently, mapping of epigenetic quantitative trait loci (epiQTL) allowed association between epialleles and phenotypic traits. Thus, quantitative epigenetics provides ample opportunities to dissect the role of epigenetic variation in trait regulation, which can be eventually utilized in crop improvement programs. Moreover, locus-specific manipulation of DNA methylation by epigenome-editing tools such as clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) can facilitate epigenetic based molecular breeding of important crop plants.

Keywords: DNA methylation; epialleles; epiRILs; epigenetics; Epigenome-Wide Association Studies

1. Introduction

Epigenetic modifications modulate gene expression without any change in genomic DNA sequences that affects multiple aspects of plant growth and development [1]. These epigenetic modifications mainly involve DNA methylation, histone modification, and small RNA (sRNA)-mediated modifications [2]. Of these epigenetic modifications, DNA methylation is relatively well studied. In plants, DNA methylation predominantly occurs in the cytosines (C) of three sequence contexts: CpG, CpHpG and CpHpH, where H represents any base other than G (i.e. A/C/T). DNA methylation at each sequence context is regulated by a particular set of enzymes named cytosine-5 DNA methyltransferases (C5-MTases) having complementary 'de novo' and 'maintenance' methylation activities [3,4]. In the *de novo* methylation process, unmethylated cytosine residues are methylated, while in methylation maintenance the preexisting methylation patterns are maintained after DNA replication [5]. Different C5-MTases including *DOMAINS REARRANGED METHYLASES* (DRMs), *METHYLTRANSFERASES* (METs) and *CHROMOMETHYLASES* (CMTs) participate in these processes. The DRMs are involved in *de novo* DNA methylation *via* RNA-directed DNA methylation (RdDM) in all three DNA sequence contexts [6]. The DRM2, an ortholog of mammalian DNMT3, is involved in CpHpH methylation of euchromatic regions [7]. The METs are involved in the maintenance of CpG methylation during DNA replication [8]. The CMTs are involved in the maintenance of CpHpG and CpHpH methylations. In Arabidopsis, CMT2 catalyzes CpHpH methylation, while CpHpG methylation is catalyzed by CMT3 and to a lesser extent by CMT2 [9,10]. The roles of DNA methylation in plant development and responses to environmental stress conditions have been discussed in detail previously [11].

The recent advances in next-generation sequencing (NGS) allow profiling of DNA methylation status across entire genomes within a species [8, 12]. Also, species-level epigenomic diversity in the natural populations of *Arabidopsis thaliana* collected from diverse locations of the globe has been determined [13]. Whole-genome bisulfite sequencing (WGBS) is particularly powerful as it constructs the genomic maps of DNA methylation at a single-base resolution level [14]. The WGBS has been utilized in the methylome analysis of several plant species including model plants *Arabidopsis thaliana* and crop plants *Zea mays and Triticum aestivum* [15-17]. The WGBS analysis in Arabidopsis has shown that 5.26% of all genomic C bases are methylated and their allocation on the genome was uneven, for instance, about 55% of CpG context was found to be methylated, which is followed by CpHpG (23%), and CpHpH (22%) contexts [18]. However, substantial variation in methylation patterns have been observed between plant species (see Table 1). For instance, CpG context methylation ranged from 32 % (*Capsella rubella*) to 93 % (*Cicer arietinum*), CpHpG methylation varied from 3.48 % (*Triticum aestivum*) to 89 % (*Cicer arietinum*), and CpHpH methylation ranged from 1.36% (*Eucalyptus grandis*) to 38% (*Cicer arietinum*). The CpG methylation is more prevalent and makes the largest portion of total DNA methylation in plants. Many factors determine the observed variation in methylation levels in different contexts, which include genome size, architecture, and distinction in the activity of methylation targeting pathways.

DNA methylation together with histone modifications and non-histone proteins delineates chromatin structure and its accessibility to transcriptional machinery. Thus, it plays an important role in gene expression regulation, transposon element (TE) silencing, genome stability, and trait inheritance [19]. In spite of having a crucial role in distinct biologicals processes, the application of DNA methylation in crop improvement remains to be fully explored. The epialleles (genetic loci that differ in chromatin state) provide an additional source of variation, which are involved in the regulation of phenotypic diversity and get inherited over generations. Various stable epialleles affecting floral morphology [20], flowering time [21], disease resistance [22], pigmentation [23], and leaf senescence [24] have been reported in different plant species. In Arabidopsis, epigenetic recombinant inbred lines (epiRILs) have been developed, which show variation and high heritability for traits like flowering time and plant height as well as stable inheritance of DNA methylation variants [25]. Utilizing this epiRIL population, epigenetic quantitative trait loci (epiQTL) controlling flowering time and primary root length were identified that showed high heritability (up to 90%) [26]. Considering that epiQTLs are stably inherited and show phenotypic diversity, they are good targets for natural/artificial selection for crop improvement.

In this review, we first discuss various epialleles controlling phenotypic traits in plants. Second, the development of epiRILs and epiQTL mapping populations in Arabidopsis and other important crop plants are described, which can be used for quantitative epigenetic studies to identify epigenetic variants controlling trait. Finally, locus-specific manipulation of DNA methylation levels by using site-specific nucleases to generate epialleles is highlighted, which can be utilized for crop trait improvement.

2. Epialleles (Natural and Mutagen Induced)

Although a number of genes/QTLs have been identified in various plants, however, missing heritability is still a major challenge for researchers and breeders where unknown components regulate phenotype rather than genes/QTLs. Epigenetic modifications are one of the major causes of missing heritability [27]. Changes in the DNA methylation status of a particular gene may affect its expression and can be trans-generationally inherited, which leads to trait variation [28]. Such stably inherited epigenetic variants are referred to as epialleles that contribute to phenotypic variation in plants.

Table 1. DNA methylation levels in different plant species.

Species (common name)	Family	Monocot/Eudicot	Genome size (Mb)	CpG (%)	CpHpG (%)	CpHpH (%)	Reference
Arabidopsis thaliana (Arabidopsis)	Brassicaceae	Eudicot	135	55.00	23.00	22.00	[15]
Beta vulgaris (beet)	Amaranthaaceae	Eudicot	758	92.00	81.00	18.80	[4]
Brassica oleracea (cabbage)	Brassicaceae	Eudicot	648	52.50	22.00	5.11	[4]
Brassica rapa (mustard)	Brassicaceae	Eudicot	485	37.20	17.28	4.44	[4]
Cajanus cajan (pigeonpea)	Fabaceae	Eudicot	833	70.23	54.60	9.87	[29]
Camellia sinensis (tea)	Theaceae	Eudicot	3100	82.00	70.00	10.00	[30]
Cannabis sativa (canabis)	Cannabaceae	Eudicot	818	75.50	65.00	8.72	[4]
Capsella rubella (pink shepherd's-purse)	Brassicaceae	Eudicot	219	32.00	9.90	34.70	[4]
Cicer arietinum (chickpea)	Fabaceae	Eudicot	738	93.00	89.00	38.00	[31]
Citrus clementina (clementine)	Rutaceae	Eudicot	370	45.83	25.13	8.26	[4]
Cucumis sativus (cucumber)	Cucurbitaceae	Eudicot	367	45.88	16.50	4.12	[4]
Eucalyptus grandis (rose gum)	Myrtaceae	Eudicot	640	37.12	19.96	1.36	[4]
Fragaria vesca (strawberry)	Rosaceae	Eudicot	240	48.35	20.63	2.32	[4]
Glycine max (soybean)	Fabaceae	Eudicot	1115	63.20	38.40	4.10	[32]
Gossypium raimondii (cotton)	Malvaceae	Eudicot	880	71.97	57.80	13.14	[4]
Lotus japonicus (birdsfoot trefoil)	Fabaceae	Eudicot	472	67.75	36.59	8.66	[4]
Malus domestica (apple)	Rosaceae	Eudicot	742	63.50	44.14	4.57	[4]
Manihot esculenta (cassava)	Euphorbiaceae	Eudicot	742	51.53	30.38	1.90	[4]
Medicago truncatula (barrel clover)	Fabaceae	Eudicot	465	59.80	16.94	5.09	[4]
Populus trichocarpa (poplar)	Salicaceae	Eudicot	500	43.95	26.78	5.01	[4]
Prunus persica (peach)	Rosaceae	Eudicot	265	50.18	19.59	3.64	[4]

Ricinus communis (castor bean)	Euphorbiaceae	Eudicot	323	64.54	37.94	11.97	[4]
Solanaceae lycopersicum (tomato)	Solanaceae	Eudicot	907	84.05	54.84	8.35	[4]
Solanaceae tuberosum (potato)	Solanaceae	Eudicot	840	70.90	42.20	15.80	[33]
Vitis vinifera (grape vine)	Vitaceae	Eudicot	487	45.95	20.43	1.15	[4]
Brachypodium distachyon (stiff brome)	Poaceae	Monocot	352	49.17	19.17	1.41	[4]
Oryza sativa (rice)	Poaceae	Monocot	430	54.70	37.30	12.00	[16]
Panicum hallii (Hall's panicgrass)	Poaceae	Monocot	550	56.28	29.97	2.43	[4]
Panicum virgatum (switchgrass)	Poaceae	Monocot	1600	53.56	35.74	3.06	[4]
Setaria viridis (green foxtail)	Poaceae	Monocot	515	44.49	23.25	1.56	[4]
Sorghum bicolor (sorghum)	Poaceae	Monocot	730	84.75	73.25	5.81	[4]
Triticum aestivum (wheat)	Poaceae	Monocot	17000	53.30	3.48	1.41	[17]
Zea mays (maize)	Poaceae	Monocot	2665	86.00	74.00	5.40	[7]

Several epialleles have been reported in the model plant Arabidopsis and crop plants like rice, maize, field mustard (details are given in Table 2). The first classical example of epiallele was reported in Arabidopsis and known as *clark-kent* (*clk*). It is a natural epimutant with an enhanced number of stamens and carpels. In this epimutant, hypermethylation of cytosine occurred at flower development locus *SUPERMAN* [20]; while hypomethylation of this locus in *clk* mutants was found unstable and reverted back to the wild type phenotype. A total of seven independent *clk* mutants were reported with similar phenotypes. Peloric mutant is another classical example of epialleles found in toadflax (*Linaria vulgaris*). Peloric mutants (radial flower) showed different flower symmetry as compared to wild type plants (bilateral flower). In this epimutant, hypermethylation occurs at the promoter of *Lcyc* gene (homolog of *CYCLOIDEA* gene of Antirrhinum, responsible for floral symmetry) and led to the silencing of the gene. This epimutation is stably inherited across the generation over a hundred years; however, loss of hypermethylation of the promoter of the Lcyc gene may regain bilateral floral symmetry of wild type [34]. In tomato, two epialleles were reported that affect fruit ripening and vitamin E accumulation. Hypermethylation at the promoter of *colorless non-ripening* (*cnr*) locus encoding SBP-box transcription factor (TF) causes ripening defective fruits in tomato [35]. Similarly, DNA methylation of *Vitamin E* (*VTE3*) gene promotor affects its expression that leads to vitamin E accumulation in tomato fruits [36]. In melon, it was shown that DNA hypermethylation in the promoter of *CmWIP1* (*WASP/N-WASP-interacting protein 1*) TF causes the transition from male to female flowers [37]. In the case of rice, six epialleles were reported that affect phenotypes such as dwarf stature, panicle architecture, leaf angle, seed size, and photosynthetic capacity (Table 2). In brassica, the epiallele *S locus protein 11/S locus* (*SP11/SCR* locus) was involved in the dominance effect that regulates the self-incompatibility [38].

Another important epigenetic phenomenon is known as paramutation. It was initially observed in the *r1* (*red1*) locus that regulates anthocyanin pigment biosynthetic pathway in maize [39], and subsequently found in *booster1* (*b1*), *purple plant1* (*pl1*), and *pericarp color1* (*p1*) loci, which also regulate

anthocyanin pigment biosynthetic pathway [23, 40, 41], and *low phytic acid1* (*lpa1-241*) that was involved in phytic acid biosynthesis [42]. Besides spontaneous mutations, small interfering RNA (siRNA) also plays a vital role in the development of epialleles in plants. In Arabidopsis, silencing of the *folate transporter 1* (*AtFOLT1*) gene is regulated by siRNAs derived from the truncated copies of *AtFOLT2* locus, which causes reduced fertility [43]. Further, methylation of transposable elements is also found to affect the phenotype substantially. For instance, hypomethylation of intronic TE (Karma retro TE) caused abnormal splicing of *DEFICIENS* (*DEF*) gene, resulting in parthenocarpy and reduced yield in oil palm [44]. Moreover, epialleles are well known to control a large number of traits including flower/fruit-related traits, sex determination, plant architecture and vitamin accumulation in different plant species (see Table 2). Epialleles also regulate the homeostasis between euchromatin and heterochromatin to maintain genome stability, as the loss of heterochromatin would expose genes to DNA methylation machinery. Most recently, it was shown that DNA methylation is inversely correlated to heterochromatin in *Arabidopsis thaliana* [45].

3. Epigenetic Recombinant Inbred Lines (epiRILs)

EpiRILs are referred to as the recombinant inbred lines that differ for DNA methylation patterns and show no genetic variation. EpiRILs represent an excellent resource to identify the effect of DNA-methylation on phenotypes [25]. Under changing climatic conditions, epigenetic modifications could play a crucial role in plant adaptation to environmental stresses [57]. epiRILs developed in Arabidopsis has been used to explore the effect of environmental factors, which revealed that stress-induced epigenetic modifications are heritable and provide phenotypic plasticity to plants to endure stress [58].

Table 2. List of some stable epialleles reported in different plant species.

Species	Gene/locus	Epigenetic variation	Phenotypic traits	References
Arabidopsis thaliana	*SUP (SUPERMAN)*	Mutagen induced	Floral morphology	[20]
	FWA (Flowering Wageningen)	Mutagen induced	Flowering time	[21]
	PAI2 (Phosphoribosyl Anthranilate Isomerise)	Trans-acting (small RNAs)	Gene expression	[46]
	BAL1	Mutagen induced	Pathogen resistance	[22]
	AG (AGAMOUS)	Mutagen induced	Flower structure	[47]
	BNS (BONSAI)	*ddm1*-induced syndrome	Stunted growth	[48]
	FOLT1(folate transporter 1)	Trans-acting (small RNAs)	Reduced fertility	[43]
	QQS (Qua-Quine Starch)	Spontaneous	Starch metabolism	[49]
	PPH (Pheophytin Pheophorbide Hydrolase)	Spontaneous	Leaf senescence	[24]
	HISN6B (Histidinol-phosphate aminotransferase 1)	Spontaneous	Hybrid incompatibility	[50]
Zea Mays	*R1* (red)	Spontaneous	Pigmentation	[39]
	B1(booster 1)	Spontaneous	Pigmentation	[40]
	PL1 (purple plant)	Spontaneous	Pigmentation	[41]

	P1 (pericarp color)	Spontaneous	Pigmentation	[23]
	Lpa1(low phytic acid1)	Paramutagenic	Phytic acid biosynthesis	[42]
Linaria vulgaris	*Lcyc* (Linaria cycliodea)	Spontaneous	Floral symmetry	[34]
Solanum lycopersicum	*CNR* (Colorless non-ripening)	Spontaneous	Ripening	[35]
	VTE3 (Vitamin E)	Spontaneous	Vitamin E content	[36]
Oryza sativa	*D1*(Drawf1)	Spontaneous	Dwarf	[51]
	SPL14 (Squamosa Promoter binding protein-Like)	Spontaneous	Panicle branching and higher grain yield	[52]
	FIE1 (*Fertilization-Independent Endosperm 1*)	Spontaneous	Dwarf	[53]
	RAV6 [Related to Abscisic Acid Insensitive 3 (ABI3)/Viviparous1 (VP1) 6]	Spontaneous	Leaf Angle and Seed Size	[54]
	AK1 (*Adenylate Kinase*)	Spontaneous	Photosynthetic capacity	[55]
	ESP (*Epigenetic Short Panicle*)	Spontaneous	Short panicle phenotype	[56]
Elaeis guineensis	*DEF1* (*DEFICIENS*)	Spontaneous	Mantled fruit	[44]
Brassica rapa	*SP11/SCR* (*S locus protein 11/S locus cystein rich*)	Trans-acting (small RNAs)	Self-incompatibility	[38]
Cucumis melo	*CmWIP1* (*WASP/N-WASP-interacting protein 1*)	Transposon Insertion	Sex determination	[37]

In Arabidopsis, two different epi-RILs have been developed [25, 59]. One epi-RIL population was derived from the crossing of *met1* mutant and its isogenic wild type [59]. The *met1* mutant is defective in DNA methyltransferase [8, 60]. In the F_2 and subsequent generations, only wild type *MET1* alleles were selected in order to prevent *de novo* DNA methylations. In each generation, progenies were advanced using single seed descent method. Similarly, another epi-RIL population was derived from the crossing of *ddm* mutant and its isogenic wild type [25]. The *ddm* mutant is defective in *DDM* locus that harbor nucleosome-remodeling ATPase required for the maintenance of C methylation [61, 62]. Due to the utilization of isogenic lines in crossing, these epiRILs have no genetic variations, however, epigenetic variations are maximum. Schematic representation of the development of epiRILs is given in Figure 1. The following points need to be considered while studying transgenerational epigenetic variations.

3.1. Persistence of Epigenetic Modification in the epiRILs

Epigenetic modifications are heritable and maintained across several generations as revealed from epiRILs developed by the crossing of mutants (*met1* or *ddm1*) with its isogenic wild types [63,64]. In the *ddm* epiRILs, epigenetic loci targeted by small RNAs were extensively remethylated, while nontargeted loci remained unmethylated [65]. In contrast, *Flowering Wageningen* (*FWA*) epiallele associated with flowering time became methylated in the subsequent generations despite being targeted by small RNA [25, 59]. The mechanism underlying remethylation process and the role of small RNAs in remethylation still remain unclear. However, a recent study provides mechanistic insights on the formation and transmission of epialleles and suggested that histone and DNA methylation marks are critical in determining the ability of RdDM target loci to form stable epialleles [66].

3.2. Phenotypic Variation and Stability in the epiRILs

Continuous variations for different traits observed in above mentioned two epi-RILs suggested that epigenetic modification was involved in the regulation of polygenic traits also. Although two epi-RILs differed for different traits. For instance, epiRILs derived from *met1* mutant showed variation for biotic (bacterial pathogen) and abiotic stress (salt) tolerance [59]; however, in the case of epiRILs derived from *ddm1* mutant, variation can be clearly seen for morphological traits like flowering time and plant height [25].

The stability of phenotypic characters in the above two epiRILs is quite different. Phenotypes in *met1* derived epiRILs are very unstable, and several lines were unable to advance to F_8 generation due to abnormal development and infertility [59]. Unlike, *ddm1* derived epiRILs were found highly stable and more than 99% lines advanced to F8 generations without any abnormality [25].

To explain phenotypic instability, Reinder et al. [59] studied the methylation pattern in *met1*-derived epiRILs. They found that some cytosine methylation sites were highly segregating even in F_8/F_9 generations and suggested that some methylations sites are very unstable and cannot be fixed by repeated selfing. Further, several ectopic and hypomethylations were observed that were different from any of the parental genotypes, suggesting de-novo methylations may be a possible reason for the phenotypic and epigenetic instability across generations in *met1* derived epiRILs [59]. In contrast, in *ddm1*-derived epiRILs, cytosine remethylation process seems to be the reason for nonparental cytosine methylation [65].

4. Development of Epigenetically Modified Population by Chemical Agents

The generation of epigenetic mutants like *met1* or *ddm1* is still not very common in non-model plants. Therefore, chemical agents can be used to induce epigenetic modifications that may serve the purpose to study the effect of epigenetics on quantitative traits. A number of chemical compounds are known to induce epigenetic modifications including DNA methylation, histone modification etc. with different modes of action [67]. For instance, chemicals like 5-Azacytidine, 5-Aza 2' deoxycytidine, Zebularine inhibit the methyltransferase activity and lead to the reduction of transfers the methyl group from S-adenosylmethionine (SAM) to the cytosine ring. Mutagenesis with these chemicals would lead to the development of hypomethylated population. On the other hand, some chemicals act as histone deacetylase inhibitors and increase the histone acetylation and reactivate the silent genes. Chemicals like trichostatin-A (TSA), Helminthosporium carbonum (HC) toxin, nicotinamide, diallyl disulfide sodium butyrate etc. are good examples of histone deacetylase inhibitors. Besides these, some chemicals like sulfamethazine (SMZ), ethionine, dihydroxypropyl adenine (DHPA) hamper the supply of methyl. These chemicals decrease the folate pool and cause methyl deficiency.
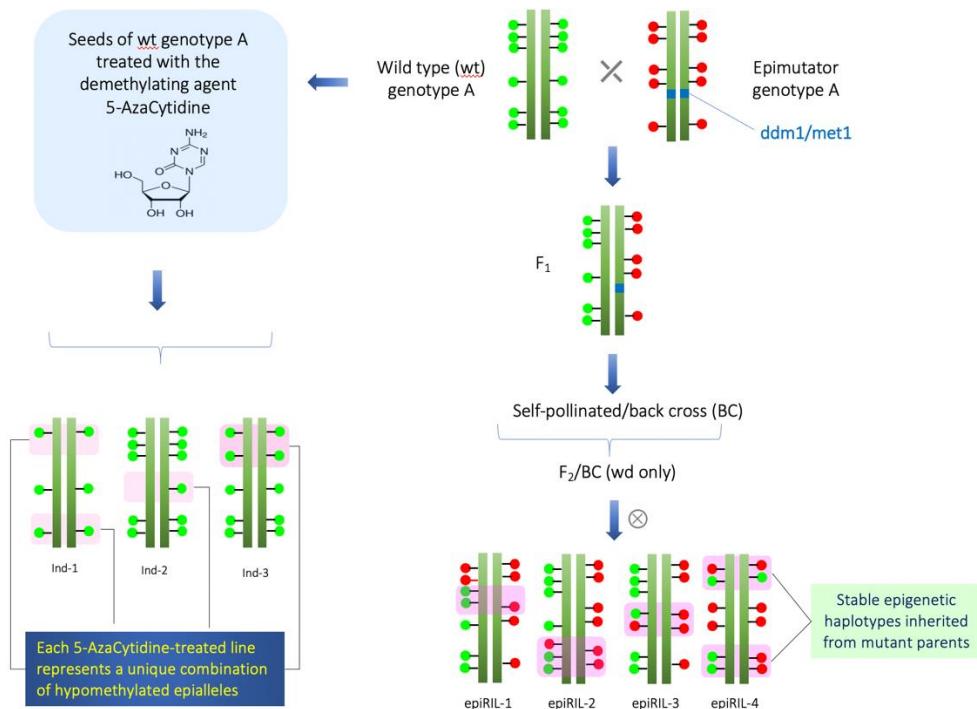
Figure 1. Construction of epiRILs (hypomethylated population) for quantitative epigenetics. In the left side, the scheme for the construction of hypomethylated population using demethylating agent 5-AzaCytidine is shown. On the right side, scheme for construction of epiRILs with stable inheritance by crossing of two parents [wild-type and epimutator parents (*met1* or *ddm1*)] with different epigenetic states is shown.. The green and red circles that overlay the genome sequence illustrates the different epigenetic states of the two parents.

The availability of a wide range of chemical mutagens would be a great asset to create epigenetically modified populations in plants. A successful example of the adoption of chemical methods to develop a hypomethylated population in Brassica, where epialleles were developed by using 5-Azacytidine, and this hypomethylated population showed variability for different phenotypic traits (5-AzaC) [68].

Transgenerational inheritance of some phenotypes has also been demonstrated. When compared to untreated controls, BraRoAZ population (hypomethylated population of *B. rapa* line R-o-18) showed a decrease immuno-staining of 5mC on pachytene chromosomes. Further, Methylation Sensitive Amplified Polymorphism (MSAP) showed high divergence as well as variability. High phenotypic variability was also observed for different seed related characters like yield, protein content, oil content, erucic acid, linoleic acid, palmitic acid. Each line in the BraRoAZ population represented a unique combination of hypomethylated epialleles. Thus, efforts should be made for the development of epigenetically modified populations using chemical agents in other crops.

5. Development of User-Friendly Epigenetic Markers

Epigenetic markers are important to study quantitative epigenetics and identify epialleles associated with traits of interest. Several epigenetic markers have been identified to be involved in biological and molecular functions in plants [27, 69-71]. However, utilization of these methylation marks into crop breeding programs is still lacking. The development of a cost-effective and easy genotyping platform for identification and selection of desirable epiallele is needed in crop plants. For the identification of epialleles, three major approaches are available and widely used in the case of human (i) bi-sulfite sequencing PCR (BSP), (ii) methylation-specific PCR (MSP), and (iii) Chop-PCR [72,73]. The first two approaches require bi-sulfite conversion of non-methylated cytosine to uracil. Bisulfite conversion is a very important and crucial step in these methods. Classical

approaches of bisulfite conversion involve lots of effort and time, and even though conversion efficiency and yield are deprived. With the advancement in technologies, now commercial kits are available with high yield and more 99% conversion efficiency [74]. These kits also facilitate the rapid conversion within 3-4 h. An exhaustive comparison of different commercially available kit for bisulfite conversion efficiency and cost-effectiveness has been done elsewhere [75]. In BSP, after bisulfite conversion, the targeted region is PCR amplified and then sequenced to identify the epialleles. However, in MSP, sequencing is not required, and the targeted region is amplified with methylation-specific primer pairs to distinguish epialleles. In chop-PCR, genomic DNA is partially digested with a methylation-sensitive restriction enzymes (MSREs) followed by PCR amplification of the targeted region. Further, quantitative measurement of DNA methylation can be done through MethylLight and methylation-sensitive high-resolution melting (MS-HRM).

Although the development and utilization of epigenetic markers are very much successful in humans [76], however, in the case of plants this area of research lags behind. Therefore, there is an urgent to develop online-platforms and databases for the assessment of epigenetic markers with full description.

6. Quantitative Epigenetic Models for Complex Trait

Several statistical methods exist to detect epigenetic variations and their impact on the phenotype or epiQTLs [27, 69-71]. The significance and accuracy of epiQTLs identification are affected by several factors like recombination, transgressive segregation, instability of epialleles, and parent off origin effect. These factors may create confounding effects during epiQTLs analysis and result in false positives or false negatives. To deal with these interrupting factors Johannes and Colome-Tatche [70] suggested that population derived from crossing between isogenic lines (dissimilar for epigenetic marks) are commendable material. Tal et al. [69] derived covariances between kinship due to epigenetic transmissibility and environmental effect. They modeled the number of chances for epigenetic reset between generations, environmental induction and estimated the heritable epigenetic variance and epigenetic transmissibility. Further, multiple testing is the major drawback of quantitative genetics because it can give several false positives. Jaffe et al. [77] developed a statistical model to deal with multiple testing corrections during genome-wide identification of epigenetic variability. These models can be useful to study the missing heritability contributed by epigenetic variation [71, 78], but did not consider the phenotypic variation contributed by epigenetic variation. Wang et al. [79] suggested a model to estimate phenotypic variation explained by epigenetic variation and their effects on phenotypic values and also the interaction of epigenetic and genetic effects (additive and dominant). This model also predicts the proportion of genetic variation contributed by epigenetic modifications.

7. EpiQTLs and Epigenome-Wide Association Study (EWAS)

As discussed, epigenetic markers are stably inherited across generations and are randomly present in the genome with high frequency. These features allow the exploitation of epigenetic markers in the identification of epiQTLs. Unlike QTLs, epiQTLs are epigenomic loci where no polymorphism for the DNA nucleotide sequence occurs but they differ for cytosine methylation levels, and these differential methylation patterns regulate phenotypic variation of quantitative traits. In Arabidopsis, Cortijo et al. [26] identified major epiQTLs explaining 60-90% heritability for quantitative traits like root length and flowering time using *ddm1*-derived epi-RILs. These epiQTLs were found reproducible and useful for artificial selection. Further, 99.9% epialleles were found stable; and based on the inheritance and recombination events epigenotype map (E-map) was constructed using mutagenic accumulation lines [80]. Another study in Brassica identified epiQTLs for seven agronomic traits using Methylation-Sensitive Amplified Fragment Length Polymorphism (MS-AFLP) and retrotransposon epimarkers [81]. High stability of epigenetic marks was found at different developmental stages, environmental conditions and transgenerational levels. In Sorghum, by implementing MSAP genotyping approach 122 methylation polymorphic loci were generated to construct E-map, which harbored methylation hotspots [82]. In soybean, co-segregation of

differentially methylated regions (DMRs) in RILs allowed the identification of methylQTL (QTLs associated with DNA methylation) [83]. Thus, the stable inheritance of epialleles across generations makes it a potential regulator of phenotypic variations in crops where genetic variation is negligible [80].

Like genome-wide association studies (GWAS), EWAS may prove a worthwhile approach to explore the impact of the epigenetic modifications on phenotype where genome-wide epimarkers are available (Figure 2). EWAS is based on linkage disequilibrium (LD) mapping, which utilizes natural population and historic recombination events and thus can accomplish high-resolution mapping. A number of EWAS studies have been conducted in humans, which identified that epigenetic modifications are associated with several human diseases like Parkinson's disease [84], coronary artery disease [85], Alzheimer's disease [86] and Type 2 diabetes [87], Moreover, to gather the extensive knowledge generated through EWAS in human, EWAS Atlas has also been developed [88]. However, there is a limited number of EWAS performed in plants, and so far, a single EWAS study identified epigenetic modification associated with a mantled abnormality in oil palm [44]. By using somatic clones (diverse for mantled abnormality and oil yield), a locus MANTLED was identified where hypomethylation in LINE retrotransposon leads the alternate splicing and premature termination.
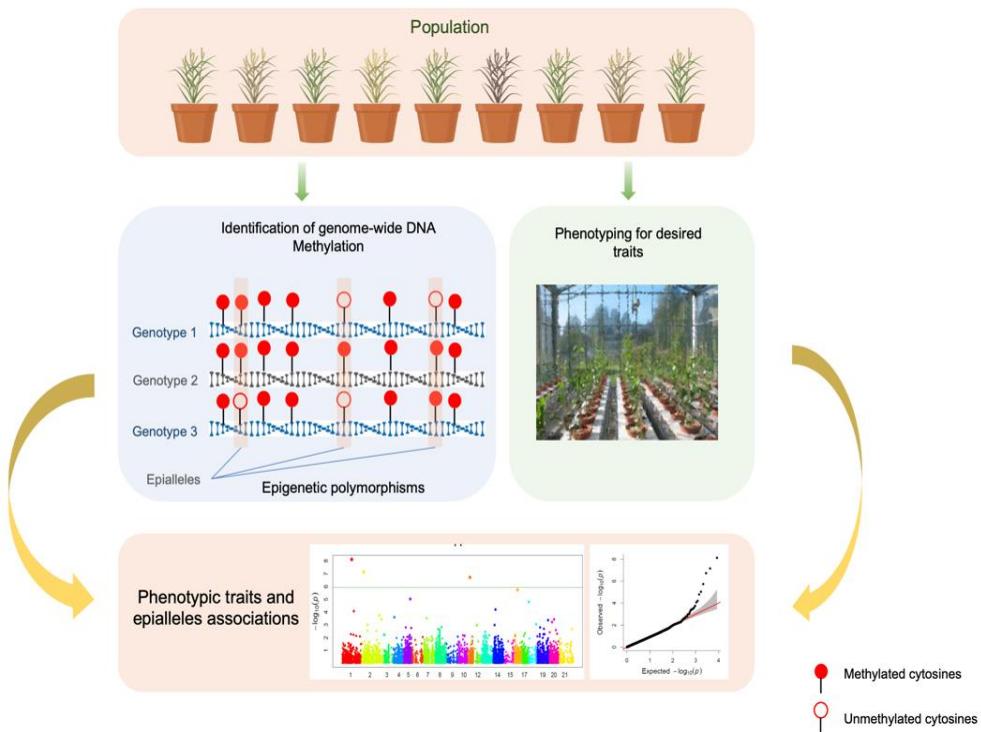
Figure 2. Schematic representation of epigenome-wide association mapping (EWAS) involving three major steps- (i) precise phenotyping of diverse germplasm, (ii) epigenotyping to explore different epialleles, (iii) statistical analysis to identify EpiQTLs.

Linkage mapping and LD mapping have their own importance and limitations. To overcome limitations, integrated genetic mapping by combining linkage and linkage-disequilibrium is recommended. Recently, an integrated linkage and linkage-disequilibrium mapping was conducted for the identification of epiQTLs in plants [89]. Using 550 F₁s and 435 natural germplasm accessions a huge number of epiQTLs (163 epiQTLs) were found to be associated with growth and wood properties in Populus. Further, 23 causal genes present within epiQTL regions showed cause and effect relationship as revealed by the coregulation of eQTN (expression quantitative trait nucleotide) and eQTM (expression quantitative trait methylation) [89].

As mentioned earlier, several epialleles have been reported in plants, and huge opportunities still exist to explore it further in various plant species. Thus, we strongly recommend that EWAS should be performed in crop plants, which will contribute to improve our understanding of epigenetic mechanisms regulating phenotypic traits, and also expedite crop improvement programs through epibreeding.

8. Epigenome Editing Using Site-Specific Nucleases

Several tools are reported that allow site-specific manipulation of DNA methylation/demethylation using programmable DNA-binding proteins [zinc finger (ZFs) proteins and CRISPR-dCas9] in plants [90]. In Arabidopsis, it was shown that ZF fused with RdDM component SU(VAR)3-9 HOMOLOG 9 (SUVH9) was able to cause target methylation to the *FWA* promoter. It caused *FWA* silencing via heritable methylation and led to the late-flowering phenotype [91]. Recently, Gallego-Bartolome et al. [92] tested the capability of several other RdDM components [i.e. RNA-dependent RNA polymerase 2 (RDR2), Sawadee Homeodomain Homolog 1 (SHH1), Microrchidia 1 (MORC1), MORC6, Defective in Meristem Silencing 3 (DMS3), and RNA-Directed DNA Methylation 1 (RDM1)] to promote targeted DNA methylation at FWA locus when fused with ZF. This study provides a theoretical framework that can be utilized to design efficient targeted DNA-methylation programs in plants. In addition to ZF nucleases, CRISPR-dCas9 was also used to target DNA methylation in plants [93]. Recently developed CRISPR-Cas9-SunTag system having the catalytic domain of tobacco DRM has been used to target a locus-specific and heritable DNA methylation that causes stable silencing of *FWA* locus in Arabidopsis [93]. Besides DNA methylation, targeted DNA demethylation has also been performed by fusing Ten-Eleven Translocation 1 (TET1) to both ZF and CRISPR-Cas9-SunTag systems in Arabidopsis [94]. The DNA demethylation achieved at the *FWA* promoter was found highly specific and heritable in nature. Further development of these tools for targeted DNA methylation and demethylation in plants other than Arabidopsis will open up new avenues to study locus-specific effects of DNA methylation and could be used for the generation of new epialleles.

9. Conclusions and Future Prospects

Strengthening crop improvement programs is crucial to feed the global population. Utilization of epigenetic information at the level of epiQTLs and epialleles may provide new prospects for crop improvement as existing breeding methods primarily focus on genetic and ignore epigenetic aspects. The advancement of new sequencing technologies like BS-seq (Bisulphite Sequencing) and MethylC-seq (MethylC-sequencing) can help to delineate the epigenetic basis of trait determination. This information will eventually enhance the inclusion of epigenetic methods in crop improvement. Furthermore, loci-specific DNA methylation patterns can be achieved in plant genomes by fusion of catalytic domains of *de novo* DNA methylation or demethylation enzymes with nucleases [i.e zinc finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats/dCRISPR-associated protein 9 (CRISPR–dCas9 systems)]. These systems have been employed to engineer epigenomes of mammalian cell lines [95,96] and plants [93,94]. Epigenome engineering will not only help in the functional validation of DNA methylation patterns regulating phenotypic traits but will also help to generate desirable traits by creating epigenomic diversity and can accelerate crop improvement by epibreeding [91].

Author Contributions: All authors planned, researched, discussed and contributed to the writing and editing of this manuscript. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: Thanks are due to the Council of Scientific and Industrial Research for providing funds (MLP-201), V.J. and V.G. thanks to the Department of Science and technology for the INSPIRE faculty award. V.J. also thanks to the Science and Engineering Research Board for the Early Career Research Award.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Feng, S.; Jacobsen, S.E.; Reik, W. Epigenetic reprogramming in plant and animal development. *Science*, **2010**, *330*, 622-627.

2. Simon, S.A.; Meyers, B.C. Small RNA-mediated epigenetic modifications in plants. *Curr. Opin. Plant Biol.* **2011**, *14*, 148-155.

3. Law, J.A.; Ausin, I.; Johnson, L.M.; Vashisht, A.A.; Zhu, J.K.; Wohlschlegel, J.A.; Jacobsen, S.E. A protein complex required for polymerase V transcripts and RNA- directed DNA methylation in Arabidopsis. *Curr Biol.* **2010** *20(10)*), 951-956.

4. Niederhuth, C.E.; Bewick, A.J.; Ji, L.; Alabady, M.S.; Kim, K.D.; Li, Q.; Rohr, N.A.; Rambani, A.; Burke, J.M.; Udall, J.A.; Egesi, C.; Schmutz, J.; Grimwood, J.; Jackson, S.A.; Springer, N.M.; Schmitz, R.J. Widespread natural variation of DNA methylation within angiosperms. *Genome boil.* **2016**, *17(1)*, 194.

5. Law, J.A.; Jacobsen, S.E. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet.* **2010**, *11(3)*, 204-220.

6. Cao, X.; Aufsatz, W.; Zilberman, D.; Mette, M.F.; Huang, M.S.; Matzke, M.; Jacobsen, S.E. Role of the DRM and CMT3 methyltransferases in RNA-directed DNA methylation. *Current Biol.* **2003**, *13(24)*, 2212–2217.

7. Gent, J.I.; Ellis, N.A.; Guo, L.; Harkess, A.E.; Yao, Y.; Zhang, X.; Dawe, R.K. CHH islands: *de novo* DNA methylation in near-gene chromatin regulation in maize. *Genome Res.* **2013**, *23*, 628–637.

8. Saze, H.; Mittelsten Scheid, O.; Paszkowski, J. Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. *Nat. Genet.* **2003**, *34*, 65–69.

9. Zemach, A.; Kim, M.Y.; Hsieh, P.H.; Coleman-Derr, D.; Eshed-Williams, L.; Thao, K.; Harmer, S.L.; Zilberman, D. The Arabidopsis nucleosome remodeler DDM1 allows DNA methyltransferases to access H1-containing heterochromatin. *Cell*, **2013**, *153(1)*, 193–205.

10. Stroud, H.; Do, T.; Du, J.; Zhong, X.; Feng, S.; Johnson, L.; Patel, D.J.; Jacobsen, S.E. Non-CG methylation patterns shape the epigenetic landscape in *Arabidopsis*. *Nat. Struct. Mol. Biol.* 2014, *21*, 64–72.

11. Zhang, H.; Lang, Z.; Zhu, J. Dynamics and function of DNA methylation in plants. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 489–506.

12. Yong, W.; Hsu, F.; Chen, P. Profiling genome-wide DNA methylation. *Epigenetics Chromatin*, **2016**, *9*, 26.

13. Kawakatsu, T.; Huang, S.C.; Jupe, F.; Sasaki, E.; Schmitz, R.J.; Urich, M.A.; Castanon, R.; Nery, J.R. Barragan, C.; He, Y. et al. Epigenomic Diversity in a Global Collection of Arabidopsis thaliana Accessions. *Cell*, **2016** *166(2)*, 492–505.

14. Laird, P.W. Principles and challenges of genomewide DNA methylation analysis. *Nat. Rev. Genet.* **2010**, *11(3)*, 191-203.

15. Cokus, S.J.; Feng, S.; Zhang, X.; Chen, Z.; Merriman, B.; Haudenschild, C.D.; Pradhan, S.; Nelson, S.F.; Pellegrini, M.; Jacobsen, S.E. Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. *Nature*, **2008**, *452(7184)*, 215–219.

16. Li, Q.; Eichten, S.R.; Hermanson, P.J.; Springer, N.M. Inheritance patterns and stability of DNA methylation variation in maize near-isogenic lines. *Genetics*, **2014**, *196(3)*, 667–676.

17. Gardiner, L.J.; Quinton-Tulloch, M.; Olohan, L.; Price, J.; Hall, N.; Hall, A. A genome-wide survey of DNA methylation in hexaploid wheat. *Genome biol.* **2015**, *16*, 273.

18. Lister, R.; O'Malley, R.C.; Tonti-Filippini, J.; Gregory, B.D.; Berry, C.C.; Millar, A.H.; Ecker, J.R. Highly integrated single-base resolution maps of the epigenome in Arabidopsis. *Cell*, **2008**, *133(3)*, 523–536.

19. Hauser, M.T.; Aufsatz, W.; Jonak, C.; Luschnig, C. Transgenerational epigenetic inheritance in plants. *Biochimica et biophysica acta*, **2011**, *1809(8)*, 459–468.

20. Jacobsen, S.E.; Meyerowitz, E.M. Hypermethylated superman epigenetic alleles in Arabidopsis. *Science*, **1997**, *277*, 1100-1103.

21. Soppe, W.J.J.; Jacobsen, S.E.; Alonso-Blanco, C.; Jackson, J.P.; Kakutani, T.; Koornneef, M.; Peeters, A.J.M. 2000. The late flowering phenotype of *fwa* mutants is caused by gain-of-function epigenetic alleles of a homeodomain gene. *Mol. Cell*, **2000**, *6*, 791-802.

22. Stokes, T.L.; Kunkel, B.N.; Richards, E.J. Epigenetic variation in *Arabidopsis* disease resistance. *Genes Dev.* **2002**, *16*, 171-182.

23. Cocciolone S.M.; Chopra, S.; Flint-Garcia, S.A.; McMullen, M.D.; Peterson, T. Tissue-specific patterns of a maize *Myb* transcription factor are epigenetically regulated. *Plant J.* **2001**, *27*, 467–478.

24. He, L.; Wu, W.; Zinta, G.; Yang, L.; Wang, D.; Liu, R.; Zhang, H.; Zheng, Z.; Huang, H.; Zhang, Q.; Zhu, J.K. A naturally occurring epiallele associates with leaf senescence and local climate adaptation in Arabidopsis accessions. *Nat. Commun.*, **2018**, *9(1)*, 460.

25. Johannes, F.; Porcher, E.; Teixeira, F.K.; Saliba-Colombani, V.; Simon, M.; Agier, N.; Bulski, A.; Albuisson, J.; Heredia, F.; Audigier, P.; et al. Assessing the impact of transgenerational epigenetic variation on complex traits. *PLoS Genet.* **2009**, 5(6), e1000530.

26. Cortijo, S.; Wardenaar, R.; Colome-Tatche, M.; Gilly, A.; Etcheverry, M.; Labadie, K.; Caillieux, E.; Hospital, F.; Aury, JM.; Wincker, P.; Roudier, F.; Jansen, R.C.; Colot, V.; Johannes, F. Mapping the epigenetic basis of complex traits. *Science*, **2014**, *343*, 1145-1148.

27. Slatkin, M. Epigenetic inheritance and the missing heritability problem. *Genetics*, **2009**, *182*, 845–850.

28. Becker, C.; Weigel, D. Epigenetic variation: origin and transgenerational inheritance. *Curr. Opin. Plant Biol.* **2012**, *15*, 562–567.

29. Sinha, P.; Singh, V.K.; Saxena, R.K.; Khan, A.W.; Abbai, R.; Chitikineni, A.; Desai, A.; Molla, J.; Upadhyaya, H.D.; Kumar, A. et al. Superior haplotypes for haplotype-based breeding for drought tolerance in pigeonpea (*Cajanus cajan* L.). *Plant Biotechnol. J.* **2020**, *10.1111/pbi.13422*.

30. Wang, L.; Shi, Y.; Chang, X. Jing, S..; Zhang, Q.; You, C.; Yuan, H.; Wang, H. DNA methylome analysis provides evidence that the expansion of the tea genome is linked to TE bursts. *Plant Biotechnol. J.* **2019**, *17(4)*, 826–835.

31. Bhatia, H.; Khemka, N.; Jain, M.; Garg, R. Genome-wide bisulphite-sequencing reveals organ-specific methylation patterns in chickpea. *Sci Rep.* **2018**, *8(1)*, 9704.

32. An, Y.C.; Goettel, W.; Han, Q.; Bartels, A.; Liu, Z.; Xiao, W. Dynamic Changes of Genome-Wide DNA Methylation during Soybean Seed Development. *Sci Rep.* **2017**, *7(1)*, 12263.

33. Wang, L.; Xie, J.; Hu, J.; Lan, B.; You, C.; Li, F.; Wang, Z.; Wang, H. Comparative epigenomics reveals evolution of duplicated genes in potato and tomato. *Plant J.* **2018**, *93(3)*, 460–471.

34. Cubas, P.; Vincent, C.; Coen, E. An epigenetic mutation responsible for natural variation in floral symmetry. *Nature*, **1999**, *401*, 157-161.

35. Manning, K.; Tor, M.; Poole, M.; Hong, Y.; Thompson, A.J.; King, G.J.; Giovannoni, J.J.; Seymour, G.B. A naturally occurring epigenetic mutation in a gene encoding an sbp-box transcription factor inhibits tomato fruit ripening. *Nat. Genet.* **2006**, *38*, 948-952.

36. Quadrana, L.; Almeida, J.; Asís, R.; Duffy, T.; Dominguez, P.G.; Bermúdez, L.; Conti, G.; Corrêa da Silva, J.V.; Peralta, I.E.; Colot, V.; Asurmendi, S.; Fernie, A.R.; Rossi, M.; Carrari, F. Natural occurring epialleles determine vitamin E accumulation in tomato fruits. *Nat. Commun.* **2014**, *5*, 3027.

37. Martin, A.; Troadec, C.; Boualem, A.; Rajab, M., Fernandez, R., Morin, H., Pitrat, M., Dogimont, C., & Bendahmane, A. (2009). A transposon-induced epigenetic change leads to sex determination in melon. *Nature*, **2009**, *461(7267)*, 1135–1138.

38. Shiba, H.; Kakizaki, T.; Iwano, M.; Tarutani, Y.; Watanabe, M.; Isogai, A.; Takayama, S. Dominance relationships between self-incompatibility alleles controlled by DNA methylation. *Nat. Genet.* **2006**, *38(3)*, 297–299.

39. Brink, R.A. A Genetic Change Associated with the *R* Locus in Maize Which Is Directed and Potentially Reversible. *Genetics*, **1956**, *41*, 872–889.

40. Patterson, G. I.; Thorpe, C.J.; Chandler, V.L. 1993 Paramutation, an allelic interaction, is associated with a stable and heritable reduction of transcription of the maize b regulatory gene. *Genetics*, **1993**, *135*, 881–894.

41. Hollick, J.B.; Patterson, G.I.; Coe, E.H.; Cone, K.C.; Chandler, V.L. Allelic interactions heritably influence the activity of a metastable maize pl allele. Genetics, **1995**, *141*, 709–719.

42. Pilu, R.; Panzeri, D.; Cassani, E.; Cerino Badone, F.; Landoni, M.; Nielsen, E.A. paramutation phenomenon is involved in the genetics of maize *low phytic acid1-241* (*lpa1-241*) trait. *Heredity*, **2009**, *102*, 236–245.

43. Durand, S.; Bouche, N.; Perez, Strand, E.; Loude,t O.; Camilleri, C. Rapid establishment of genetic incompatibility through natural epigenetic variation. Curr. Biol. 2012, 22, 326-331.

44. Ong-Abdullah, M.; Ordway, J.M.; Jiang, N.; Ooi, S.E.; Kok, S.Y.; Sarpan, N.; Azimi, N.; Hashim, A.T.; Ishak, Z.; Rosli, S.K. et al. Loss of Karma transposon methylation underlies the mantled somaclonal variant of oil palm. *Nature*, **2015**, *525(7570)*, 533–537.

45. Zhang, Y.; Wendte, J.M.; Ji, L.; Schmitz, R.J. (2020). Natural variation in DNA methylation homeostasis and the emergence of epialleles. *Proc. Natl. Acad. Sci. USA*, **2020**, *117*(9), 4874–4884.

46. Bender, J.; Fink, G.R.. Epigenetic control of an endogenous gene family is revealed by a novel blue fluorescent mutant of Arabidopsis. *Cell*, **1995**, *83*, 725-734.

47. Jacobsen, S.E.; Sakai, H.; Finnegan, E.J.; Cao, X.; Meyerowitz, E.M. Ectopic hypermethylation of flower specific genes in Arabidopsis. *Curr. Biol.* **2000**, *24*, 179-86.

48. Saze, H.; Shiraishi, A.; Miura, A.; Kakutani, T. Control of genic DNA methylation by a jmjc domain-containing protein in *Arabidopsis thaliana*. *Science*, **2008**, *319*, 462-465.

49. Silveira, A. B.; Trontin, C.; Cortijo, S.; Barau, J.; Del Bem, L.E.; Loudet, O.; Colot, V.; Vincentz, M. Extensive natural epigenetic variation at a de novo originated gene. *PLoS Genet.* **2013**, *9(4)*, e1003437.

50. Blevins, T.; Wang, J.; Pflieger, D.; Pontvianne, F.; Pikaard, C.S. Hybrid incompatibility caused by an epiallele. *Proc. Natl. Acad. Sci. USA*, **2017**, *114(14)*, 3702-3707.

51. Miura, K.; Agetsuma, M.; Kitano, H.; Yoshimura, A.; Matsuoka, M.; Jacobsen, S.E.; Ashikari, M. A metastable *dwarf1* epigenetic mutant affecting plant stature in rice. *Proc. Natl. Acad. Sci. USA*, **2009,** *106*, 11218-11223.

52. Miura, K.; Ikeda, M.; Matsubara, A.; Song, X.J.; Ito, M,.; Asano, K.; Matsuoka, M.; Kitano, H.; Ashikari, M. *Osspl14* promotes panicle branching and higher grain productivity in rice. *Nat. Genet.* **2010**, *42*, 545-549.

53. Zhang, L.; Cheng, Z.; Qin, R.; Qiu, Y.; Wang, .JL.; Cui, X.; Gu, L.; Zhang, X.; Guo, X.; Wang, D. et al. Identification and characterization of an epi-allele of *fie1* reveals a regulatory linkage between two epigenetic marks in rice. *Plant Cell*, **2012**, *24*, 4407-4421.

54. Zhang, X.; Sun, J.; Cao, X.; Song, X. Epigenetic Mutation of RAV6 Affects Leaf Angle and Seed Size in Rice. *Plant Physiol*. **2015**, *169*(3), 2118–2128.

55. Wei, X.; Song, X.; Wei, L.; Tang, S.; Sun, J.; Hu, P.; Cao, X. An epiallele of rice AK1 affects photosynthetic capacity. *J. Integr. Plant Biol.* **2017**, *59(3)*, 158-163.

56. Luan, X.; Liu, S.; Ke, S.; Dai, H.; Xie, X.M.; Hsieh, T.F.; Zhang, X.Q. Epigenetic modification of ESP, encoding a putative long noncoding RNA, affects panicle architecture in rice. *Rice*, **2019**, *12(1)*, 20.

57. Mirouze, M.; Paszkowski. J. Epigenetic contribution to stress adaptation in plants. *Curr. Opin. Plant Biol*. **2011,** *14(3)*, 267-274.

58. Kooke, R.; Keurentjes, J.J.B. Epigenetic variation contributes to environmental adaptation of *Arabidopsis thaliana*. *Plant Signal. Behav.* **2015**, *10*, e1057368.

59. Reinders J, Wulff BB, Mirouze M, Marı´-Ordonez A, Dapp M, Rozhon W, Bucher E, Theiler G, Paszkowski J. Compromised stability of DNA methylation and transposon immobilization in mosaic Arabidopsis epigenomes. *Genes Dev.* **2009**, *23*, 939–950.

60. Kankel, MW.; Ramsey, DE.; Stokes, T.L.; Flowers, S.K.; Haag, J.R.; Jeddeloh, J.A.; Riddle, N.C.; Verbsky, M.L.; Richards, E.J. Arabidopsis *MET1* cytosine methyltransferase mutants. *Genetics*, **2003**, *163*, 1109–1122.

61. Jeddeloh, J.A,.; Stokes, T.L.; Richards E.J. Maintenance of genomic methylation requires a SWI2/SNF2-like protein. *Nat Genet.* **1999**, *22*, 94–97.

62. Lippman, Z.; Gendrel, A.V.; Black, M.; Vaughn, M.W.; Dedhia, N.; McCombie, W.R.; Lavine, K.; Mittal. V.; May, B.; Kasschau, K.D. et al. Role of transposable elements in heterochromatin and epigenetic control. *Nature*, **2004**, *430*, 471–476.

63. Vongs, A, Kakutani, T, Martienssen, RA, Richards, E.J. *Arabidopsis thaliana* DNA methylation mutants. *Science,* **1993**, *260*, 1926–1928.

64. Kakutani, T.; Munakata, K.; Richards, E.J.; Hirochika, H. Meiotically and mitotically stable inheritance of DNA hypomethylation induced by ddm1 mutation of *Arabidopsis thaliana*. *Genetics,* **1999**, *151*, 831–838.

65. Teixeira, F.K.; Heredia, F.; Sarazin, A.; Roudier, F.; Boccara, M.; Ciaudo, C.; Cruaud, C.; Poulain, J.; Berdasco, M.; Fraga, M.F. et al. A role for RNAi in the selective correction of DNA methylation defects. *Science*, **2009**, *323*, 1600–1604.

66. Li, J.; Yang, D. L.; Huang, H.; Zhang, G.; He, L.; Pang, J.; Lozano-Durán, R.; Lang, Z.; Zhu, J.K. Epigenetic memory marks determine epiallele stability at loci targeted by de novo DNA methylation. *Nat. Plants*, **2020**, *6(6)*, 661–674.

67. Zhang, H.; Wang, B.; Duan, C.G.; Zhu, J.K. Chemical probes in plant epigenetics studies. *Plant Signal Behav.* **2013**, *8(9)*, e25364.

68. Amoah, S.; Kurup, S.; Lopez, C.M.R.; Welham, S.J.; Powers, S.J.; Hopkins, C.J.; Wilkinson, M.J.; King, G.J. A Hypomethylated population of *Brassica rapa* for forward and reverse Epi-genetics. *BMC Plant Biol.* **2012**, *12*, 193.

69. Tal, O.; Kisdi, E.; Jablonka, E. Epigenetic contribution to covariance between relatives. *Genetics*, **2010**, *184*, 1037–1050.

70. Johannes, F.; Colome-Tatche, M. Quantitative epigenetics through epigenomic perturbation of isogenic lines. *Genetics* **2011**, *188*, 215–227.

71. Furrow, R.E.; Christiansen, F.B.; Feldman, M.W. Environment-sensitive epigenetics and the heritability of complex diseases. *Genetics*, **2011**, *189*, 1377–1387.

72. O'Sullivan, E.; Goggins, M. DNA methylation analysis in human cancer. *Methods Mol Biol.* **2013**, *980*, 131-156.

73. Estival, A.; Sanz, C.; Ramirez, J.L.; Velarde, J.M.; Domenech, M.; Carrato, C.; de Las Peñas, R.; Gil-Gil, M.; Sepúlveda, J.; Armengol, R. et al. Pyrosequencing versus methylation-specific PCR for assessment of MGMT methylation in tumor and blood samples of glioblastoma patients. *Sci. Rep*, **2019**, *9*(1), 11125.

74. Munson, K.; Clark, J.; Lamparska-Kupik, K.; Smith, S.S. Recovery of bisulfite-converted genomic sequences in the methylation-sensitive QPCR. *Nucleic Acids Res*. **2007**, *35(9)*, 2893-2903.

75. Hernández, H.G.; Tse, M.Y.; Pang, S.C.; Arboleda, H.; Forero, D.A. Optimizing methodologies for PCR-based DNA methylationn analysis. *Biotechniques*, **2013**, *55(4)*, 181-197.

76. Kanherkar, R.R.; Bhatia-Dey, N.; Csoka, A.B. Epigenetics across the human lifespan. *Front Cell Dev Biol*. **2014**, *2*, 49.

77. Jaffe, A.E.; Feinberg, A.P.; Irizarry, R.A.; Leek, J.T. Significance analysis and statistical dissection of variably methylated regions. *Biostatistics*, **2012**, *13*, 166–178.

78. Roux, F.; Colome-Tatche, M.; Edelist. C.; Warenaar, R.; Guerche, P.; Hospital, F.; Colot, V.; Jansen, R.C.; Johannes, F. Genome-wide epigenetic perturbation jump-starts patterns of heritable variation found in nature. *Genetics*, **2011**, *188*, 1015–1017.

79. Wang, Z.; Wang, Z.; Wang, J.; Sui, Y.; Zhang, J.; Liao, D.; Wu, R. A quantitative genetic and epigenetic model of complex traits. *BMC Bioinformatics*, **2012**, *13*, 274.

80. Hofmeister, B.T.; Lee, K.; Rohr, N.A.; Hall, D.W.; Schmitz, R.J. Stable inheritance of DNA methylation allows creation of epigenotype maps and the study of epiallele inheritance patterns in the absence of genetic variation. *Genome Biol*. **2017**, *18*(1), 155.

81. Long, Y.; Xia, W.; Li, R.; Wang, J.; Shao, M.; Feng, J.; King, G.J.; Meng, J. Epigenetic QTL mapping in *Brassica napus*. *Genetics*, **2011**, *189* 1093–1102.

82. Duan, Y.; Qian, J.; Sun, Y.; Yi, Z.; Yan, M. Construction of methylation linkage map based on MSAP and SSR markers in *Sorghum bicolor* (L.). *IUBMB Life*, **2009**, *61*(6), 663-669.

83. Schmitz, R.J.; He, Y.; Lopez, O.V.; Khan, S.M.; Joshi, T.; Urich, M.A.; Nery, J.R.; Diers, B.; Xu, D.; Stacey, G., Ecker, J.R. Epigenome-wide inheritance of cytosine methylation variants in a recombinant inbred population. *Genome Res*. **2013**, *23*, 1663-1674.

84. Moore, K.; McKnight, A.J.; Craig, D.; O'Neill, F. Epigenome-Wide Association Study for Parkinson's Disease. *NeuroMolecular Med*. **2014**, *16*(4), 845-855.

85. Sharma, P.; Garg, G.; Kumar, A.; Mohammad, F.; Kumar, S.R.; Tanwar, V.S.; Sati, S.; Sharma, A.; Karthikeyan, G.; Brahmachari, V. et al. Genome wide DNA methylation profiling for epigenetic alteration in coronary artery disease patients. *Gene*, **2014**, *541*, 31–40.

86. Gasparoni, G.; Bultmann, S.; Lutsik, P. Kraus, T.; Sordon, S.; Vlcek, J.; Dietinger, V.; Steinmaurer, M.; Haider, M.; Mulholland, C.B. et al. DNA methylation analysis on purified neurons and glia dissects age and Alzheimer's disease-specific changes in the human cortex. *Epigenetics Chromatin*, **2018**, *11*(1), 41.

87. Cardona, A.; Day, F.R.; Perry, J.; Loh, M.; Chu, A.Y.; Lehne, B.; Paul, D.S.; Lotta, L.A.; Stewart, I.D. Kerrison, N.D. et al. Epigenome-Wide Association Study of Incident Type 2 Diabetes in a British Population: EPIC-Norfolk Study. *Diabetes*, **2019** *68*(12), 2315–2326.

88. Li, M.; Zou, D.; Li, Z.; Gao, R.; Sang, J.; Zhang, Y.; Li, R.; Xia, L.; Zhang, T.; Niu, G.; Bao, Y.; Zhang, Z. EWAS Atlas: a curated knowledgebase of epigenome-wide association studies. *Nucleic Acids Res*. **2019**,47 (D1), D983-D988.

89. Lu, W.; Xiao, L.; Quan, M.; Wang, Q.; El-Kassaby, Y.A.; Du, Q.; Zhang, D. Linkage-linkage disequilibrium dissection of the epigenetic quantitative trait loci (epiQTLs) underlying growth and wood properties in Populus. *New Phytol*. **2020**, 225(3), 1218-1233.

90. Gallego-Bartolomé, J. DNA methylation in plants: mechanisms and tools for targeted manipulation. *New Phytol*. **2020**, *227*(1), 38-44.

91. Johnson, L.M.; Du, J.; Hale, C.J.; Bischof, S.; Feng, S.; Chodavarapu, R.K.; Zhong, X.; Marson, G.; Pellegrini, M.; Segal, D.J. et al. 2014. SRA- and SET-domain-containing proteins link RNA polymerase V occupancy to DNA methylation. *Nature*, **2014**, *507*, 124– 128.

92. Gallego-Bartolomé, J.; Liu, W.; Kuo, P.H.; Feng, S.; Ghoshal, B.; Gardiner, J.; Zhao, J.M.; Park, S.Y.; Chory, J.; Jacobsen, S.E. Co-targeting RNA Polymerases IV and V Promotes Efficient De Novo DNA Methylation in Arabidopsis. *Cell*, **2019**, *176(5)*, 1068-1082.

93. Papikian, A.; Liu, W.; Gallego-Bartolomé, J.; Jacobsen, S.E. Site-specific manipulation of Arabidopsis loci using CRISPR-Cas9 SunTag systems. *Nat. Commun*. **2019,** *10*, 729.

94. Gallego-Bartolomé, J.; Gardiner, J.; Liu, W.; Papikian, A.; Ghoshal, B.; Kuo, H.Y.; Zhao, J.M.; Segal, D.J.; Jacobsen, S.E. Targeted DNA demethylation of the *Arabidopsis* genome using the human TET1 catalytic domain. *Proc. Natl. Acad. Sci. USA*, **2018**,. *115*(9), E2125-E2134.

95. Amabile, A.; Migliara, A.; Capasso, P.; Biffi, M.; Cittaro, D.; Naldini, L.; Lombardo, A. Inheritable Silencing of Endogenous Genes by Hit-and-Run Targeted Epigenetic Editing. *Cell*, **2016**, *167*(1), 219–232.e14.

96. Liu, X.S.; Wu, H.; Ji, X.; Stelzer, Y.; Wu, X.; Czauderna, S.; Shu, J.; Dadon, D.; Young, R.A.; Jaenisch, R. Editing DNA Methylation in the Mammalian Genome. *Cell*, **2016**, *167*(1), 233–247.e17.