

# Hidden genetic nature of epigenetic natural variation in plants

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**Transcriptional gene silencing (TGS) is an epigenetic mechanism that suppresses the activity of repetitive DNA elements via accumulation of repressive chromatin marks. We discuss natural variation in TGS, with a particular focus on cases that affect the function of protein-coding genes and lead to developmental or physiological changes. Comparison of the examples described has revealed that most natural variation is associated with genetic determinants, such as gene rearrangements, inverted repeats, and transposon insertions that triggered TGS. Recent technical advances have enabled the study of epigenetic natural variation at a whole-genome scale and revealed patterns of inter- and intraspecific epigenetic variation. Future studies exploring non-model species may reveal species-specific evolutionary adaptations at the level of chromatin configuration.**

## Transcriptional gene silencing suppresses activity of repetitive DNA elements

In addition to transcriptional activators and repressors, gene expression is also orchestrated by epigenetic mechanisms (see [Glossary](#)) controlling chromatin structure and organization [1–3]. Repetitive DNA sequences are one of the major targets of epigenetic regulation and are usually stably suppressed by TGS [4,5]. Functional TGS, acting via deposition of repressive chromatin marks (e.g., DNA methylation) is essential for maintaining genome integrity, regulating gene expression and proper timing of specific developmental processes [6–9].

In plants, newly inserted and transcriptionally active repeat copies trigger RNA-directed DNA methylation (RdDM), a process in which small interfering RNAs (siRNAs) with perfect homology to the target sites guide *de novo* deposition of cytosine methylation in CG, CHG, and CHH sequence contexts (where H is A, C, or T) [4]. *De novo* DNA methylation in CG and CHG serves as a template for replication-coupled maintenance methylation by METHYLTRANSFERASE1 (MET1) and CHROMOMETHYLASE3 (CMT3), respectively. CHG methylation depends on the presence of histone H3 lysine 9 dimethylation (H3K9me2) deposited by the histone methyltransferase KRYPTONITE (KYP) [10,11]. Repetitive genomic regions are turned into

compact heterochromatin by the chromatin-remodeling factor DECREASED IN DNA METHYLATION1 (DDM1). Generally, the maintenance TGS takes on a major role once silencing is established; however, *de novo* DNA methylation is essential for the silencing of newly inserted repeats or incidentally activated elements [12–15].

Recently, high-throughput genomic analyses have enabled the expansion of in-depth TGS studies to natural populations of model and non-model species. This development has been reviewed in several publications [16,17]. In this review, we focus on the origin of epigenetic natural variation leading to morphological, developmental, or physiological phenotypes. Our survey indicates that epigenetic natural variation is frequently coupled with and also likely to be caused by DNA sequence variation, in *cis* or *trans*.

## RdDM ensures stability of TGS across natural populations

Silencing of transposable elements (TEs) is robust in different thale rock cress (*Arabidopsis thaliana*) strains with a relatively small number of expressed transposons [18–21]. An exception is the non-long terminal repeat retroposon *SADHU1*, which is expressed in Col-0 but silent in Cvi-0 and *Ler-1* strains [18]. However, the fourteen full-length copies of *SADHU* in Col-0 contribute unequally to the transcripts and are differentially methylated. This natural variation in TGS within the genome is determined

## Glossary

**Chromatin:** complex of DNA and associated proteins, mainly histones.

**Epiallele:** genetically identical but epigenetically different loci. One variant is typically transcribed, whereas the other is associated with repressive chromatin marks and silenced.

**Epigenetics:** studies heritable information that is stored in chromatin structure, DNA methylation, and post-translational modifications of histones.

**5-Methyl-deoxycytosine (5mdC):** an epigenetic DNA modification that in plants can occur in all cytosine sequence contexts. It is typically associated with TGS when present at a gene promoter.

**Paramutation:** an interaction between a paramutagenic and a paramutable version of a gene leading to heritable silencing of the originally expressed copy. There is accumulating evidence that paramutation is caused by the action of the RdDM machinery.

**RNA-directed DNA methylation (RdDM):** plant-specific TGS pathway leading to *de novo* DNA methylation guided by siRNA molecules.

**Small interfering RNA (siRNA):** predominantly 21–24 nt-long RNA molecules that lead to degradation or translational inhibition of homologous mRNA. They are often generated from transcription of repetitive DNA and provide sequence specificity to the RdDM machinery.

**Transcriptional gene silencing (TGS):** an epigenetic mechanism suppressing transcription from repetitive DNA sequences through specific chromatin modifications.

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in *cis* [22], which is likely to reflect the differential robustness of the TGS or sensitivity to RdDM.

Intraspecific comparisons of epigenetic mark and gene expression profiles for *A. thaliana*, maize (*Zea mays*), and rice (*Oryza sativa*) accessions revealed 100 to 1000 differentially DNA-methylated regions, but the overall patterns were conserved [23–25]. A comparison of *Arabidopsis* and rice accessions revealed that these differentially methylated regions are associated with accession-specific siRNA profiles [23]. Surprisingly, nearly half of the regions were related to protein-coding sequences. In rice, 10% of differentially methylated genes also showed differential expression [24]. This indicates a possible role of siRNAs in accession-specific gene regulation [23]. Indeed, siRNA-targeted TEs affect expression of neighboring genes and the repressive effects are stronger for those targeted by uniquely matching siRNAs [26,27].

The composition of siRNA populations among different species belonging to the *Arabidopsis* genus seems to be more diverse than that observed among different strains of *A. thaliana*. In *A. thaliana*, the fraction of siRNAs that uniquely match TEs is higher than in the closely related northern rock cress (*Arabidopsis lyrata*) [28]. Generally, uniquely matching siRNAs correlate more consistently with DNA methylation than multiply matching ones, suggesting their greater efficiency in directing *de novo* DNA methylation and TE silencing [29]. Indeed, several retrotransposons in *A. lyrata* were found to have a higher transcription rate relative to their *A. thaliana* homologs [30]. Given that RdDM suppresses transposition [15], it is possible that different siRNA populations and silencing efficiencies in *A. thaliana* and *A. lyrata* contribute to lower and higher TE numbers, respectively [31]. The difference in TE numbers between the two species is surprising given that they are closely related, have a similar genome size, and show preferential clustering of repetitive elements in pericentromeric chromosomal regions [32,33].

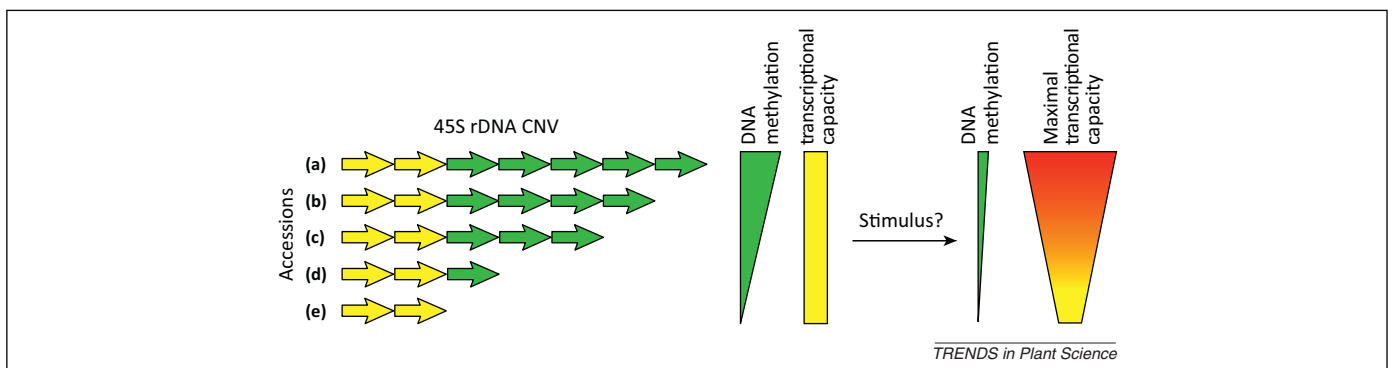
Closely related species such as those in the genus *Arabidopsis* may hybridize in natural populations. Although stable hybrids often have high fitness, throughout the first generations interspecific hybridization seems to represent a ‘genomic shock’ that may transiently weaken TGS [30,34,35]. The genomic shock is often buffered by small RNAs and most likely represents a transient phase

during which a new equilibrium in TE silencing and gene expression is established [36].

### DNA methylation of 45S rDNA repeats correlates with their copy number variation (CNV)

Besides transposons, 45S ribosomal DNA (rDNA) copies, forming the nucleolus organizer regions (NORs), represent one of the largest tandem repetitive tracks in plant genomes and are dynamic in terms of chromosomal positions and repeat copy number [37–39]. The two NORs of *A. thaliana* are subject to epigenetic control [40]. Most *Arabidopsis* accessions have NORs in which only a fraction of the rDNA copies are DNA methylated; however, other accessions with fully methylated or non-methylated repeats at NORs have been found [41,42]. This epigenetic variation is copy number dependent: 45S rDNA in accessions with few copies is generally hypomethylated, whereas at least part of the arrays in high-copy accessions is hypermethylated [42]. We hypothesize that this intriguing pattern might have an adaptive meaning: the methylated copies could serve as a backup that may become demethylated and, subsequently, transcriptionally active under conditions that require enhanced metabolic activity (Figure 1). Evidence for such regulation comes from *Z. mays*, where the heterochromatin proximal to the nucleolus organizing secondary constriction contains rDNA that may become active in rearranged chromosomes that have lost the secondary constriction [43,44]. Further support may be provided by a recent study showing that *Arabidopsis* accessions from North Sweden have longer 45S rDNA arrays than those from South Sweden [39], with prevailing milder environmental conditions. However, a study of crosses between *A. thaliana* accessions carrying differentially methylated NORs suggests that the amount of methylation is under complex regulation [42]. Parental patterns are ‘memorized’ (carried over) in the crosses to some extent and probably reflect predominantly *cis* determination of this DNA methylation. Minor effects have been assigned to specific genomic regions that include multiple, so far not experimentally validated, candidate genes [39,42].

Nevertheless, epigenetic regulation of NORs in *trans* has been implicated in nucleolar dominance, a mechanism in which one parental rDNA set is preferentially silenced



**Figure 1.** Natural copy number variation (CNV) of 45S ribosomal DNA (rDNA) repeats correlates with the amount of DNA methylation. Arrows indicate the relative number of 45S rDNA copies. Yellow and green arrows correspond to unmethylated and methylated copies, respectively. DNA methylation is mainly associated with higher copy numbers, thus restricting the number of active copies (yellow arrows). We hypothesize that, on specific conditions or stimuli, DNA methylation can be reduced in the long rDNA arrays and, thus, maximize their transcriptional output.

in interspecific hybrids [40]. Natural variation in nucleolar dominance has been found in Swedish rock cress (*Arabidopsis suecica*), a natural hybrid between *A. thaliana* and *Arabidopsis arenosa* (sand rock cress). Typically, the NORs derived from *A. thaliana* become transcriptionally suppressed via RdDM [45]. However, in some natural *A. suecica* accessions, biparental expression of NORs has been found [46]. Generating synthetic *A. suecica* using *A. thaliana* parents with different 45S rDNA copy numbers and corresponding DNA methylation patterns may elucidate to what extent these features affect the establishment of nucleolar dominance.

### Spontaneous mutations of TGS control genes lead to changes in DNA methylation and heterochromatin content

Chromosomal regions containing a high density of DNA methylation and histone H3K9me2 form heterochromatin [47–49]. Large heterochromatic segments are organized into chromocenters (CCs) that frequently associate with each other at the nuclear periphery or around the nucleolus [50,51]. Screening for natural variation in cytologically detectable heterochromatin has revealed significant differences among 21 *Arabidopsis* accessions. There, the CC area correlated positively with the latitude and negatively with the annual dose of solar radiation at the original locations [52]. Genetic mapping with the southern accession Cvi-0 (Cape Verde Islands) and the northern accession Ler-1 (Poland), representing parents with small and large CCs, respectively, identified two causal genes, *PHYTOCHROME-B* (*PHYB*) and *HISTONE DEACETYLASE 6* (*HDA6*). The most likely causal *PHYB*<sup>Cvi</sup> polymorphisms are two point mutations leading to non-synonymous substitutions in the PAS-A and PAS-B domains, which are important for light reception and *PHYB* translocation into the nucleus. A reduced amount of CC heterochromatin has been hypothesized to be a result of reduced physical interaction between *PHYB*<sup>Cvi</sup> and *CRY2*, previously identified to control decondensation of heterochromatin during floral transition in a currently unknown manner [53]. *HDA6* has already been implicated in TGS and heterochromatin control [54,55]. The difference between the accessions in this case comprise two single nucleotide polymorphisms (SNPs) that disturb the perfect identity of two simple tandem repeats in the *HDA6* 5' untranslated region (5'-UTR) [53]. Thus, under natural conditions, the degree of heterochromatinization seems to be regulated in response to light, which is likely to be by histone deacetylation and light-sensing pathways.

Genetic screens for mutation-induced loss of centromeric DNA methylation identified *DDM1* and *MET1*, two major players in TGS [56]. A screen of approximately 90 natural *Arabidopsis* accessions revealed reduced centromeric DNA methylation in the Bor-4 accession [57] due to a deletion of the *VARIANT IN METHYLATION* (*VIM1*) gene (Figure 2). *VIM1* is a SRA-SET domain protein that binds to hemimethylated DNA substrate and recruits *MET1* to methylate the opposite strand [58]. Full DNA demethylation observed in the *vim1 vim2 vim3* triple mutant revealed that the *VIM* proteins have a partially redundant role in DNA methylation and repeat silencing [42,59].

Thus, mutations in genes involved in TGS may lead to *in-trans* chromatin changes with genome-wide effects.

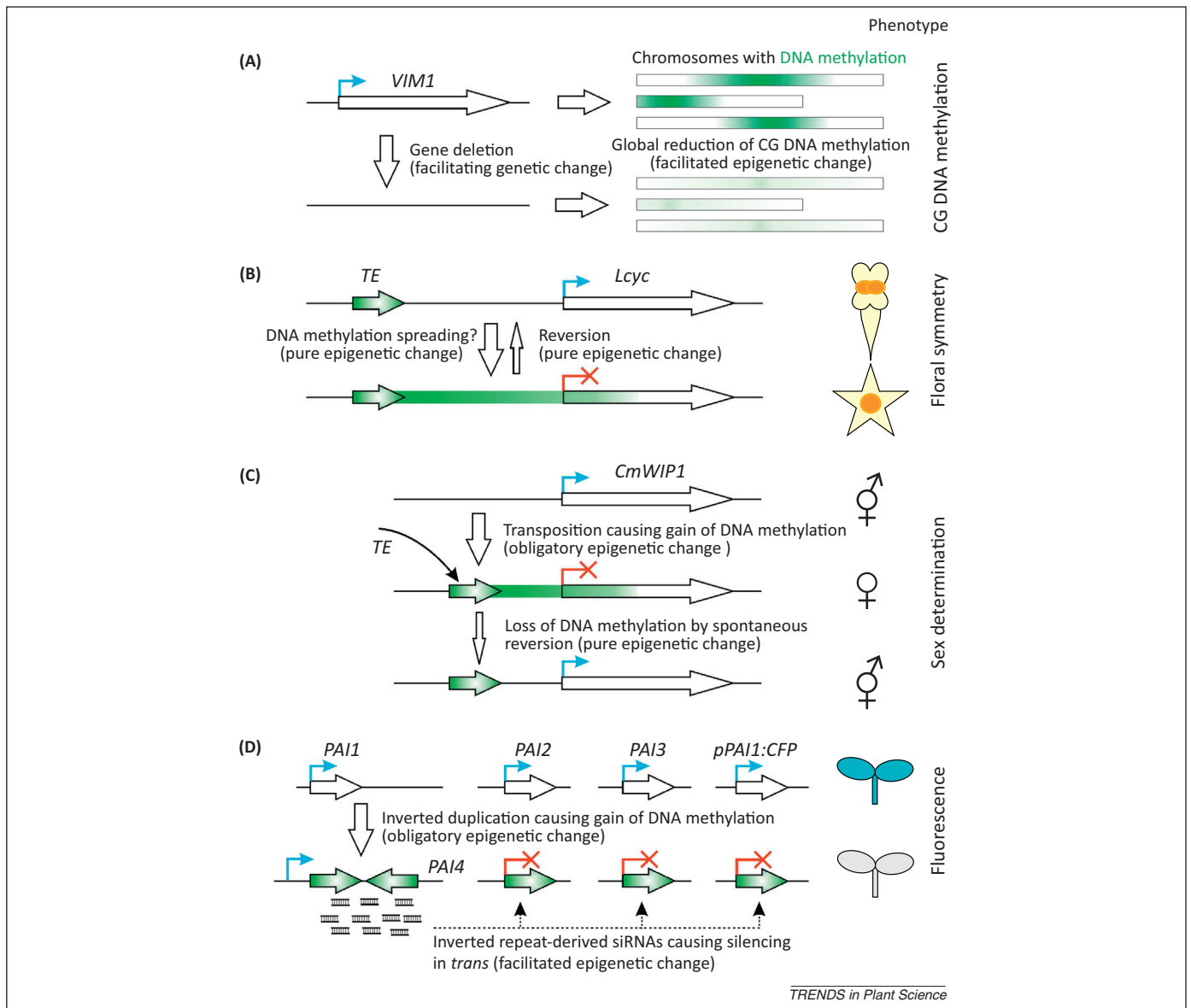
### Gene-body DNA methylation variation and TGS control of protein-coding genes

Approximately 20% of protein-coding genes in *A. thaliana* contain DNA methylation at CG sites in exonic and/or intronic regions [60]. The function of this methylation is not fully understood, but it seems to be more frequent at stably expressed and evolutionarily conserved single-copy genes [20,60,61]. Although extensive natural variation in gene-body methylation is found among *Arabidopsis* accessions, it has not been associated with specific phenotypes until now [19,20].

Naturally occurring TGS variation can affect the expression of protein-coding genes, sometimes resulting in developmental or physiological phenotypes [6,62–65]. These phenotypes were described before their epigenetic basis was revealed, indicating that a systematic search for epigenetic natural variation affecting protein-coding genes might identify many more cases [20].

Gene duplication and rearrangement led to TGS at the *PHOSPHORIBOSYLANTHRANILATE ISOMERASE* (*PAI*) gene encoding enzymes in the tryptophan biosynthesis pathway [62]. Most *A. thaliana* accessions (e.g., Col-0) contain *PAI1*, *PAI2*, and *PAI3*, but a few (e.g., Ws-2) carry an additional copy, *PAI4*, in tail-to-tail orientation with *PAI1* (Figure 2). The *PAI1–PAI4* inverted-repeat locus is DNA hypermethylated by RdDM. Loss of the *PAI1–PAI4* inverted repeat led to a reduction of DNA methylation at *PAI2* and *PAI3*, confirming the role of the inverted repeat as the *trans* genetic trigger of the epigenetic silencing [62]. Silencing of a housekeeping gene such as *PAI* is dangerous because it may impair plant performance and, therefore, is expected to create a strong selection toward bypassing the silencing. Indeed, the Ws-2 accession does not exhibit a tryptophan mutant-like phenotype because the promoter of a head-to-head-oriented gene is used to drive *PAI*<sup>Ws-2</sup>. Plants that do not immediately overcome deleterious silencing effects are expected to be under strong purifying selection in wild populations.

Another example of silencing by a duplicated locus has recently been described for paralogs of the *FOLATE TRANSPORTER* (*FOLT*) gene [65]. A recombinant inbred population of accessions Col-0 and Shahdara (Sha) showed strong linkage disequilibrium for a combination of specific regions on Col-0 chromosome 4 and Sha chromosome 5. The low frequency of plants in the segregating population that have this genomic combination resembles the situation in some hybrid incompatibility systems (for review see, e.g., [66]). Col-0 contains only one active *FOLT1* gene, whereas Sha bears two paralogs, *FOLT1* and *FOLT2*, but only *FOLT2* is expressed. Silencing of *FOLT1*<sup>Sha</sup> is due to promoter DNA hypermethylation and is triggered by *FOLT2*<sup>Sha</sup>. The *FOLT2*<sup>Sha</sup> locus has a complex structure with one full-length and at least two truncated *FOLT2* copies that are likely to produce siRNAs causing *FOLT1*<sup>Col-0</sup> silencing *in trans*. Thus, the combination of an inactive *FOLT1*<sup>Sha</sup> (on chromosome 5) with a chromosome 4 from Col-0 lacking *FOLT2* in one genome leads to complete lack of *FOLT* expression and lethality. A similar incompatibility based



**Figure 2.** Examples of facilitated, pure, and obligatory natural epigenetic variation. **(A)** Facilitated epigenetic variation affecting global CG DNA methylation due to deletion of the *VIM1* gene in *Arabidopsis* accession Bor-4. **(B)** Pure epigenetic variation at the *Lcyc* locus of toadflax (*Linaria vulgaris*). An active *Lcyc* gene confers flowers with unilateral symmetry, whereas a silenced *Lcyc* gene, presumably as a result of the spread of DNA methylation from an upstream transposable element (TE), leads to the development of peloric flowers with radial symmetry. The phenotype occasionally reverts back toward radial flowers by pure epigenetic change. **(C,D)** Obligatory epigenetic variation. **(C)** Insertion of the *hAT* transposon upstream of the *CmWIP1* gene led to its DNA methylation and silencing, resulting in abortion of male sexual organs. Rare reversions by pure epigenetic change may partially restore their development. **(D)** An inverted duplication of the *PAI1* gene caused hypermethylation of this locus and silencing of the original promoter. In addition, three homologs and a reporter construct driven by the *PAI1* promoter were silenced in *trans* by small interfering RNAs (siRNAs). Expression from the *PAI1-PAI4* locus is achieved using a promoter from a gene located upstream (not shown).

on the interaction of the *FOLT* genes was detected in five other mapping populations that had one parent from Russia or Central Asia, suggesting that *FOLT2* rearrangement is common in Eastern *Arabidopsis* accessions. The *PAI* and *FOLT* examples show how structural rearrangements may plunge protein-coding genes under TGS control.

Another way in which protein-coding genes can become controlled by TGS is their proximity to transposons or repetitive DNA sequences [21,67]. One of the best-studied examples is the *FLOWERING WAGENINGEN A* (*FWA*) gene containing a DNA hypermethylated *SHORT INTERSPERSED ELEMENT* (*SINE*) in the 5' regulatory region. Loss of *SINE* methylation in a *ddm1* background leads to *FWA* expression during vegetative growth, causing

delayed flowering [6,68]. Natural *SINE* demethylation occurs in the central cell and the endosperm and causes maternal *FWA* allele expression [69–71]. Comparative analysis of *FWA* in several *Arabidopsis* species has revealed interspecific variation in the *SINE* structure [72]. The most complex *SINE* in *A. thaliana* contains two short and two long tandem repeats, whereas *A. lyrata* and *A. arenosa* harbor only two to four species-specific short repeats. In meadow rock cress (*Arabidopsis halleri*), a *SINE* lacking internal repeats was sufficient to accumulate DNA methylation; however, a higher number of tandem repeats seems to ensure stronger silencing and to control *FWA* expression more efficiently [72]. Another stunning example is associated with sex determination

in melon (*Cucumis melo*). A *hAT* transposon insertion close to the gene encoding the transcription factor *CmWIP1* induced DNA methylation and transcriptional downregulation of the *CmWIP1* gene [64] (Figure 2). This resulted in aborted anthers and enhanced growth of the female gametophyte. The occasional presence of revertant flowers correlated with a lower degree of DNA methylation and partially restored anther development. Natural and induced loss of the *hAT* transposon led to normal development of both floral organs. Therefore, the *hAT* insertion is required for establishment and maintenance of *CmWIP1* promoter methylation and abolishment of male sexual organ development in melon (Figure 2).

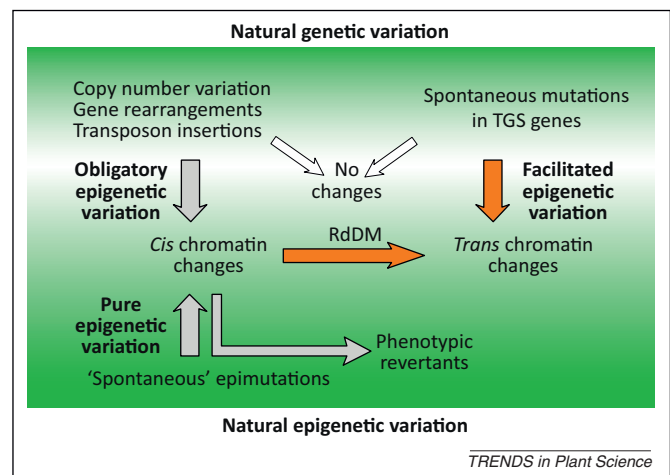
Extreme examples of epigenetic natural variation in protein-coding genes are represented by natural epialleles [63,73–75]. A region responsible for a change from bilateral to radial (peloric) floral symmetry in toadflax (*Linaria vulgaris*) has been mapped to the *cis*-regulatory region of the *CYCLOIDEA-like* (*Lcyc*) gene [63] (Figure 2). In peloric flowers, *Lcyc* was DNA hypermethylated and silenced, presumably by the spread of DNA methylation from an upstream transposon. Phenotypic reversion from peloric to bilateral flowers correlated with reduced DNA methylation. The presence of peloric, intermediate, and revertant flowers within a single plant strongly indicated that *Lcyc* may be an epiallele [63]. Epialleles with an identical sequence of seven tandem repeats have been described for the maize *b1* locus [76]. In some strains, this array is not methylated and allows expression of the pigment gene located 100 kb downstream, resulting in violet coloration, whereas in other strains with methylated repeats the *B1* transcription is suppressed and plants remain green. Combining both epialleles in a cross leads to silencing of the active epiallele by paramutation [76,77]. Thus, paramutation can be considered as an example of epigenetic natural variation in natural populations. However, it is likely that, in the long term, paramutation will act against paramutable alleles and will silence them.

Experimental work has demonstrated how DNA methylation can spread into a protein-coding gene and silence it via the RdDM pathway [78]. However, stimuli initiating the spread of silencing signals in natural populations have yet to be discovered. Laboratory work has demonstrated that plant chromatin can be modified by stress conditions [79–81]; however, to date there is no convincing experimental evidence for long-term inheritance of such changes (reviewed in [82]). Recent analysis of DNA methylation variation among genetically almost identical plants, separated by only 30 generations, has identified a handful of differentially methylated individual cytosines [16,83]. These *de novo* differentially methylated cytosines rarely formed clusters of larger differentially methylated regions. Given that individual methylation patterns seem to be less important than DNA methylation density [84], most of the modifications at single cytosine residues may represent local oscillations without any functional and/or phenotypic consequences. Extensive variation in the methylation of individual cytosines has been observed among accessions of *A. thaliana*, maize, and rice and hundreds to thousands of differentially methylated regions, of size ranging between 0.2 to several kbp, have been found [20,24,25].

Single-base pair resolution in rice and *Arabidopsis* revealed that most changes occurred in the CG methylation context [20,24]. In *Arabidopsis*, these could be associated with gene bodies and, to a lesser extent, with other genomic regions that were also methylated in other sequence contexts [20]. Approximately 20% of the differentially methylated regions were associated with genetic variation and, therefore, could reflect local (*cis*) methylation quantitative trait loci (QTLs) in *Arabidopsis* [20]. However, the question of whether methylation QTLs have any effect on plant phenotypes and/or performance remains to be tested. The prevalence of genetically identical and yet differentially methylated regions can be explained by the *trans*-acting activity of RdDM (e.g., see the *PAI* example described above and in Figure 2) and, therefore, such regions represent distant (*trans*) methylation QTLs.

### Concluding remarks: genetic variation is the major source of epigenetic variation affecting the function of protein-coding genes

On the basis of the above-described examples, we suggest a model for natural epigenetic variation (Figure 3 and Table 1) based on an earlier proposal [84]. Three types of epigenetic variation are defined by their origin and predominant *cis* or *trans* effects as pure, facilitated, and obligatory. Pure epigenetic natural variation occurs without any obvious linkage to genetic differences and forms genetically identical but epigenetically different epialleles. To date, pure epigenetic natural variation leading to phenotypic consequences has been detected for only a few loci in plants (*Lcyc*, *B*). By contrast, epigenetic natural variation induced by DNA sequence changes seems more common. Facilitated natural epigenetic variation is a two-step process. First, a genetic mutation in a TGS control gene occurs (e.g., *VIM1*, *HDA6*) and creates a facilitating change. Second, under the right genetic configuration (e.g., appearance of



**Figure 3.** Origin of natural epigenetic variation. DNA mutations may result in obligatory natural epigenetic variation. No such changes are observed for pure natural epigenetic variation. When effective, both types lead to chromatin changes in *cis* (gray arrows) that can silence homologous sequences in *trans* (orange arrows) via RNA-directed DNA methylation (RdDM). Spontaneous mutations in genes controlling transcriptional gene silencing may result in facilitated natural epigenetic variation that leads to chromatin changes in *trans* and can be independent of RdDM.

Table 1. Examples of genomic loci showing natural variation in transcriptional gene silencing

Source	Target	Species	Epigenetic variation				DNA sequence variation	Chromatin change		Phenotype	Refs
			Obligatory	Facilitated	Pure			<i>cis</i>	<i>trans</i>		
45S rDNA	45S rDNA	<i>A. thaliana</i>	+	?	?		CNV	+	-	None in wild type	[41,42]
FWA	FWA	Arabidopsis genus	+	-	-		Intrarepeat CNV	+	-	Late flowering	[6,68]
PAI1-PAI4	PAI1-PAI4, PAI2, PAI3	<i>A. thaliana</i>	+	+	(PAI2, PAI3)		CNV, rearrangement	+	-	Transgene silencing	[62]
FOLT2	FOLT1, FOLT2	<i>A. thaliana</i>	+	+	(FOLT1)		CNV, rearrangement	+	-	Lethality	[65]
TE near <i>CmWIP1</i>	<i>CmWIP1</i>	<i>C. melo</i>	+	-	(+ revertant flowers)		TE insertion	+	-	Abortion of anther development	[64]
TE near <i>a-m2-7991A1</i>	<i>a-m2-7991A1</i>	<i>Z. mays</i>	+	-	-		TE insertion	+	?	Pigmentation	[67]
<i>VIM1</i> deletion	CG DNA methylated cytosines	<i>A. thaliana</i>	-	+	-		Gene deletion	-	+	Partial loss of DNA methylation	[42,57]
<i>PHYB</i> mutation	Pericentromeric heterochromatin	<i>A. thaliana</i>	-	+	-		Point mutations	-	+	Less heterochromatin	[52]
<i>HDA6</i> mutation	Pericentromeric heterochromatin	<i>A. thaliana</i>	-	+	-		Point mutations	-	+	Less heterochromatin	[52]
<i>Lycy</i> locus	<i>Lycy</i>	<i>L. vulgaris</i>	-	-	+		Not detected	+	-	Peloric flowers	[63]
<i>Cnr</i>	<i>Cnr</i>	<i>Solanum lycopersicum</i>	-	-	+		Not detected	+	-	Colorless fruits	[75]
<i>P-pr</i>	<i>P-pr</i>	<i>Z. mays</i>	-	-	+		Not detected	+	-	Pigmentation	[73]
Tandem repeat 100 kb upstream	<i>B</i> gene	<i>Z. mays</i>	-	-	+		Not detected	+	-	Pigmentation	[74,76]

homozygous mutants in cases of recessive loss-of-function alleles) such mutations lead to altered or lost protein function, facilitating *in-trans* chromatin changes at the target loci. Obligatory natural epigenetic variation results directly from genetic changes such as transposon insertions, non-synonymous substitutions, CNV, and gene rearrangements (Figures 2 and 3 and Table 1), acts preferentially in *cis* and affects genes and repeats (e.g., *NORs*, *PAI*, *FOLT*, *CmWIP1*). Gene CNV and structural rearrangements are relatively common in natural populations [85], suggesting that the resulting epigenetic variation may occur frequently. However, it has to be taken into account that not every genetic change of this type must necessarily result in heritable chromatin modifications. *Cis* chromatin changes caused by pure or obligatory natural epigenetic variation may subsequently lead to *trans* silencing of homologous regions (e.g., *PAI*, *FOLT*), presumably by RdDM. It must also be noted that several types of natural epigenetic variation may act at the same locus. For example, DNA methylation at the *CmWIP1* locus, gained in obligatory way, may be lost by a pure epigenetic change (Figure 2) and result in a partially reverted phenotype [64].

Natural variation in DNA methylation was detected at thousands of loci among strains of *A. thaliana*, maize, and rice [20,24,25]. However, the ratio between the total number of changes and those resulting in developmental or physiological phenotypes ( $n = <20$ ) (Table 1) suggests that most of these epigenetically differentially marked regions have little or no effects on genome and gene function. Hence, natural epigenetic variants affecting gene function are at least an order of magnitude less frequent than genetic mutations [86,87]. Among natural epigenetic variation, facilitated natural variation is the least frequent. In the three known cases, only one or two accessions were affected. Nevertheless, facilitated variation may have the strongest effect on transposon amplification, because it affects many loci genome wide. Pure and obligatory natural epigenetic variations affecting gene expression are more frequent; however, mapping the causal loci is not straightforward because they frequently, and to various extents, follow non-Mendelian inheritance [41,42,77]. Our survey suggests that obligatory natural epigenetic variation leading to morphological or developmental phenotypes is 30% more frequent than pure variation (Table 1). This may be an underestimation, because only low-resolution information is available on the structure of some loci that are considered to be true epialleles (e.g., *Lycy*) [63]. However, many recently discovered cases of obligatory and pure epigenetic natural variation [20,24,25] still need to be tested for their possible effects on gene function.

In summary, TGS suppresses repetitive elements, buffers genetic variation, and regulates gene expression in developmental and environmental contexts. In addition, it can be seen as an important contribution to the creation of potentially heritable diversity of gene expression patterns in natural populations. However, transgenerational experiments have indicated that inheritance of novel epigenetic patterns follows complex rules and is not common in plants [82]. The examples referred to here show that natural epigenetic variation is frequently induced in response to specific DNA sequence alterations, such as gene

rearrangement or nearby transposon insertion, and may further act in *trans* by RdDM. Recent progress in plant genomics and the rapidly increasing potential to access the epigenomes of many populations and non-model species should enable epigenetic natural variation to be explored on a previously unprecedented scale and its relationship with genetic components to be investigated at a larger scale and in greater depth.

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