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Review

DNA Methylation in Rice and Relevance for Breeding

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Abstract: The challenge of sustaining food security in the context of global changes is at the heart of plant research. Environmental stresses, in particular, are known to impact genome stability and epigenetic mechanisms. Epigenetic pathways are well characterized in plants, particularly in the dicotyledon model plant *Arabidopsis thaliana*, but an increasing number of epigenetic and epigenomic studies are also performed on rice (*Oryza sativa*). Rice represents a major food crop of worldwide importance and is also a good model for monocotyledons owing to its relatively small genome size and fully sequenced well-annotated genome. Today, the main regulators of DNA methylation are identified in rice. Moreover, compared to *Arabidopsis*, rice has an important evolutionary history due to human selection since its domestication. DNA methylation may be involved in both adaptation and agronomic performances and thus, a better understanding of epigenetic regulations in rice should contribute to improving the adaptation of crops to a changing environment. In this review, we expose the current knowledge on DNA methylation in rice and future perspectives to be considered.

Keywords: epigenetics; epigenomics; *Oryza sativa*; *Arabidopsis thaliana*; DNA methylation; transposable elements; siRNAs

1. Introduction

Epigenetics is defined as the study of chromatin marks including DNA methylation and histones post-translational modifications. Chromatin accessibility modulates DNA replication and repair, expression of genes and transposable elements (TEs) activity in both plants and animals. In plants, cytosine methylation occurs at three contexts (CG, CHG, and CHH; where H is A, T, or C) whereas in mammals mainly CG dinucleotides are methylated [1]. In addition, genome-wide DNA methylation profiles are different between plants and mammals. Indeed, mammalian genomes are strongly methylated: for example in human embryonic stem cells 72–85% of CGs are methylated [2] compared to *Arabidopsis thaliana* where the methylation levels are 24% for CGs, 6.7% for CHGs, and 1.7% for CHHs, respectively [3]. DNA methylation is predominantly found at TEs and repeats, ensuring the maintenance of TEs silencing. Furthermore, DNA methylation is involved in important developmental processes and stress responses in both plants and animals [4,5]. Epigenetic regulatory mechanisms affect the reproductive development, flowering regulation, and stress responses and thus could potentially play a role in crop improvement [6].

Asian rice (*Oryza sativa*) is one of the most important food crops worldwide and is the best model for cereal genomics. Interest for rice research also resides in the rich source of genetic diversity of the species. Whether epigenetic mechanisms and epigenomic variations have accumulated during the long history of selection and domestication in rice and could contribute to adaptation and agronomic traits is a major question in rice research. Epigenetic regulations have been dissected in great detail in *A. thaliana*, but are still poorly characterized in rice, although recent studies have shed some light on epigenetic

regulation in this crop and we refer the reader to excellent recent reviews [7,8]. Here, we first discuss the relevance of epigenetic regulations for breeding through recent examples of epigenetic control of agricultural traits in rice, and we then focus our review on recent work deciphering the epigenetic regulators involved in the maintenance and establishment of DNA methylation in this species.

2. Epigenetic Regulations Are Involved in Agricultural/Adaptive Traits

In both *Arabidopsis* and rice, the crucial role of DNA methylation is well established in various developmental processes such as seed/embryo development, gametophyte development, and flowering time control [9–14]. Furthermore, epimutations (DNA methylation variations) can be heritable or reversible and thus, might allow phenotypic variation and quick response to environmental changes [15,16]. In *A. thaliana*, a massive analysis of 1107 methylomes from the 1001 Genomes collection demonstrates that the intraspecies epigenomic diversity extent can be correlated with both climate and geographical origin [17]. These natural and spontaneous DNA methylation variations induce alterations of gene transcription and can induce the emergence of new adaptive traits.

Indeed, in plants, many analyses have demonstrated the role of DNA methylation in stress responses [18] and in rice the number of epigenetic studies in stress conditions is increasing. For example, Secco et al. [19] have shown the impact of inorganic phosphate (Pi) deficiency on gene transcription and DNA methylation patterns in rice and in *Arabidopsis*. They identified more differentially methylated regions (DMRs) in response to Pi starvation in rice compared to *Arabidopsis*. These DNA methylation changes occur mainly at TEs located near to Pi starvation-induced (*PSI*) genes. They observed that these specific TEs are hypermethylated in response to Pi starvation, suggesting a mechanism repressing their activity in order to limit their deleterious effects and to facilitate *PSI* gene transcription. These differences could be explained by significant differences of TE density (15% of the genome in *Arabidopsis* vs. 40% in *O. sativa*).

Rice productivity is also affected by two major stresses, salinity and water deficit. Garg et al. [20] identified DMRs associated with differential expression of genes involved in abiotic stress responses in three cultivars—one stress sensitive and two drought and salinity tolerant, respectively—in normal conditions. These DMRs could explain the resistance phenotype. In addition, Wang et al. [21] demonstrated that, under drought conditions, a drought-resistant genotype has a more stable methylome than a drought-sensitive genotype, suggesting again the influence of DNA methylation in abiotic stress response. Interestingly, drought-induced epimutations seem to be non-random and are inherited from generation to generation [22]. Finally, new questions emerge on agricultural practices and on the impact of exposition to pesticides or heavy metal in the soil. Recent studies [23,24] demonstrated that DNA methylation patterns are affected in rice exposed to these substances.

Epigenomic diversity is strongly influenced by TE content and activity [25]. TEs contribute to plant evolution [26] and could influence agricultural traits [27,28]. A picture is emerging where TE polymorphisms and their associated epigenetic marks could contribute to the evolution of gene networks [29] and play a key role in adaptation [30]. Beneficial roles of TEs in rice are reviewed by Song and Cao [31]. One example recently discovered in rice is the natural epiallele *Epi-rav6* [22]. The hypomethylation of a MITE (Miniature Inverted-Repeat Transposable Element) inserted in the *RAV6* promoter induces an over-expression of the gene, resulting in an increase in leaf angle. Undoubtedly, TEs represent a new source of adaptive traits for crop breeding.

Overall, these observations highlight the importance of epigenetic mechanisms in stress responses and raise the question of the molecular actors involved. Furthermore, the seminal work on *Arabidopsis* epigenetic recombinant inbred lines [32,33] has shown that basic knowledge on epigenetic mutation is instrumental if one wants to introduce epigenetic diversity using crosses between mutant and wild type plants. Growing literature on rice epigenetic mutants starts to build a picture of epigenetic regulations in this species. Here, we focus on mutants affecting DNA methylation, as the best-studied epigenetic mark so far.

3. Main Regulators of DNA Methylation in Rice

The methylome (genome-wide DNA methylation) is monitored by DNA methyltransferases (DNA METs), and for some part maintained during replication and thus transmitted across cell divisions. A more dynamic part of the methylome is controlled by 24-nucleotide, small interfering RNAs (siRNAs) via an RNA-directed DNA methylation (RdDM) pathway involving DNA MET activity. In plants, at least three classes (or three major classes) of DNA MET genes have been identified: DNA methyltransferases (*METs*), plant specific chromomethyltransferases (*CMTs*), and domain rearranged methyltransferases (*DRMs*). Genes directly or indirectly involved in DNA methylation are listed in Table 1 and studies on the corresponding rice mutants are detailed. Of note, most of these mutants have been produced by callus culture (*Tos17* insertion, T-DNA, or RNA interference (RNAi) techniques) also known to affect DNA methylation [34] and TE activity. Results should therefore be interpreted with caution.

In *A. thaliana*, MET1, the ortholog of the mammalian Dnmt1 [35], is the major CG methylase and ensures the maintenance of CG methylation [11]. In rice, two closely related putative *MET1* genes are present: *OsMET1-1* and *OsMET1-2* [36]. The loss-of-function mutant *Osmet1-2* presents strong developmental defects in seed development and vegetative growth [37]. The global CG methylation level is reduced by 76% in the homozygous *Osmet1-2* mutant compared to the wild type (WT). CHG and CHH methylation are also affected (6.6 and 43%, respectively). However, the genome-wide CG methylation level in the *Arabidopsis met1* mutant is decreased by 98%, suggesting a redundant function between OsMET1-1 and OsMET1-2 in rice. Nevertheless, *Osmet1-1* mutants do not show discernible developmental phenotype [38] and thus, OsMET1-1 seems to have a minimal and/or redundant function in the maintenance of CG methylation. Consistently, *OsMET1-2* is expressed at higher levels than *OsMET1-1* [37]. Gene expression is largely altered in *Osmet1-2* (13% misregulated genes) while it is only slightly affected in *A. thaliana met1* mutant (2%), suggesting that a large proportion of genes are regulated directly or indirectly by DNA methylation in rice. Lastly, both transcriptional activity of TEs and 24 nucleotide (nt) siRNA production are disturbed in *Osmet1-2* mutant, indicating that OsMET1-2 could be involved in transcriptional silencing of TEs [37].

CMTs are plant-specific DNA METs, characterized by the presence of a chromo (chromatin organization modifier) domain and a bromo-adjacent homology (BAH) domain in the N-terminal region. In *Arabidopsis*, three *CMT* genes have been identified: *CMT2* is known to establish CHH methylation [39] while *CMT3* is a major CHG MET [1,40,41]. *CMT1* is only weakly expressed and its function is still unknown [42]. In rice, there are three *CMT* genes: *OsCMT2*, *OsCMT3a*, and *OsCMT3b*. *OsCMT3a* is the only functional *CMT3* ortholog and is involved in the maintenance of DNA hypermethylation at CHG sites during DNA replication [43]. The loss-of-function *Oscmt3a* mutation affects the expression of genes and TEs [43]. TEs are predominantly transcriptionally activated and transgenerational TE mobility is observed in mutants confirming the role of OsCMT3a in their control. In contrast to *Arabidopsis cmt3*, *Oscmt3a* mutant displays pleiotropic developmental phenotypes: early flowering, short stature, and low fertility. *Oscmt3b* mutant does not present any morphological abnormality and *OsCMT3b* is expressed only in panicles, suggesting that OsCMT3b could play a minor role in CHG methylation. Finally *OsCMT2* is closely related to *CMT2* [43], suggesting that OsCMT2 may play a role in CHH methylation, although no *Oscmt2* mutant is described yet.

Table 1. Genes involved in DNA methylation in rice.

	Proteins	Locus ID	Mutation	Expression	Description/Phenotype	Functions	References
Maintenance of DNA methylation	OsMET1-2 (DNA METHYLTRANSFERASE 1)	LOC_Os 07g08500	T-DNA insertion (*Tos17*)	KO	All germinated seedlings undergo quick necrotic death	Maintain DNA methylation at CG sites during DNA replication. Two copies *MET1-1* and *MET1-2*	[37,44]
	OsDRM1a	LOC_Os 11g01810	/	/	Downregulated by jasmonic acid	Not expressed, lack of methyltransferase motifs	[45]
	OsDRM1b	LOC_Os 12g01800	/	/	Downregulated by jasmonic acid		
	OsCMT3 (CHROMOMETHY LTRANSFERASE)	LOC_Os 10g01570	T-DNA insertion (*Tos17*)	KO	No difference in the vegetative phase. Early reproductive stage, 15% shorter stature and decreased fertility	Maintain DNA methylation at CHG sites during DNA replication	[43,44]
Chromatin remodeler	OsDDM1a (DECREASE in DNA METHYLATION)	LOC_Os 09g27060	RNAi mutants	KD	93% identity between both DDM1 homologs; dwarf phenotype; hypomethylation in later generations of selfed progenies	Remodeling histones ATPases. Maintenance of cytosine methylation; Required for maintenance of TE silencing	[46,47]
	OsDDM1b (DECREASED in DNA METHYLATION)	LOC_Os 03g51230	RNAi mutants	KD	/	Maintenance of cytosine methylation	[46,47]
RdDM	OsDRM2 (DOMAINS REARRANGED METHYLTRANSFERASE)	LOC_Os 03g02010	Gene targeting through homologous recombinaison	KO	Reduction of vegetative growth and semi-dwarf phenotype. Reduction in the de novo methylation at transposons and 5S repeat sequences	De novo DNA methylation at CHH sites directed by siRNAs. Major DRM1/2-type methyltransferase gene in rice	[44,45,48,49]
	OsDCL3a (DICER LIKE PROTEIN 3)	LOC_Os 01g68120	RNAi mutant	KD	Pleiotropic phenotypes affecting agricultural traits: plant height, angle of flag leaf, smaller panicles. Similar phenotypes as RNAi mutants of AGO4ab-1 and RDR2-2	Biogenesis of 24-nt long miRNAs (lmiRNAs) which can direct DNA methylation (*cis* and *trans*); 24 nt siRNA biogenesis	[50–52]
	OsDCL3b (DICER LIKE PROTEIN 3)	LOC_Os 10g34430	RNAi mutant	KD	/	Panicle and early seed-specific and require for 24 nt phased small RNAs. *DCL3a* is expressed at a much higher level than *DCL3b*	[50,53]
	OsDCL4 (DICER LIKE PROTEIN 4)	LOC_Os 04g43050	/	KO	Severe spikelet defects including thread-like lemma and male sterility	Biogenesis of 21 nt siRNA in panicles and seedlings	[53,54]
	OsRDR1 (RNA DEPENDENT RNA POLYMERASE 1)	LOC_Os 02g50330	T-DNA insertion (*Tos17*)	KO	Ephemeral phenotypic fluctuations occurred only under some abiotic stress conditions	Role in the production and amplification of exogenous, virus-derived siRNAs (vsiRNAs) in infected plants and in some abiotic stress responses. Role in maintaining the intrinsic locus-specific CHH methylation patterns	[55]

Table 1. *Cont.*

	Proteins	Locus ID	Mutation	Expression	Description/Phenotype	Functions	References
RdDM	OsRDR2 (RNA DEPENDENT RNA POLYMERASE 2)	LOC_Os 04g39160	RNAi mutant	KD	Similar phenotypes as RNAi mutants of AGO4ab-1 and OsDCL3a.	Role not studied yet but could be similar to AtRDR2 (according to its expression pattern)	[50]
	OsRDR6 (RNA DEPENDENT RNA POLYMERASE 6)	LOC_Os 01g34350	SNP (G -> T)	Temperature dependent	Spikelet defects	Biogenesis of 21 nt and 24 nt siRNAs (different from *Arabidopsis*) and resistance against virus	[53,56]
	AGO4a/AGO4b	LOC_Os 01g16870/LOC_Os 04g06770	RNAi mutants	KD	Similar phenotypes as RNAi mutants of OsDCL3a and RDR2-2	High similarity with *Arabidopsis* AGO4	[50]
	OsAGO1s (4 OsAGO1 homologs OsAGO1a, OsAGO1b, OsAGO1c, OsAGO1d)	LOC_Os 02g45070/LOC_Os 04g47870/LOC_Os 02g58490/LOC_Os 06g51310	RNAi mutants	KD	Various developmental defects	miRNA mediated gene regulation	[50]
	WAF1 (WAVY LEAF1)	LOC_Os 07g06970	NMU mutagenesis	KO	Seedling lethality due to defects of SAM maintenance or pleiotropic phenotypes in leaf morphology and floral development. Phenotypes similar to *sho1* and *sho2* mutants deficient in DCL4 and AGO7, respectively	Methylates 3′ terminal nucleotide of siRNAs; HEN1 (HUA ENHANCER 1) homolog	[57]
5-meC DNA glycosylase/lyases	OsROS1a	LOC_Os 01g11900	knock-in targeting	KO	Severe underdeveloped endosperm phenotype	There are 4 ROS1 orthologs (ROS1a-d). ROS1a is the most expressed gene compared to ROS1b-d. ROS1a and *Arabidopsis DME* gene could have analogous functions in the endosperm	[58]
	DNG701 (OsROS1c)	LOC_Os 05g37350	T-DNA insertion, RNAi	KO; KD; OE	The progeny of *ros1c* mutant present two seed phenotypes, normal seeds and wrinkled seeds	ROS1a and ROS1c could play different roles in seed development. Could be involved in the control of transposition.	[58,59]
	DML3a (DEMETER LIKE 3) and DML3b	LOC_Os 04g28860/LOC_Os 02g29380	/	/	/	/	[10,58]

DDM1: decrease in DNA methylation; KD: knock-down; KO: knock-out; NMU: N-nitroso-N-methylurethane; OE: over-expressed; SAM: shoot apical meristem; t-DNA: transfer DNA.

DRM is required for the maintenance of non-CG methylation and for de novo methylation in all the three contexts CG, CHG, and CHH [60]. In *Arabidopsis*, de novo methylation is established by the RdDM pathways where siRNAs guide DRM2 to the region to be methylated [61]. DRM2, which is homologous to mammalian Dnmt3, is also functionally redundant with CMT3 in the maintenance of non-CG methylation at many loci [62]. *DRM2* is expressed at much higher levels than *DRM1* and suggests that DRM2 is the predominant de novo DNA MET in *Arabidopsis* [63]. In rice, homozygous *Osdrm2* mutants show severe developmental defects such as a semi-dwarfed phenotype, reductions in tiller number, abnormal panicle architecture, and complete sterility [45]. The genome-wide DNA methylation level is reduced to 17% in *Osdrm2* compared to the WT and the RdDM pathway is deficient. In contrast to *Arabidopsis*, OsDRM2 is the major CHH MET in rice [46]. Heterologous expression of *OsDRM2* in yeast confirmed that OsDRM2 could methylate DNA de novo [48]. In rice, two other *DRM* genes were identified respectively, *DRM1a* and *DRM1b*. These two genes are not expressed and might not encode functional DNA METs [45].

In addition to DNA METs, DNA methylation is regulated by chromatin factors. DDM1 (DECREASE IN DNA METHYLATION 1), a SWItch/Sucrose Non-Fermentable (SWI2/SNF2)-like chromatin remodeling protein, is necessary to maintain DNA methylation in *Arabidopsis* [64,65]. Two genes are orthologous of *DDM1* in rice designated as *OsDDM1a* and *OsDDM1b*. These two genes share 93% similarity and their expression patterns are also similar. Interestingly, only the double mutants *Osddm1a* and *Osddm1b* show severe developmental defaults and a complete sterility, suggesting functional redundancy [46,47]. The global DNA methylation level is reduced by 54% in the double mutant *Osddm1a Osddm1b* compared to the WT. Genome-wide analyses of DNA methylation have shown that OsDDM1 is involved in both CG and CHG methylation at euchromatic and heterochromatic regions but is also involved in CHH methylation of small TEs such as MITEs mainly located in euchromatic regions [46]. Transcriptomic analyses in *Arabidopsis ddm1* mutants have shown an important deregulation of gene transcription [39,66] suggesting that OsDDM1 could also play a role in gene regulation and ensure normal plant development.

In plants, DNA demethylation is ensured by 5-meC DNA glycosylase enzymes that are encoded by four genes in *Arabidopsis*: *REPRESSOR OF SILENCING* (*ROS1*) [67], *DEMETER* (*DME*) [68], *DEMETER-LIKE2* (*DML2*) AND *DEMETER-LIKE3* (*DML3*) [69]. In rice, phylogenetic analyses identified six DNA glycosylases: 4 ROS1 homologs (ROS1a–d) and two DML3 (DML3a and DML3b) homologs and no DME homolog [10]. Expression analysis showed that *ROS1a* is the most expressed gene compared to *ROS1b–d* and *DML3a–b* in the five tissues tested (seedling leaf, seedling root, anther, pistil, and immature seed) [58]. Maternal null *ros1a* mutants present severe underdeveloped endosperm phenotype, reminiscent of the *dme* mutant phenotype in *Arabidopsis,* suggesting that ROS1a and DME could have analogous functions in the endosperm. Even if the DNA glycosylase function of ROS1a is not demonstrated yet, maternal and paternal null *ros1a* alleles are not transmitted to the next generation, suggesting that DNA methylation level play important roles in gametophyte development. Characterization of the *ROS1c/DNG701* gene [59] showed that ROS1c is required for the demethylation of the *Tos17* retrotransposon in rice calli, therefore suggesting it could be involved in the control of transposition. In addition, the progeny of the knockout mutant *ros1c* present a proportion of 10% of wrinkled seeds that could be due to the impact of the mutation on the endosperm hypomethylation. The cause of this phenotype and its low penetrance is yet unknown. It is less pronounced in the *ros1a* mutant, suggesting that ROS1a and ROS1c could play different roles in seed development.

Altogether these studies have shown that rice mutants affected in the DNA methylation machinery present severe developmental phenotypes in both vegetative and reproductive stages in contrast to *Arabidopsis*. In addition, a clear difference of DNA methylation patterns at the chromosome-wide level is observed between both species [70]. Here again, these differences could be explained by significant differences of TE density. Plant species with a high TE content (*O. sativa*, *Zea mays*, etc.) seem to require more robust DNA methylation mechanisms than species with a low TE content such as *Arabidopsis*.

In maize for instance, genetic perturbation of the methylome lead to developmental phenotypes and some mutant combination cannot be recovered due to lethality [71].

4. Establishment of De Novo DNA Methylation

TEs are mobile genetic elements able to proliferate in their host genomes. They represent a main source of genomic diversity and an evolutionary force in both plants and animals. Host genomes have established strong regulations and young TE copies are silenced by epigenetic marks such as cytosine methylation, ensuring a stable repression of TE expression and preventing their proliferation. As mentioned above, de novo DNA methylation can be initiated via the RdDM mechanism, a plant-specific pathway through which siRNAs target homologous DNA regions to methylate it. The RdDM pathway is well characterized in *Arabidopsis* [72,73] but largely unstudied in rice. In this part, we will detail RdDM pathways based on the model plant *A. thaliana* (Figure 4a) to highlight a comprehensive overview of what have been identified in rice (Figure 4b).

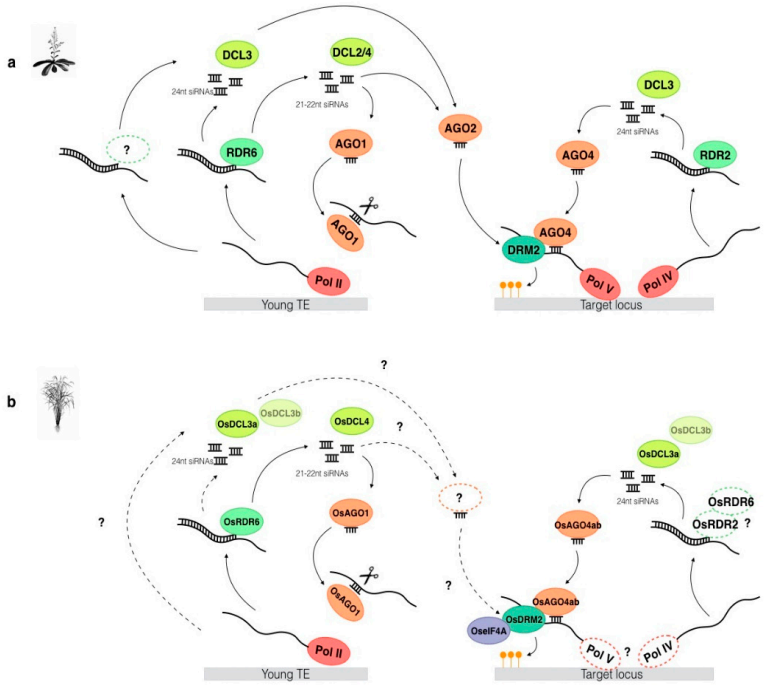

Figure 1. RNA-directed DNA methylation (RdDM) pathways in *Arabidopsis* and in rice. (**a**) In *Arabidopsis*, the "canonical" RdDM pathway (right panel) is initiated by the RNA polymerase IV (Pol IV) that generates a single strand RNA (ssRNA) of the target locus which is a template for the RNA-dependent RNA polymerase 2 (RDR2) to generate a double strand RNA (dsRNA). DICER-like 3 (DCL3) cleaves dsRNAs to 24 nucleotide (nt) siRNAs then loaded into ARGONAUTE 4 (AGO4). AGO4-bound siRNAs can base-pair with the nascent RNA polymerase V (Pol V) transcript or with DNA leading to the recruitment of DRM2 to establish de novo methylation of the target locus and to mediate transcriptional gene silencing (TGS) of transposable elements (TEs). The "non-canonical" pathway (left panel) incorporate components that are typically associated with post-transcriptional gene silencing (PTGS). A young TE copy, future RdDM target, is transcribed by RNA polymerase II (Pol II) to produce mRNAs. Some of these Pol II transcripts can be converted into dsRNAs by RDR6 and processed by DCL2 and DCL4 into 21–22 nt siRNAs, resulting in AGO1-mediated PTGS by the cleavage of TE mRNAs. These dsRNAs can also initiate de novo DNA methylation by three independent pathways: RDR6-DCL3-RdDM, RDR6-RdDM, and DCL3-RdDM involving AGO2, Pol V, and DRM2. These non-canonical pathways enhance methylation and reinforce TGS. A transition from

PTGS to TGS occurs when high levels of Pol II and RDR6-dependent dsRNAs saturate DCL2 and DCL4 enzymes and become available for processing by DCL3, which produces 24 nt siRNAs that trigger canonical RdDM and TGS of TEs. (**b**) In rice, only some genes involved in RdDM pathways are functionally characterized. DCLs, AGOs, and RDRs are encoded by one of the largest multigene families and some genes, like *OsAGO4a* and *OsAGO4b* or *OsRDR2* and *OsRDR6* seem to have functional redundancy. However, *OsDCL3a* and *OsDCL3b* seem to have some similar roles, *OsDCL3b* being mostly involved in panicle and early seed development. Dotted lines represent hypothetical pathways or hypothetical proteins, not yet characterized.

Main classes of regulators involved in this complex mechanism include: RNAi machinery ensured by Dicer-like (DCL), Argonaute (AGO), and RNA-dependent RNA polymerases (RDRs) genes families, de novo MET DRMs and two plant-specific RNA polymerases Pol IV and Pol V. The "canonical" RdDM pathway is initiated by Pol IV which generates a single strand RNA (ssRNA) of the target locus which is a template for RDR2 to generate a double strand RNA (dsRNA). DCL3 cleaves dsRNAs to 24 nucleotides siRNAs (24 nt siRNAs) which are stabilized by methylation at their 3′-OH groups by HUA ENHANCER 1 (HEN1) and loaded into AGO4. AGO4-bound siRNAs can base-pair with the nascent Pol V transcript or with DNA [74], leading to the recruitment of DRM2 to establish de novo methylation of the target locus and to mediate transcriptional gene silencing (TGS) of TEs.

In addition to the canonical pathway, various "non-canonical" forms of RdDM have recently been identified in *Arabidopsis* [73]. These mechanisms partly incorporate components that are typically associated with post-transcriptional gene silencing (PTGS). Initially, future RdDM targets—for instance, a young TE copy—are transcribed by Pol II to produce mRNAs. Some of these Pol II transcripts can be converted to dsRNAs by RDR6 and processed by DCL2 and DCL4 into 21–22 nt siRNAs, resulting in AGO1-mediated PTGS by the cleavage of TE mRNAs. However, these dsRNAs can also initiate de novo DNA methylation by three independent pathways: RDR6–DCL3–RdDM, RDR6–RdDM, and DCL3–RdDM involving AGO2, Pol V, and DRM2. These non-canonical pathways enhance methylation and reinforce TGS. A transition from PTGS to TGS occurs when high levels of Pol II- and RDR6-dependent dsRNAs saturate DCL2 and DCL4 enzymes and become available for processing by DCL3, which produces 24 nt siRNAs that trigger canonical RdDM and TGS of TEs. Finally, RdDM mechanisms seem to be able to compensate for each other [75].

So far, only some of the rice RdDM machinery components have been functionally characterized by using RNAi mutants and expression analyses [50]. In plants, DCLs, AGOs, and RDRs are encoded by small multigenic families and curiously in rice these families gather 32 genes, one of the largest numbers of these genes among the plant species analyzed so far. These results suggest that there is a complex and robust network of siRNAs in rice [76]. Nevertheless, only a few genes have been fully identified as partners in the RdDM mechanism. For example, *OsDCL3a* is involved in 24 nt siRNA processing [51]. RdDM mutants display several developmental alterations as lower plant height and smaller panicles compared to WT. RNAi mutants of OsAGO4a and OsAGO4b, homologs of *Arabidopsis* AGO4 [52], and RNAi mutant of OsRDR2 have similar phenotypes suggesting that OsRDR2 and OsAGO4ab are involved in the same pathway. The function of *OsRDR2* is not clearly established yet its expression pattern is similar to *RDR2* in *Arabidopsis* at earlier stages of flower development, suggesting a similar role [50]. OsRDR6 is involved in the biogenesis of 21 and 24 nt siRNAs [53,56] suggesting a possible redundancy between OsRDR2 and OsRDR6. In addition, the loss-of-function of OsRDR1 causes alteration of CHH methylation indicating a possible role in the RdDM pathway [55]. Finally, *WAVY LEAF1* (*WAF1*) has been identified as an ortholog of *Arabidopsis HEN1*. Rice *waf1* mutants show strong pleiotropic phenotypes and do not survive 10 days after germination. Abe et al. [57] have shown that the siRNAs abundance was decreased in this mutant compared to WT. Finally, as in *Arabidopsis*, WAF1 is required for the stabilization of siRNAs.

RNA polymerases are composed of at least 12 subunits, forming a large holoenzyme [77]. Nuclear RNA Polymerases D and E (NRPD and NRPE) are specific subunits of Pol IV and Pol V, respectively, and are derived from the duplication of Pol II subunit, NRPB [78]. Orthologs of *NRPD* and *NRPE* have

been identified in rice [79] and present the same domain structure as in *Arabidopsis*. No study has been published yet on Pol IV and Pol V in rice nevertheless similarities of structure suggest a similar role.

Interestingly, no rice knockout mutant has been described for any of the early actors of the RdDM pathway, so far [76]. The difficulty of obtaining such mutants suggests that they could be sterile and weak alleles or RNAi lines could be of interest. This might also underline the more drastic effect of affecting the RdDM pathway in this species than in *A. thaliana*. Recently, Bousios and Gaut [80] discussed the importance of studying epigenetic mechanisms in a wide range of species. Indeed, due to the singularities of the *A. thaliana* genome (small genome with weak TE activity), the generalization of the current epigenetic model to plants at large may not be fully relevant. There is therefore a need for a comprehensive study of these mechanisms in another model species, such as rice.

5. Conclusions and Perspectives

Food security and climate changes are serious global concerns and it is now well established that epigenetic regulations are strongly influenced by the environment and could provide a reversible yet heritable source of variation for rapid adaptation. Rice is one of the most important food crops worldwide and its evolution is explained by a long history of selection and domestication. Natural epimutations have accumulated in this species and we propose that they could contribute to adaptation and to agronomic traits in this species. Over recent years, the number of rice epigenetic studies has significantly increased. In response to different stresses, rice epialleles have been identified and are correlated with the appearance of new adaptive traits. Nevertheless, the underlying mechanisms are still unclear and lots of questions remain unanswered. Massive comparative studies of rice varieties will provide an opportunity to improve our knowledge of epigenetic regulations and also to identify new agronomically interesting epialleles. Finally, the fact that epigenetic mechanisms are closely linked to TE content and are thus species-specific, stresses the importance of expanding these epigenetic studies to a large number of crop species.

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