Dynamics and function of DNA methylation in plants

Huiming Zhang[1,2]*, *Zhaobo Lang*[1,2] and *Jian-Kang Zhu*[1,2,3]*

Abstract | DNA methylation is a conserved epigenetic modification that is important for gene regulation and genome stability. Aberrant patterns of DNA methylation can lead to plant developmental abnormalities. A specific DNA methylation state is an outcome of dynamic regulation by de novo methylation, maintenance of methylation and active demethylation, which are catalysed by various enzymes that are targeted by distinct regulatory pathways. In this Review, we discuss DNA methylation in plants, including methylating and demethylating enzymes and regulatory factors, and the coordination of methylation and demethylation activities by a so-called methylstat mechanism; the functions of DNA methylation in regulating transposon silencing, gene expression and chromosome interactions; the roles of DNA methylation in plant development; and the involvement of DNA methylation in plant responses to biotic and abiotic stress conditions.

DNA methylation at the 5′ position of cytosine contributes to the epigenetic regulation of nuclear gene expression and to genome stability[1,2]. Epigenetic changes, including DNA methylation, histone modifications and histone variants and some non-coding RNA (ncRNA) changes, influence chromatin structure and, in turn, the accessibility of genetic information. As a result, DNA methylation is important to many biological processes, and disruption of DNA methylation can lead to developmental abnormalities in plants and mammals, such as failure in tomato fruit ripening and embryo lethality in mice[1,3,4].

DNA methylation is conserved in plants and mammals, and precise patterns of genomic DNA methylation are crucial for development. In both plants and mammals, DNA methylation is catalysed by conserved DNA methyltransferases using *S*-adenosyl-L-methionine as the methyl donor, whereas active DNA demethylation involves a base excision repair pathway[5–9]. An RNA-directed DNA methylation pathway is crucial for de novo methylation in plants but is less important in mammals[10,11]. In contrast to mammals, which initiate active DNA demethylation with oxidation and/or deamination of 5-methylcytosine (5-mC), plants directly excise the 5-mC base utilizing 5-mC DNA glycosylases[5,6,8].

In this Review, we discuss recent discoveries and the current understanding of the regulation and function of DNA methylation in plants. The mechanisms underlying the generation of specific DNA methylation patterns are best understood in the model plant *Arabidopsis thaliana*, in which mutations in components of the DNA methylation and demethylation machineries and regulatory factors are generally not lethal. However, DNA methylation appears to be more crucial for development and environmental-stress responses in plants that have more complex genomes. Recent findings have uncovered important regulatory mechanisms of plant DNA methylation, such as the initial triggering of de novo DNA methylation by ncRNA, the targeting of active DNA demethylation by the novel protein complex IDM (increased DNA methylation) and the balancing of DNA methylation and demethylation by a methylation-sensing genetic element. We also discuss the important roles of DNA methylation dynamics in regulating transposon silencing, in gene expression, in chromosome interactions, in plant development and in plant responses to biotic and abiotic environmental stimuli, as well as in fruit ripening, root nodulation and other developmental processes.

DNA methylation dynamics

A specific DNA methylation state reflects the outcome of the dynamic regulation of establishment, maintenance and active-removal activities. These activities are catalysed by various enzymes that are targeted to specific genomic regions by distinct pathways. Plant DNA methylation occurs in all cytosine sequence contexts: CG, CHG and CHH (H represents A, T or C)[12,13]. In *A. thaliana*, genome-wide DNA methylation is characterized by heavy methylation in heterochromatin, which is enriched with transposable elements (transposons) and other repetitive DNA sequences[12,14]. Interspersed transposon-associated DNA methylation also exists in euchromatic chromosome arms[12].

[1]*Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, Shanghai, China.*

[2]*CAS Center for Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai, China.*

[3]*Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN, USA.*

**e-mail: hmzhang@sibs.ac.cn; jkzhu@sibs.ac.cn*

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Establishment of DNA methylation by the RNA-directed DNA methylation pathway. In plants, de novo DNA methylation is mediated through the RNA-directed DNA methylation (RdDM) pathway, which involves small interfering RNAs (siRNAs) and scaffold RNAs in addition to an array of proteins (FIG. 1).

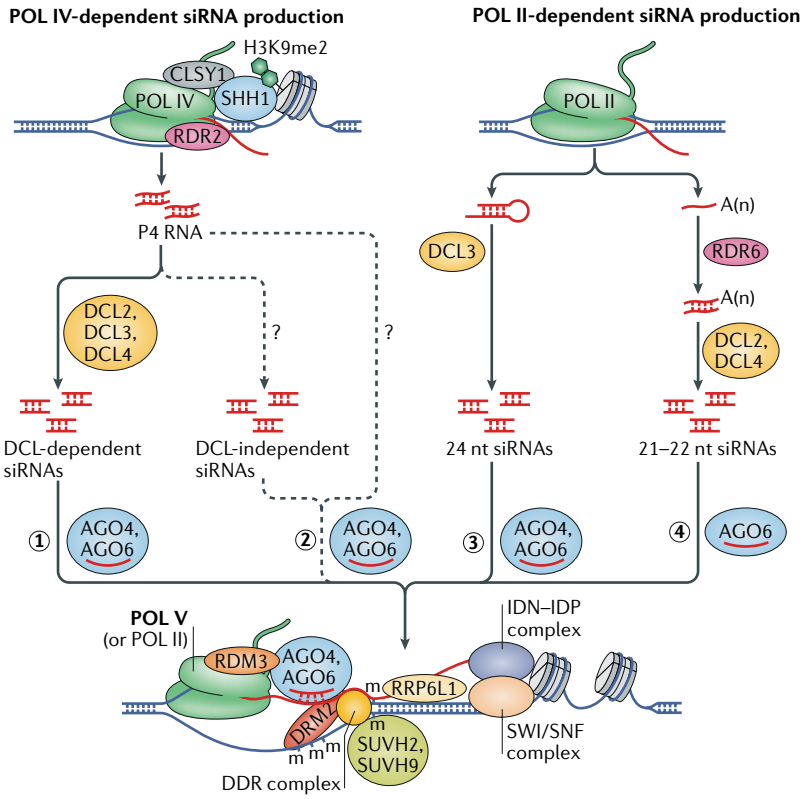

Fig. 1 | **RNA-directed DNA methylation pathways in *Arabidopsis thaliana*.** In the canonical RNA-directed DNA methylation (RdDM) pathway[11] (step 1), RNA POLYMERASE IV (POL IV) generates non-coding RNAs (P4 RNAs) that serve as templates for RNA-DEPENDENT RNA POLYMERASE 2 (RDR2)-mediated production of double-stranded RNAs, which are cleaved by DICER-LIKE PROTEIN 3 (DCL3), DCL2 and DCL4 to yield mainly 24-nucleotide small interfering RNAs (siRNAs). Subsequently, siRNAs are bound by ARGONAUTE 4 (AGO4) or AGO6 and pair with POL V-transcribed scaffold RNAs to recruit DOMAINS REARRANGED METHYLASE 2 (DRM2), which methylates (m) the DNA. POL IV is recruited to RdDM loci by SAWADEE HOMEODOMAIN HOMOLOGUE 1 (SHH1), which binds dimethylated histone H3 lysine 9 (H3K9me2)[29,30]. The chromatin remodeller SNF2 DOMAIN-CONTAINING PROTEIN CLASSY 1 (CLSY1) interacts with POL IV and is required for POL IV-dependent siRNA production[30,31]. The majority of RdDM targets remain methylated in the *dcl1–dcl2–dcl3–dcl4* quadruple mutant, implying DCL-independent RdDM may be mediated by DCL-independent siRNAs or directly by P4 RNAs[43] (step 2). At some RdDM loci, POL II can produce 24-nucleotide siRNAs and scaffold RNAs[45] (step 3). At some activated transposons, POL II and RDR6 collaboratively produce precursors of 21-nucleotide or 22-nucleotide siRNAs that mediate DNA methylation similarly to 24-nucleotide siRNAs[47–49] (step 4). AGO4 and/or AGO6 directly associate with POL V, and the association is enhanced by RNA-DIRECTED DNA METHYLATION 3 (RDM3)[19,20]. Production of scaffold RNAs by POL V requires the DDR complex, consisting of the chromatin remodeller DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1, DEFECTIVE IN MERISTEM SILENCING 3 and RDM1, which associates with both AGO4 and DRM2 and may bind single-stranded methylated DNA[18,32–36]. The DDR complex interacts with SUPPRESSOR OF VARIEGATION 3-9 HOMOLOGUE PROTEIN 2 (SUVH2) and SUVH9, which bind to pre-existing methylated cytosines and can recruit POL V[35,37]. The retention of nascent POL V-transcribed RNA on the chromatin may be facilitated by the RNA-binding proteins RRP6-LIKE 1 (RRP6L1)[21] and the INVOLVED IN DE NOVO 2 (IDN2)–IDN2 PARALOGUE (IDP) complex, which interacts with a SWITCH/SUCROSE NONFERMENTING (SWI/SNF) chromatin-remodelling complex[22–28].

According to the current understanding of canonical RdDM in *A. thaliana*[7,11,15,16], the production of 24-nucleotide siRNAs is initiated through transcription by RNA POLYMERASE IV (POL IV), which is followed by RNA-DEPENDENT RNA POLYMERASE 2 (RDRP2; also known as RDR2)-dependent copying of the transcript to generate a double-stranded RNA (dsRNA) and by DICER-LIKE PROTEIN 3 (DCL3)-dependent cleavage of the dsRNA into siRNAs. The siRNAs are loaded onto ARGONAUTE (AGO) proteins, mainly AGO4 and AGO6, and pair with complementary scaffold RNAs, which are nascent transcripts produced by POL V. AGO4 interacts with the DNA methyltransferase DOMAINS REARRANGED METHYLASE 2 (DRM2)[17], which catalyses de novo DNA methylation in a sequence-independent manner. This reaction may be assisted by RNA-DIRECTED DNA METHYLATION 1 (RDM1), which associates with both AGO4 and DRM2 and may bind single-stranded methylated DNA[18] (FIG. 1).

In addition to the sequence-specific pairing between siRNAs and scaffold RNAs, protein interactions between AGO4 and the AGO hook-containing proteins DNA-DIRECTED POL V SUBUNIT 1 (also known as NRPE1) and RDM3 are also important for RdDM. NRPE1 is the largest subunit of POL V, and RDM3 is a POL V-associated putative transcription elongation factor[19,20]. POL V-transcribed ncRNAs must remain on the chromatin to function as scaffold RNAs; this association seems to be facilitated by RRP6-LIKE 1 (RRP6L1), which is a homologue of the yeast and mammalian ribosomal RNA-processing 6 (RRP6) proteins that can function in RNA retention[21]. In addition, the siRNA–scaffold RNA pairing may be stabilized by the INVOLVED IN DE NOVO 2 (IDN2)–IDN2 PARALOGUE (IDP) complex, which binds RNA and interacts with the SWI/SNF chromatin-remodelling complex that contains SWI/SNF COMPLEX SUBUNIT SWI3B and participates in POL V-mediated transcriptional silencing by altering nucleosome positioning[22–28].

The recruitment of POL IV and POL V to RdDM target loci can be facilitated by pre-existing chromatin modifications. POL IV is recruited by SAWADEE HOMEODOMAIN HOMOLOGUE 1 (SHH1), which binds dimethylated histone H3 lysine 9 (H3K9me2) through its Tudor domain[29,30]. SHH1 also interacts with SNF2 DOMAIN-CONTAINING PROTEIN CLASSY 1 (CLSY1), which is a chromatin-remodelling protein associated with POL IV and is required for POL IV-dependent siRNA production[30,31] (FIG. 1). The association of POL V with chromatin for scaffold-RNA production requires the chromatin-remodelling DDR, which comprises the chromatin-remodelling protein DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), the putative structural maintenance of chromosomes protein DEFECTIVE IN MERISTEM SILENCING 3 and RDM1 (REFS[18,32–35]) (FIG. 1). The DDR complex physically interacts with SUPPRESSOR OF VARIEGATION 3-9 HOMOLOG PROTEIN 2 (SUVH2) and SUVH9, which are SUPPRESSOR OF VARIEGATION (SU(VAR)) 3-9 histone methyltransferase family proteins but lack histone methyltransferase activity[36,37] (FIG. 1). SUVH2 and SUVH9 recognize methylated cytosine through their

AGO hook
A protein motif containing Gly–Trp or Try–Gly repeats, which mediate protein interactions with ARGONAUTE (AGO) proteins.

Ribosomal RNA-processing 6 (RRP6). A conserved single-stranded RNA nuclease with both negative and positive functions in RNA accumulation.

Trans-acting siRNA genes
Genes that encode transcripts that are cleaved by microRNAs, synthesized into double-stranded RNA and then cleaved again to produce *trans*-acting small interfering RNAs (siRNAs).

SET and RING finger-associated (SRA) domains and are required for the proper genome-wide chromatin occupancy of POL V, and were therefore proposed to recruit POL V through pre-existing DNA methylation[37]. However, the tethering of SUVH9 by a zinc-finger to unmethylated DNA is sufficient to recruit POL V and to establish DNA methylation and gene silencing[37].

Given that POL V can generate ncRNAs with different 5′ ends from the same locus, it seems to initiate transcription independently of promoters[38]. Genome-wide POL V or POL IV chromatin occupancy mapping did not reveal consensus promoter motifs[29,39]. Some POL V-transcribed ncRNAs have 7-methylguanosine caps at the 5′ ends[38], indicating that POL V-generated transcripts can be subjected to certain RNA-processing activities that are known to modify POL II-transcribed mRNAs. Nevertheless, POL V-generated transcripts are devoid of polyadenylation at their 3′ ends and thus are different from mRNAs[38]. Unlike POL V transcripts, which are long enough to be detected by regular PCR[38], POL IV-transcribed ncRNAs (P4 RNAs) are mostly 26–50 nucleotides in length and were thus identified only recently by small-RNA deep sequencing in *A. thaliana* with mutant *dcl2*, *dcl3* and *dcl4* (*dcl* triple mutant) and in *A. thaliana* with mutant *dcl1*, *dcl2*, *dcl3* and *dcl4* (*dcl* quadruple mutant)[40–44], in which the cleavage of POL IV-dependent and RDR2-dependent dsRNAs into 24-nucleotide siRNAs is presumably blocked. P4 RNAs accumulate in *dcl* triple mutants and can be processed into 24-nucleotide siRNAs by exogenous DCL3. Because P4 RNAs are small, each 24-nucleotide siRNA could be produced from one slicing of a precursor P4 RNA[42].

In addition to the canonical POL IV–RDR2–DCL3 pathway that generates 24-nucleotide siRNAs, paralogues of these proteins can also produce siRNAs that trigger non-canonical RdDM (FIG. 1). POL II-mediated transcription can not only generate 24-nucleotide siRNAs and scaffold RNAs but also recruits POL IV and POL V to promote siRNA production at some RdDM target loci[45]. POL II also has spatially distinct associations with different AGO proteins when compared with POL V[46]. At *Trans*-acting siRNA genes and at some regions of transcriptionally active transposons, RdDM depends on POL II and RDR6 rather than on POL IV and RDR2 (REFS[47–49]). RDR6-dependent RdDM can be mediated either through 21-nucleotide or 22-nucleotide siRNAs, which are produced by DCL2 and DCL4, or through 24-nucleotide siRNAs produced by DCL3 (REFS[49,50]).

Genome wide, most siRNAs in *A. thaliana* are 24-nucleotide siRNAs, which disappear almost completely in the *dcl* quadruple mutant; however, DNA methylation at approximately two-thirds of RdDM target regions still remains[43,44], indicating the existence of DCL-independent RdDM that may be mediated by some DCL-independent siRNAs or directly by P4 RNAs (FIG. 1). RNase III enzymes other than DCL proteins could dice dsRNAs[51], and in wild-type plants, they may work with DCLs in processing POL II, POL IV or POL V transcripts into siRNAs.

Genetic screens have identified some pre-mRNA splicing factors whose mutations reduce the levels of POL IV-dependent siRNAs to varying degrees[52–55], although it remains largely unclear how these splicing factors affect siRNA levels. Similarly, mutations in two splicing factors, STABILIZED 1 and RDM16, reduce the accumulation of POL V-dependent scaffold RNAs[53,54]. Presumably, some of the splicing factors that normally bind pre-mRNAs may interact with the non-coding transcripts generated by POL IV and POL V and affect their processing or stability, thus influencing siRNA or scaffold RNA abundance.

Maintenance of DNA methylation. Maintenance of plant DNA methylation depends on the cytosine sequence context and is catalysed by DNA methyltransferases that are regulated by different mechanisms (FIG. 2). CG cytosine methylation is maintained by METHYLTRANSFERASE 1 (MET1) (FIG. 2a). MET1 is an orthologue of the mammalian DNA (cytosine-5)-methyltransferase 1 (DNMT1), which recognizes hemi-methylated CG dinucleotides following DNA replication and methylates the unmodified cytosine in the daughter strand[9,56]. Compared with mouse and human DNMT1, *A. thaliana* MET1 lacks the cysteine-rich CXXC domain that is thought to help DNMT1 distinguish a hemi-methylated CG from a non-methylated CG[57,58]. Similar to the model in which DNMT1 is recruited by the E3 ubiquitin-protein ligase UHRF1 (REFS[59,60]), MET1 has been proposed to be recruited to DNA by VARIANT IN METHYLATION proteins, which are UHRF1 orthologues required to maintain CG methylation[61,62].

Maintenance of CHG methylation in *A. thaliana* is catalysed by the DNA methyltransferase CHROMOMETHYLASE 3 (CMT3) and to a much lesser extent by CMT2 (REFS[63,64]) (FIG. 2a). The protein structure of the maize CMT3 homologue CHROMOMETHYLASE 1 (MET2A) demonstrated that its bromo-adjacent homology (BAH) and chromo domains bind to H3K9me2 (REF.[65]). Preventing the CMT3–H3K9me2 interaction not only disrupts the binding of CMT3 to the nucleosome but also leads to a complete loss of CMT3 activity[65]. Mutations in the *A. thaliana* H3K9-specific methyltransferase SUVH4 and its paralogues SUVH5 and SUVH6 abolish H3K9me2 and substantially reduce CHG methylation[66–71]. Methylated CHG is bound by the SRA domain of SUVH4 and recruits it to carry out H3K9 methylation[72]. Therefore, the methylated CHG and H3K9me2 modifications reinforce each other through regulatory feedback loops (FIG. 2a).

CHH methylation is maintained by DRM2 or CMT2, depending on the genomic region. Through RdDM, DRM2 maintains CHH methylation at RdDM target regions, which are preferentially located at evolutionarily young transposons and short transposons and at other repeat sequences in euchromatic chromosome arms as well as at the edges of long transposons, which are usually located in heterochromatin[36,73,74]. By contrast, CMT2 catalyses CHH methylation at histone H1-containing heterochromatin, where RdDM is inhibited. Methylation by CMT2 is impaired by mutations in DECRESED DNA METHYLATION 1 (DDM1), which is a chromatin-remodelling protein that is also required for maintaining DNA methylation in symmetric cytosine sequence

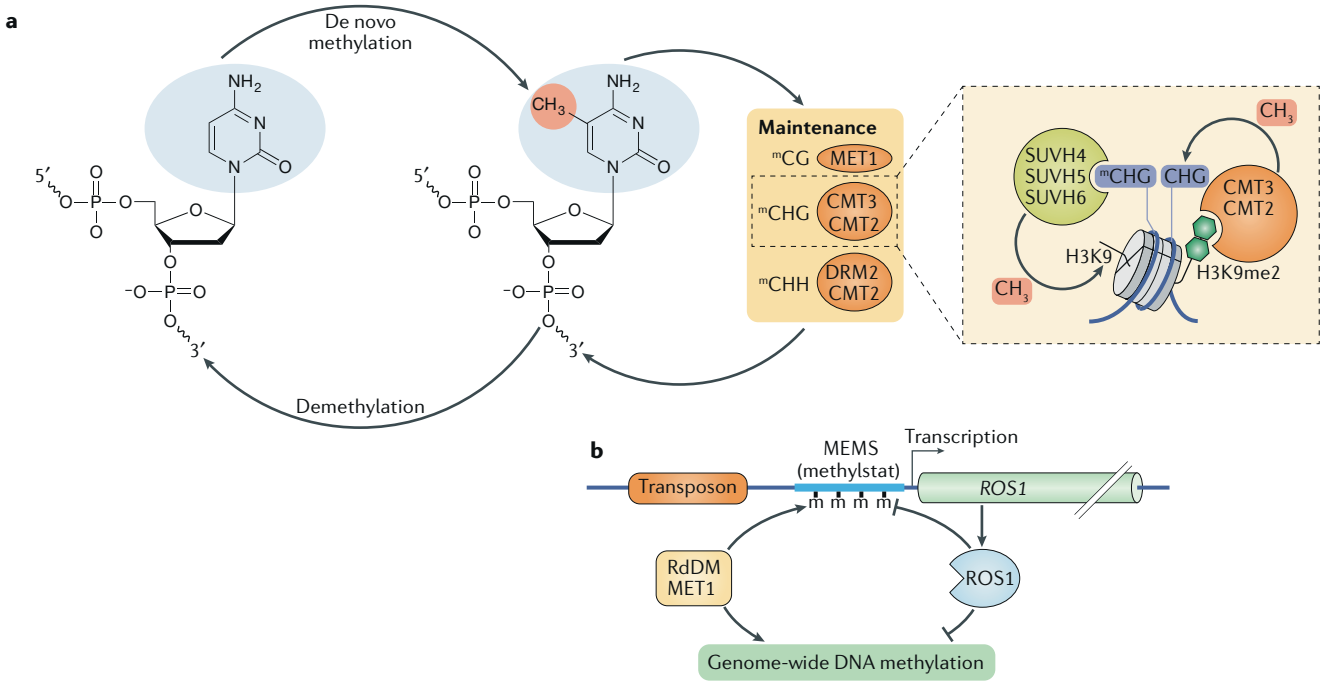

Fig. 2 | **Dynamic regulation of DNA methylation in plants. a** | De novo DNA methylation can occur in all cytosine contexts. Following DNA replication, methylation in the symmetric CG context is maintained by METHYLTRANSFERASE 1 (MET1), whereas CHG (H represents A, T or C) methylation is maintained by CHROMOMETHYLASE 3 (CMT3) or CMT2 (REFS[63,64]). Methylated CHG recruits the histone H3 lysine 9 (H3K9)-specific methyltransferases SUPPRESSOR OF VARIEGATION 3-9 HOMOLOGUE PROTEIN 4 (SUVH4), SUVH5 and SUVH6, and, in turn, dimethylated H3K9 (H3K9me2) facilitates CMT3 and CMT2 function, thereby forming a reinforcing loop between CHG methylation and H3K9 methylation[65–72]. Methylation in the asymmetric CHH context is maintained by DOMAINS REARRANGED METHYLASE 2 (DRM2) through RNA-directed DNA methylation (RdDM) or by CMT2 (REFS[74,75]). DNA methylation can be removed by active demethylation that is initiated by DNA demethylases or by passive demethylation owing to failure in methylation maintenance following DNA replication[77–82]. **b** | The methylation (m) of DNA methylation monitoring sequence (MEMS) within the promoter region of REPRESSOR OF SILENCING 1 (ROS1; a major DNA demethylase in *Arabidopsis thaliana*) is required for *ROS1* gene expression. DNA methylation at the MEMS is regulated by RdDM and MET1, and by ROS1 itself, and thus helps the plant to monitor cellular DNA methylation status to achieve a dynamic balance between DNA methylation and demethylation by fine-tuning *ROS1* transcription, and accordingly is termed a 'methylstat'[106–109].

contexts[74,75]. Maintenance of asymmetric methylation may also be affected by MET1 and CMT3 because MET1-dependent methylation can be recognized by SUVH2 and SUVH9 for recruitment of POL V at some RdDM loci[37] and because CMT3-dependent CHG methylation increases H3K9me2 levels, which facilitates CMT2-catalysed non-CG methylation[64]. Although CHH cytosines can be methylated only by DRM2 and CMT2, these two enzymes can also methylate cytosines in other contexts.

Active DNA demethylation. Lack of DNA methyltransferase activity or shortage of a methyl donor following DNA replication results in failure to maintain methylation, which is known as passive DNA demethylation[76–79]. DNA methylation can also be erased enzymatically, which is referred to as active DNA demethylation. In contrast to the DNA methylation reaction, which is catalysed by a single DNA methyltransferase enzyme, active DNA demethylation requires a team of enzymes, with the enzyme initiating the process referred to as a DNA demethylase. In plants, a family of bifunctional 5-mC DNA glycosylases–apurinic/apyrimidinic lyases initiates active DNA demethylation through a base excision repair pathway[80–82] (FIG. 3a). Active DNA demethylation in mammals also involves a DNA glycosylase and thus base excision repair. However, the plant DNA glycosylases can recognize and directly remove the 5-mC base, whereas in mammals, the 5-mC must be oxidized before the DNA glycosylase can catalyse base removal[8,83].

A. thaliana has a family of four bifunctional 5-mC DNA glycosylases, including REPRESSOR OF SILENCING 1 (ROS1), TRANSCRIPTIONAL ACTIVATOR DEMETER (DME), DEMETER-LIKE PROTEIN 2 (DML2) and DML3 (REFS[80,82]), which can excise 5-mC from all cytosine sequence contexts[81,84–87]. ROS1, DML2 and DML3 are expressed in all vegetative tissues, whereas DME is preferentially expressed in companion cells of the female and male gametes, that is, in the central cell of the female gametophyte and in the vegetative cell of the male gametophyte[86,88].

During DNA demethylation, these bifunctional enzymes act first as DNA glycosylases to hydrolyse the glycosylic bond between the base and the deoxyribose and then as apurinic or apyrimidinic lyases to cut the DNA backbone and produce an abasic site (FIG. 3a). The excision of the 5-mC base is followed by β-elimination

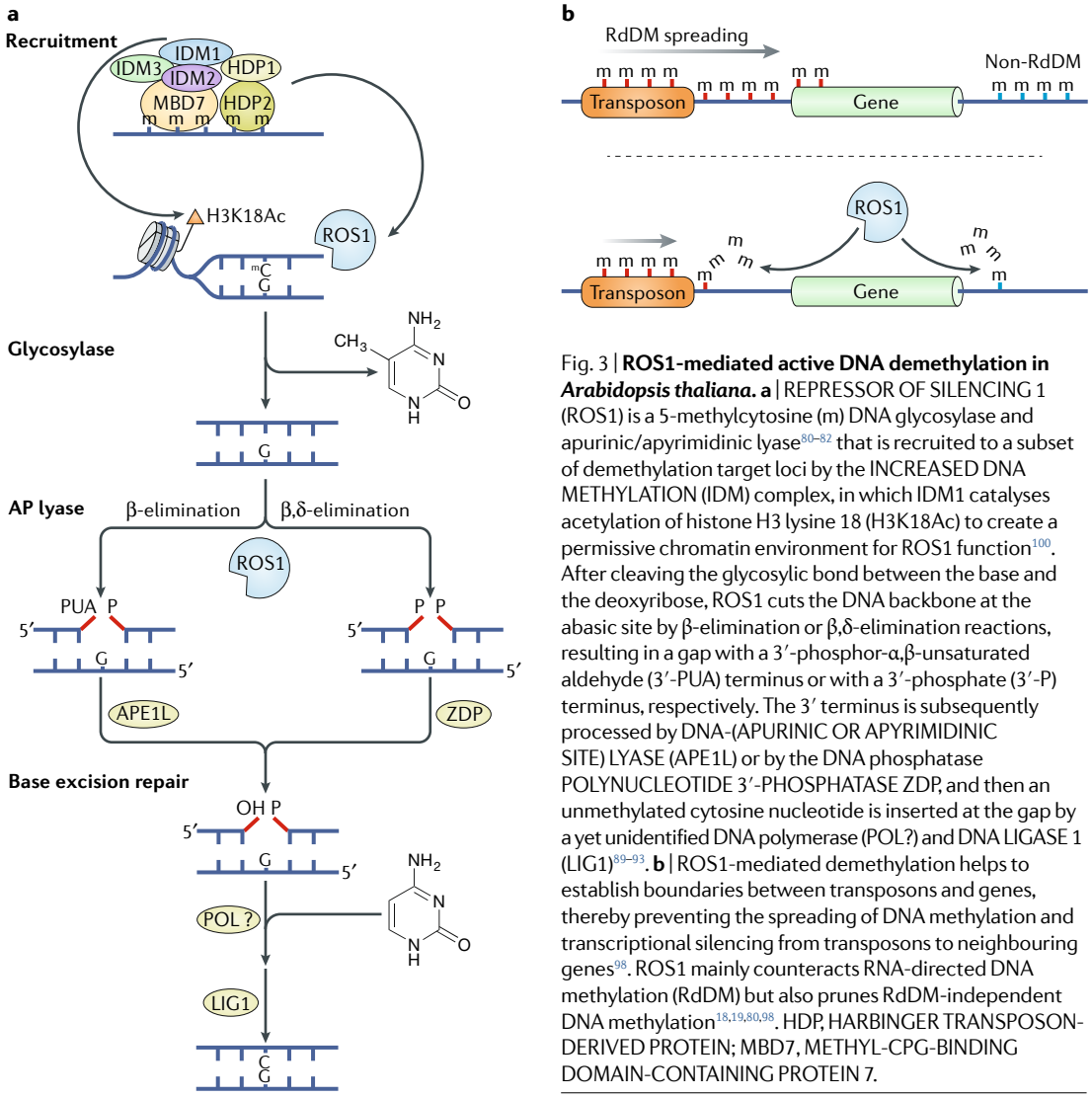

Fig. 3 | **ROS1-mediated active DNA demethylation in *Arabidopsis thaliana*. a** | REPRESSOR OF SILENCING 1 (ROS1) is a 5-methylcytosine (m) DNA glycosylase and apurinic/apyrimidinic lyase[80–82] that is recruited to a subset of demethylation target loci by the INCREASED DNA METHYLATION (IDM) complex, in which IDM1 catalyses acetylation of histone H3 lysine 18 (H3K18Ac) to create a permissive chromatin environment for ROS1 function[100]. After cleaving the glycosylic bond between the base and the deoxyribose, ROS1 cuts the DNA backbone at the abasic site by β-elimination or β,δ-elimination reactions, resulting in a gap with a 3′-phosphor-α,β-unsaturated aldehyde (3′-PUA) terminus or with a 3′-phosphate (3′-P) terminus, respectively. The 3′ terminus is subsequently processed by DNA-(APURINIC OR APYRIMIDINIC SITE) LYASE (APE1L) or by the DNA phosphatase POLYNUCLEOTIDE 3′-PHOSPHATASE ZDP, and then an unmethylated cytosine nucleotide is inserted at the gap by a yet unidentified DNA polymerase (POL?) and DNA LIGASE 1 (LIG1)[89–93]. **b** | ROS1-mediated demethylation helps to establish boundaries between transposons and genes, thereby preventing the spreading of DNA methylation and transcriptional silencing from transposons to neighbouring genes[98]. ROS1 mainly counteracts RNA-directed DNA methylation (RdDM) but also prunes RdDM-independent DNA methylation[18,19,80,98]. HDP, HARBINGER TRANSPOSON-DERIVED PROTEIN; MBD7, METHYL-CPG-BINDING DOMAIN-CONTAINING PROTEIN 7.

or β,δ-elimination reactions, which result in a gap that terminates with a 3′-phosphor-α,β-unsaturated aldehyde or with a 3′ phosphate, respectively. Subsequently, the apurinic/apyrimidinic endonuclease DNA-(APURINIC OR APYRIMIDINIC SITE) LYASE (APE1L) and the DNA phosphatase POLYNUCLEOTIDE 3′-PHOSPHATASE ZDP function downstream of the β-elimination and β,δ-elimination reactions, respectively, to generate a 3′ OH group so the gap can be filled by DNA polymerase and ligase enzymes[89–91] (FIG. 3a). *A. thaliana* DNA LIGASE 1 (LIG1) is probably the ligase in the active DNA demethylation pathway, as indicated by its subcellular colocalization with ROS1, ZDP and APE1L, and by the observation that LIG1 is essential for demethylation and activation of the maternally imprinted genes *FLOWERING WAGENINGEN* and *MEA* (*MEDEA*) in the endosperm[92,93].

ROS1 was shown in vitro to randomly slide at a specific target site[94], but in cells on a global scale, DNA demethylases exhibit target specificity, which depends on distinct chromatin characteristics and on recruiting proteins. Demethylation by DME favours small, AT-rich transposons in euchromatic regions, leading to altered expression of nearby genes[88,95–97]. ROS1 also targets transposons, which tend to be near genes[98]. This suggests that ROS1-mediated demethylation helps to establish boundaries between transposons and genes and to prevent the spreading of DNA methylation and transcriptional silencing from transposons[98]. ROS1 is particularly important for counteracting DNA methylation established by the RdDM pathway, although it also demethylates RdDM-independent regions[18,19,80,98] (FIG. 3b). It remains unclear whether small RNAs, which determine target specificity in RdDM, also mediate the targeting of ROS1 to certain loci, even though the RNA-binding protein ROS3 is important for demethylation at several ROS1-dependent genomic regions[99]. ROS1 targeted transposons and other genomic regions are characterized by enrichment of acetylated H3K18 and of H3K27me3 and depletion of H3K27me and H3K9me2 (REF.[98]). At a subset of ROS1 target loci, a chromatin environment that is permissible for active demethylation is established by INCREASED DNA METHYLATION 1 (IDM1), which is a histone acetyltransferase that binds methylated DNA and acetylates histone H3 at chromatin sites lacking H3K4me2 and

H3K4me3 (REF.[100]), that is, sites not yet associated with active transcription.

Targeting ROS1 to some genomic regions is mediated by an anti-silencing protein complex, IDM, which consists of IDM1, IDM2, IDM3, METHYL-CPG-BINDING DOMAIN-CONTAINING PROTEIN 7 (MBD7), HARBINGER TRANSPOSON-DERIVED PROTEIN 1 (HDP1) and HDP2 (REFS[100–103]) (FIG. 3a). IDM2 is an α-crystallin domain-containing protein that interacts with IDM1 and is required for IDM1-dependent H3K18 acetylation in planta[101]. MBD7 preferentially binds to highly methylated, CG-dense regions and physically interacts with IDM2, as well as with the IDM2 paralogue IDM3, which also interacts with IDM1 and is required to prevent gene repression and DNA hypermethylation[102,104]. Although the IDM complex is proposed to ensure IDM1 targeting to methylated DNA loci, how IDM1-catalysed histone acetylation helps to recruit ROS1 for active DNA demethylation remains undetermined.

Coordination between DNA methylation and demethylation. ROS1 antagonizes RdDM to prevent DNA hypermethylation at specific loci, and *ROS1* gene expression is reduced in all known RdDM mutants[17,72,105–107]. These observations revealed that DNA methylation and active demethylation activities are coordinated. A recent study of the *A. thaliana* DNA methylome showed that ROS1 activity counteracts RdDM at over 2,000 genomic regions. These regions were hypermethylated in *ros1-4* mutant plants but not in double mutant *ros1-4* and *nrpd1-3* plants, which have defective functioning of both ROS1 and the largest subunit of POL IV, DNA-DIRECTED POL IV SUBUNIT 1 (NRPD1)[98]. The *nrpd1-3* mutant displayed considerably lower levels of *ROS1* gene expression and DNA hypermethylation in many genomic regions. Methylome analysis suggested that genomic hypermethylation in the *nrpd1-3* mutant is at least partly due to suppressed *ROS1* expression.

In addition to RdDM mutants, *met1* mutants also showed suppressed *ROS1* gene expression[108]. The *ROS1* gene promoter contains a 39 bp sequence in which methylation is decreased in *met1* and RdDM mutants. Because hypomethylation in this particular sequence is accompanied by *ROS1* gene repression, it appears that this sequence, termed the DNA methylation monitoring sequence (MEMS)[106], may serve as a general indicator of RdDM and MET1 activities and may thus allow the coordination between DNA methylation and active DNA demethylation through transcriptional regulation of *ROS1* (FIG. 2b). Consistent with this model, DNA hypermethylation of MEMS occurred in loss-of-function *ros1* mutants, showing that MEMS is also targeted by ROS1 (REF.[106]). The hypermethylation of MEMS in *ros1* mutants is accompanied by increased *ROS1* expression[106]. Upstream of MEMS, the *ROS1* gene promoter contains a helitron transposon that may help attract DNA methylation factors and render the promoter responsive to DNA methylation. The specific transcription factors that promote *ROS1* transcription by DNA methylation have not been identified.

Like a thermostat that senses and maintains a stable temperature, MEMS can be considered as a 'methylstat' sequence that maintains homeostasis of ROS1-dependent DNA methylation levels in plant cells[106,107]. For instance, in *met1-3* plants, in which *ROS1* expression is considerably decreased, CG hypomethylation at 5 S ribosomal DNA sequences is compensated by a progressive increase in CHH methylation levels in successive generations as a result of cumulative RdDM, eventually resulting in re-establishment of transcription silencing[108]. By contrast, decoupling *ROS1* expression from regulation by RdDM causes widespread methylation loss and abnormal phenotypes that worsen progressively over generations[109]. Methylation-sensitive regulation of demethylase gene expression has also been observed in rice, maize and *A. lyrata*[107,110,111]. Thus, this methylstat may be a conserved mechanism for regulating DNA methylation dynamics in plants. The methylstat mechanism may also exist in non-plant organisms with DNA methylation, including in mammalian cells, where it could help explain the genome-wide hypomethylation concurrent with locus-specific hypermethylation in cancer cells and in ageing humans[112,113].

Molecular functions of DNA methylation

DNA methylation, in combination with histone modifications and non-histone proteins, defines chromatin structure and accessibility. DNA methylation therefore helps to regulate gene expression, transposon silencing, chromosome interactions (FIG. 4) and trait inheritance (Supplementary Box 1).

Gene regulation. Gene-associated DNA methylation in plants can occur in the promoter or within the transcribed gene body. Promoter DNA methylation usually inhibits gene transcription, although in some cases it promotes gene transcription, such as in the *ROS1* gene in *A. thaliana* and in hundreds of genes that inhibit fruit ripening in tomato[3,8,12,106,107]. Promoter DNA methylation directly represses transcription by inhibiting the binding of transcription activators or promoting the binding of transcription repressors or indirectly represses transcription by promoting repressive histone modifications such as H3K9me2 and inhibiting permissive histone modifications such as histone acetylation[114,115] (FIG. 4a). How promoter methylation activates gene transcription is less understood. Presumably, DNA methylation may enhance the binding of some transcription activators or may inhibit the binding of some transcription repressors. DNA methylation at promoters is often a result of the spreading of methylation machineries from nearby transposons and other repeats. The gene-adjacent transposons and repeats are also targeted by active DNA demethylation machineries to protect the genes from transcriptional silencing[98]. In the case of genes activated by promoter DNA methylation, active demethylation causes transcriptional silencing of the genes[3,106].

In *A. thaliana*, only approximately 5% of the genes are methylated in promoter regions. As a result, DNA methylation does not regulate the transcription of many genes, and most mutants with decreased or increased DNA methylation do not have severely impaired growth or development[11]. By contrast, crop plants with larger genomes can have a higher transposon content and more transposons that are close to genes; consequently, there

α-Crystallin domain
A motif of approximately 100 amino acids that is characteristic of evolutionarily conserved small heat shock proteins.

Helitron
A major class of eukaryotic transposons that transpose through rolling-circle replication.

are more genes with promoter methylation[3]. Therefore, DNA methylation has more important roles in gene regulation in several crop plants than in *A. thaliana*, and DNA methylation mutants in these crop plants are generally either lethal or have severe growth and developmental defects[3,116–119].

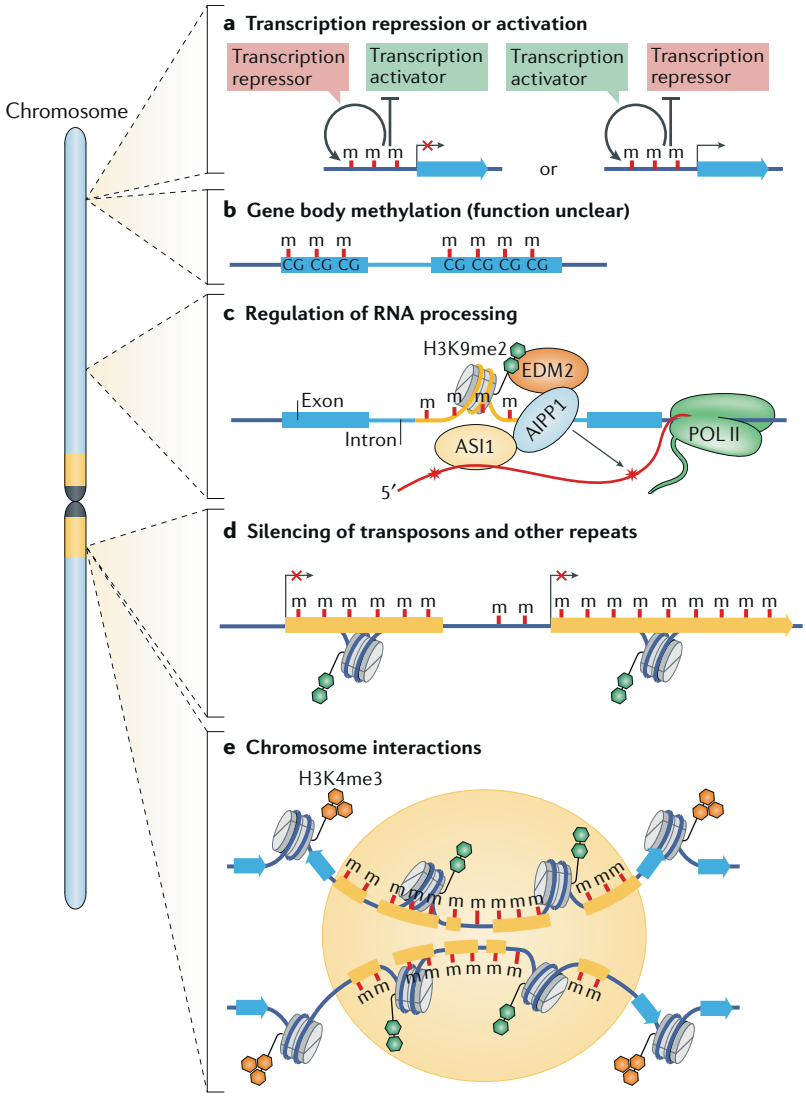

Fig. 4 | **Cellular functions of DNA methylation in plants.** DNA methylation controls gene expression, transposon silencing and chromosome interactions. **a** | DNA methylation (m) in gene promoters usually represses transcription, but in some cases, it can increase transcription[3,8,12,106,107]. **b** | DNA methylation in gene bodies mainly exists in the CG context[12,13,120,121], and its function remains to be elucidated. **c** | DNA methylation in some heterochromatin introns attracts the ANTI-SILENCING 1 (ASI1)–ASI1-IMMUNOPRECIPITATED PROTEIN 1 (AIPP1)–ENHANCED DOWNY MILDEW 2 (EDM2) complex to promote the selection of alternative distal polyadenylation sites (red asterisks) during mRNA processing[132–136]. ASI1 associates with chromatin and binds RNA; EDM2 recognizes dimethylated histone H3 lysine 9 (H3K9me2) in the intronic heterochromatin. **d** | DNA methylation is also important for silencing transposons and other DNA repeats, which are mainly located in pericentromeric heterochromatin[12,120]. **e** | DNA methylation is involved in chromosome interactions among pericentromeric regions and at some interactive heterochromatin islands, which are repressive chromatin regions located on the otherwise euchromatic chromosome arms and are characterized by abundant transposons and small RNAs[145,146]. In all panels, transposons and other repeats are in yellow and genes are in blue. Black and yellow chromosomal regions represent centromeric and pericentromeric regions, respectively. POL II, RNA POLYMERASE II.

The gene bodies of over one-third of *A. thaliana* genes are methylated[12]. In contrast to transposons and repeats, which are usually heavily methylated in all three cytosine contexts, DNA methylation in gene bodies has very little non-CG methylation[12,13,120,121] (FIG. 4b). Gene body methylation (gbM) preferentially occurs at exons and is absent from the transcription start and stop sites[121]. As a conserved feature in most angiosperms, genes with gbM tend to be longer than unmethylated genes and are generally constitutively expressed[12,121,122]. In two angiosperms, *Eutrema salsugineum* and *Conringia planisiliqua*, genome-wide loss of gbM was attributed to the loss of CMT3 (REFS[123,124]). Levels of gbM decreased in *A. thaliana* with reduced histone H3.3 levels, and this correlated with increased density of the linker histone H1, suggesting that gbM is facilitated by histone H3.3, which inhibits histone H1-dependent chromatin folding and consequently increases chromatin accessibility to DNA methylases[125].

Gene body CG methylation is almost completely absent in the *A. thaliana met1-3* mutant, in which steady-state mRNA levels of gbM genes do not appear to be globally increased relative to unmethylated genes[12]. Additionally, natural variation in gbM does not correlate with global gene expression levels in *A. thaliana* populations[126]. On the other hand, a comparison between the grass *Brachypodium distachyon* and rice (*Oryza sativa* Japonica group) showed that gbM is strongly conserved among orthologues of the two species and affects a biased subset of long, slowly evolving genes[121]. Thus, the biological importance of gbM in angiosperms seems to be species dependent. Considering that enrichment of the histone variant H2A.Z in gene bodies correlates with gene responsiveness to environmental and developmental stimuli and that the genomic distributions of H2A.Z and DNA methylation in *A. thaliana* are anti-correlative, gbM was proposed to reduce gene expression variability by excluding H2A.Z from nucleosomes[127]. In addition, gbM in plants may prevent aberrant transcription from internal cryptic promoters[128]. Indeed, in mouse cells, intragenic DNA methylation protects the gene body from spurious Pol II entry and cryptic transcription initiation[129]. It was also suggested that gbM increases pre-mRNA splicing efficiency in plants[127], which is consistent with the observation that a small portion of alternative exon–intron junctions are affected by the global loss of CG methylation in the *O. sativa met1-2* mutant[130].

Some gene introns harbour transposons or other repeats, which are heavily methylated in all cytosine sequences and regulate mRNA processing, for example, alternative polyadenylation. Loss of DNA methylation in a long interspersed nuclear element retrotransposon in the intron of the homeotic gene *DEFICIENS* causes alternative splicing and premature termination and consequently the generation of the unproductive mantled somaclonal variant of oil palm[131]. An intron of the *A. thaliana* INCREASE IN BONSAI METHYLATION 1 (*IBM1*; also known as *JMJ25*) gene, which encodes a histone H3K9 demethylase, contains a heterochromatic repeat element that is recognized by a newly discovered protein complex that promotes distal polyadenylation of *IBM1* transcripts[132–136] (FIG. 4c). This protein complex consists of ANTI-SILENCING 1 (ASI1),

Box 1 | **DNA methylation in tissue culture**

Tissue culture is important for clonal propagation and for the genetic transformation of plants. Tissue culture includes dedifferentiation of explants to undifferentiated masses of cells, known as calli, and regeneration of new plants from the calli. Plant DNA methylation patterns change substantially during tissue culturing[138,235–242], indicating that the cells undergo extensive epigenetic reprogramming. *Arabidopsis thaliana* shoot regeneration requires auxin-mediated induction of the expression of the gene *WUS*, which encodes the transcription factor WUSCHEL and whose promoter DNA is otherwise methylated[239–241]. DNA hypomethylation achieved by dysfunction of the DNA methyltransferases CHROMOMETHYLASE 3 (CMT3) or METHYLTRANSFERASE 1 (MET1) leads to direct induction of *WUS* expression by a cytokinin-rich shoot-inducing medium without the need for pre-incubation on auxin-rich callus-inducing medium, resulting in accelerated de novo shoot regeneration[241,242]. DNA methylation, therefore, may be manipulated to enable or expedite the tissue culture process, which could be important for the many plants for which tissue culture and regeneration are still very difficult.

ENHANCED DOWNY MILDEW 2 (EDM2) and ASI1-IMMUNOPRECIPITATED PROTEIN 1 (AIPP1). ASI1 is an RNA-binding protein that contains a BAH domain that may mediate its chromatin association with the heterochromatin region within the *IBM1* intron[132,133]. EDM2 contains a composite plant homeodomain (PHD) that recognizes both the transcription-repressing H3K9me2 and transcription-activating H3K4me3 modifications, which together characterize introns that contain heterochromatin repeats[134]. AIPP1 interacts with both ASI1 and EDM2, thereby promoting the formation of the complex, which also promotes distal polyadenylation of many other genes that similarly harbour intronic heterochromatin[135], although the mechanism by which the complex promotes alternative polyadenylation is unknown. Mutation of ASI1, EDM2 or AIPP1 indirectly causes gene silencing owing to the loss of full-length, functional transcripts of IBM1. ASI1 also associates with AIPP2, which has a PHD domain, AIPP3, which has a BAH domain, and the POL II carboxy-terminal domain phosphatase CARBOXY-TERMINAL PHOSPHATASE-LIKE 2 (REF.[135]). Intriguingly, mutations in the three proteins had opposing effects on gene regulation compared with mutations in the ASI1–AIPP1–EDM2 complex[135].

Transposon silencing. Transposons can threaten genome stability through the relocation of DNA transposons or the insertion of new copies of retrotransposons.

In *A. thaliana*, pericentromeric heterochromatin and some transposon-containing or repeat-containing euchromatin regions are heavily methylated in all cytosine contexts[12,120] (FIG. 4d). RdDM maintains asymmetric (CHH) methylation in short transposons and along the edges of long transposons; asymmetric methylation in the internal regions of heterochromatic, long transposons is dependent on DDM1 and is catalysed by CMT2 (REFS[64,74]). In the maize genome, active genes and inactive transposons are interspersed and are often separated by RdDM-dependent CHH methylation islands, which are short regions with elevated CHH methylation. Loss of CHH methylation islands frequently leads to transcription activation accompanied by CG and CHG hypomethylation in nearby transposons, suggesting that RdDM in maize is required to prevent silenced transposons from being activated by euchromatin of nearby active genes[137]. In sugar beets, asymmetric methylation appears to be preferentially involved in silencing DNA transposons, which show higher CHH methylation levels than retrotransposons and genes[138]. Interestingly, pericentromeric regions in *A. thaliana* and tomato display biased CHH methylation, with low levels of methylated CCG and over-representation of methylated CAA, CTA or CAT; by contrast, asymmetric methylation in euchromatin regions is context-independent in the two dicots[139]. In the monocots maize and rice, the context-dependent bias in CHH methylation is dispersed throughout the chromosomes[139].

Transposon derepression is common in *A. thaliana* mutants defective in DNA methylation; however, transposition has been observed for only a few transposons, possibly owing to suppression by post-transcriptional mechanisms. Dysfunction of MET1 or CMT3 infrequently caused transposon mobilization, whereas double mutations of these two DNA methylases or dysfunction of DDM1 resulted in strong DNA hypomethylation in both CG and CHG contexts and was accompanied by elevated levels of transposition[140–142]. Compared with wild-type plants, the *nrpd1* mutant showed more frequent transposition of the retrotransposon *ONSEN* in response to heat stress[143]; however, transposition has not been reported in mutants defective in CMT2 or RdDM factors in non-stressed conditions, indicating that CHH methylation does not function alone in suppressing transposon mobilization in *A. thaliana*. In rice, the ROS1 homologue DNA GLYCOSYLASE/LYASE 701 influenced transposition of the retrotransposon *Tos17*, indicating that DNA hypomethylation through active demethylation can also facilitate transposon mobilization[144].

Chromosome interactions. DNA methylation influences the epigenetic state of chromatin and thus can be involved in chromosome interactions. In the *A. thaliana* nucleus, all five chromosomes interact in a structure termed KNOT[145]. Chromosomal regions that form the KNOT structure comprise interactive heterochromatin islands (IHIs), which are repressive chromatin regions located in euchromatic chromosome arms and are characterized by abundant transposons and robust enrichment of small RNAs[145,146]. IHI interactions are not affected in *met1* and *ddm1* mutants, both of which show extensive DNA hypomethylation in all cytosine contexts, as well as in the *suvh4–suvh5–suvh6* triple mutant, which is defective in H3K9 methylation[146]. Thus, DNA methylation and H3K9me2 may be dispensable for chromosome interactions at IHIs. In addition, ectopic IHI loci were observed in *met1* and *ddm1* mutants[146]. Therefore, it seems that potential chromosome interactions are suppressed by DNA methylation, although the mechanisms underlying the appearance of the new IHIs remain

Homeotic gene
A gene that controls pattern formation during development.

Mantled
A type of abnormality in oil palm male floral organs in which they transform into supernumerary carpels.

unclear. Similarly, the frequency of chromosomal interactions at some RdDM regions is increased in mutants defective in RdDM, indicating that RdDM prevents certain genomic regions from forming chromosome interactions in wild-type plants[147]. Moreover, enhanced chromosome interaction was observed between POL V-dependent DNA methylation sites and distal genes that are repressed by RdDM, indicating that chromosome interactions may have a regulatory function in gene expression[147].

In addition to the KNOT, strong chromosome interactions occur among pericentromeric regions, including the heterochromatin knob on the short arm of chromosome 4 (REFS[145,146]) (FIG. 4e). Unlike IHIs, chromosome interactions at pericentromeric regions are impaired by the loss of DNA methylation in *met1* and *ddm1*, as shown by the reduced interactions among pericentromeric regions, the increased interactions between pericentromeres and euchromatic chromosome arms and a clear shift in the locations of the interactive portions within pericentromeric regions in mutant compared with wild-type nuclei[146]. Pericentromeric interaction patterns are similar in the *suvh4–suvh5–suvh6* triple mutant and *met1* or *ddm1* mutants, except that no shift in pericentromeric interaction regions was observed in the former. Chromosome interaction patterns are not changed by dysfunction of CMT3 (REF.[146]), indicating that the altered chromosome interaction patterns in *suvh4–suvh5–suvh6* mutant result from the loss of H3K9me2 rather than the loss of CHG methylation, even though DNA methylation is a major epigenetic determinant of plant chromosome interactions at pericentromeric regions.

DNA methylation in plant development

DNA methylation levels in different tissues or cell types are tightly controlled during growth and development and throughout a plant's life cycle, reflecting important roles of DNA methylation in plant physiology (BOX 1; FIG. 5).

Imprinting and seed development. *A. thaliana* plants use a double-fertilization strategy that depends on the multicellular nature of male and female gametophytes. Each of the two sperm cells in pollen fertilizes the egg cell and the central cell in the female gametophyte, thereby producing the embryo and the endosperm, respectively. Rice and *A. thaliana* endosperms display global DNA hypomethylation compared with the embryos[95–97,148]. In *A. thaliana*, this is partially attributed to DME-dependent active demethylation in the central cell (the companion cell of the female gamete) before fertilization[95,97,149] (FIG. 5a). Transcriptional repression of *MET1* also occurs during female gametogenesis but does not seem to contribute to the extensive demethylation, as genome-wide CG hypomethylation was not observed in the wild-type endosperm, as would be expected with lower MET1 activity, and DNA methylation is almost fully recovered in the *dme* mutant endosperm[97,149].

DME-mediated DNA demethylation also occurs in the male gamete companion cell (the vegetative cell) and is concurrent with considerable downregulation of DDM1 (REFS[97,150]) (FIG. 5a). Consequently, siRNAs are produced from demethylated and de-silenced transposons and travel from the vegetative cell to the sperm cells, where they reinforce RdDM[97,150,151]. *A. thaliana* POL V and DRM2, but not POL IV, which is necessary for siRNA production in the canonical RdDM pathway, were also detected in egg cells[152]; thus, transposon siRNAs that accumulate in the sperm cells may also reinforce transposon silencing after fertilization of egg cells. Later, during seed development, global levels of CHH methylation increase; subsequently, during germination, they decrease owing to passive demethylation, indicating a potential role of DNA methylation in seed dormancy[153–156]. Although CHH methylation levels in the male sexual lineage are generally lower than in somatic cells, hundreds of RdDM-dependent hypermethylation loci were identified; this sexual-lineage-specific methylation was shown to be important for meiosis[157].

The maternal genome in the endosperm is less methylated than the paternal genome, particularly in the CG context[95,96,148,158,159]. This parent-of-origin-specific methylation strongly correlates with a corresponding parent-of-origin-specific gene expression (gene imprinting) at many loci in the endosperm[158–161]. A common feature of maternally expressed genes (MEGs) is that the maternal allele is hypomethylated, whereas the paternal allele is methylated and repressed (FIG. 5b). At certain MEGs, such as *MEDEA* in *A. thaliana*, the paternal allele is silenced by the repressive histone modification H3K27me3 instead of by DNA methylation[81,162]. Dysfunction of either maternal DME or paternal MET1 disrupts the imprinting of MEGs, demonstrating that some MEGs can be attributed to allele-specific repression of DNA methylation[163–166].

The maternal alleles of paternally expressed genes (PEGs) are typically marked by H3K27me3 (FIG. 5b). This histone modification is thought to underlie the silencing of maternal alleles that exhibit DNA hypomethylation[158,163,167]. As recently shown in maize endosperms, PEGs are marked by H3K27me3 on the silenced, DNA-hypomethylated maternal allele and by the active modification H3K36me3 on the expressed, DNA-hypermethylated paternal allele. By contrast, endosperm-specific MEGs are marked near the transcription start sites by DNA methylation in the paternal allele and by the active modification H3K4me3 in the maternal allele[168].

Vegetative growth and pattern formation. Plant meristems harbour the stem cells that are the source of all tissues and organs. In *A. thaliana*, transcript levels of RdDM factors are higher in meristem tissues than in tissues that grow mostly by cell expansion, such as those in the hypocotyl or differentiated leaves[169]. Comparison of various cell types in the root meristem revealed that DNA methylation levels are highest in columella cells, possibly because these cells have less condensed pericentromeric chromatin, which allows greater accessibility to RdDM factors[170]. Although no obvious meristem defects have been reported for *A. thaliana* RdDM mutants, rice and maize RdDM mutants display strong developmental abnormalities[116–118,171] that likely reflect crucial roles for these factors in meristem function.

Columella cells
A layer of cells that form the root cap and protect the growing root tip.

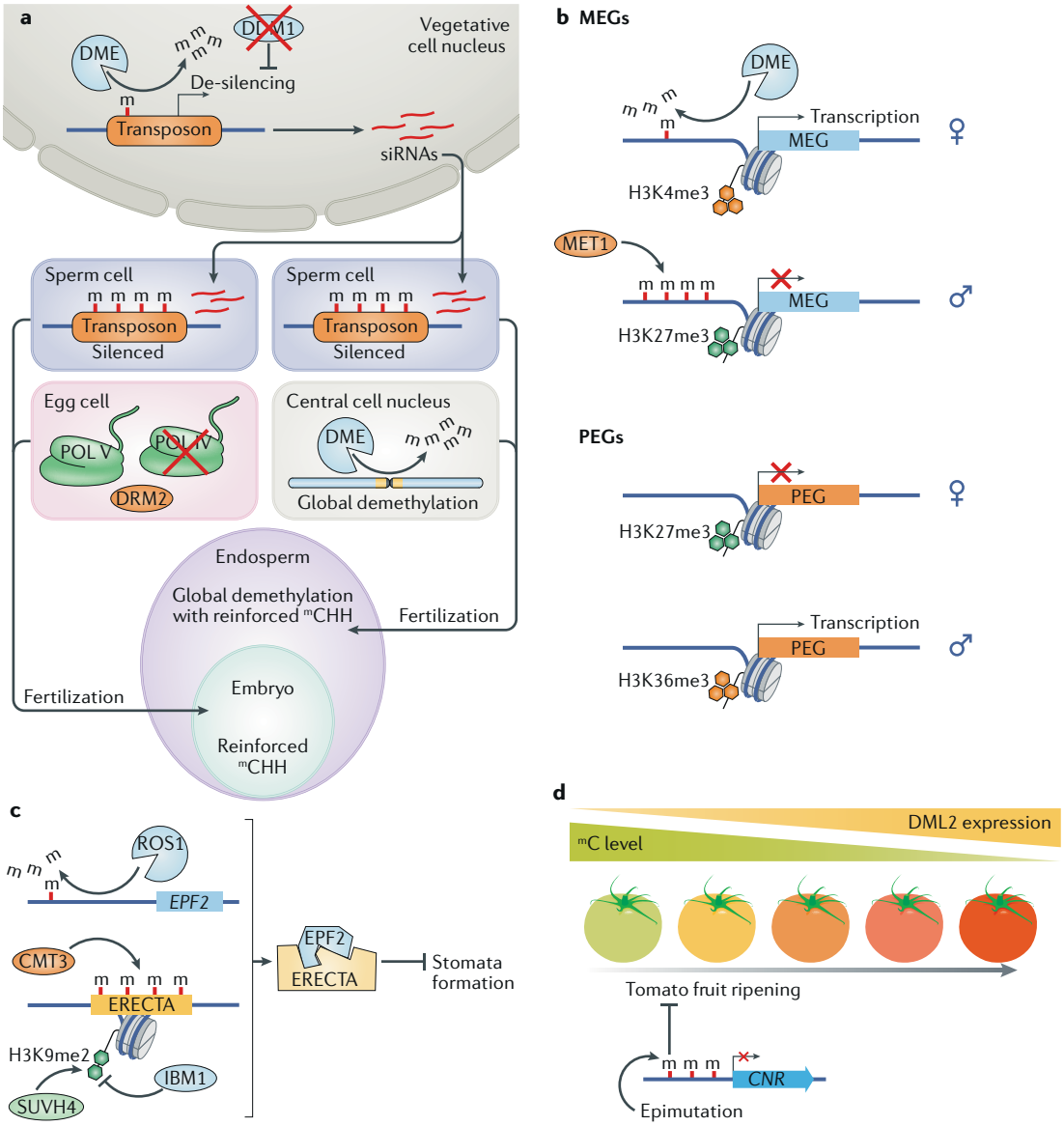

Fig. 5 | **Roles of DNA methylation in plant growth and development. a** | In the *Arabidopsis thaliana* male gamete companion cell (vegetative cell), transposons are derepressed by TRANSCRIPTIONAL ACTIVATOR DEMETER (DME)-mediated active DNA demethylation and by downregulation of the chromatin remodeller DECREASED DNA METHYLATION 1 (DDM1)[97,150]. Transposon transcripts are processed into small interfering RNAs (siRNAs), which travel from the vegetative cell to the two sperm cells, where they reinforce transposon silencing through DNA methylation (m)[97,150,151]. One of the sperm cells fertilizes the female gamete companion cell (central cell), which undergoes DME-mediated global demethylation, and together, they yield the endosperm that also shows global demethylation but has reinforced CHH (where H represents A, T or C) methylation at transposons[95,97]. The other sperm cell fertilizes the egg cell, where RNA POLYMERASE V (POL V) and the DNA methyltransferase DOMAINS REARRANGED METHYLASE 2 (DRM2), but not POL IV, are detected[152], and together, they produce the embryo, which shows reinforced CHH methylation. **b** | In the endosperm, gene imprinting occurs at maternally expressed genes (MEGs) or paternally expressed genes (PEGs). The maternal allele of MEGs is commonly characterized by DNA hypomethylation and trimethylation of histone H3 lysine 4 (H3K4me3), whereas the paternal allele of MEGs is silenced by DNA hypermethylation or H3K27me3 (REFS[81,162]). The paternal allele of PEGs is active and characterized by H3K36me3, whereas the maternal allele can be silenced by H3K27me3 (REF.[168]). **c** | The DNA demethylase REPRESSOR OF SILENCING 1 (ROS1) prunes DNA methylation at the promoter of the gene that encodes EPIDERMAL PATTERNING FACTOR 2 (EPF2), which is a ligand that represses stomata formation[174]. Three LRR RECEPTOR-LIKE SERINE/THREONINE-PROTEIN KINASE ERECTA genes that encode EPF2 receptors are negatively regulated by CHROMOMETHYLASE 3 (CMT3)-catalysed CHG methylation and SUPPRESSOR OF VARIEGATION 3-9 HOMOLOG PROTEIN 4 (SUVH4)-catalysed histone H3K9 dimethylation[175]. Mutations in ROS1 or the H3K9 demethylase INCREASE IN BONSAI METHYLATION 1 (IBM1) cause the silencing of *EPF2* or the ERECTA genes, respectively, resulting in over-production of stomatal lineage cells in *A. thaliana*. **d** | During tomato fruit ripening, expression of the DNA demethylase DME-LIKE 2 (DML2) gradually increases, leading to progressive loss of 5-methylcytosine (mC) DNA methylation at hundreds of loci, including at many genes involved in ripening such as *CNR*, which encodes COLOURLESS NON-RIPENING and whose epimutation is known to inhibit ripening[3,119]. MET1, METHYLTRANSFERASE 1.

Many developmental genes in rice are repressed by SET DOMAIN GROUP PROTEIN 711 (SDG711)-dependent H3K27me3 deposition after leaves develop from the shoot apical meristem[172]. Unlike endosperm H3K27me3, which seems to antagonize DNA methylation, SDG711-dependent H3K27me3 coexists with *O. sativa* DRM2-catalysed non-CG DNA methylation in gene bodies. SDG711 physically interacts with DRM2, whose mutation reduces SDG711 binding to chromatin and H3K27me3 deposition at the repressed genes[172]. In maize, maintenance DNA methyltransferases are differentially regulated during leaf growth, resulting in distinct CG and CHG methylation patterns among the division zone, transition zone, elongation zone and mature zone, which collectively represent the spatial gradient of cells in the leaf[173]. Differential DNA methylation in maize leaves mainly occurs at or near gene bodies, including in some genes involved in chromatin remodelling, cell cycle progression and growth regulation. Although non-CG gbM positively correlates with SDG711-dependent gene repression in rice[172], maize genes with differential gbM do not have altered transcript levels. By contrast, methylation in promoter regions is negatively correlated with gene expression[173], indicating DNA methylation has an important role in leaf growth in maize.

DNA methylation is important for pattern formation of some leaf epidermal cells in *A. thaliana*. ROS1 dysfunction causes promoter hypermethylation and repression of the gene encoding EPIDERMAL PATTERNING FACTOR 2 (EPF2), which is a peptide ligand that represses stomata formation, resulting in over-production of stomatal lineage cells in *A. thaliana*[174]. Similarly, dysfunction of the H3K9 demethylase IBM1 results in increased levels of H3K9me2 and CHG DNA methylation and repression of three LRR RECEPTOR-LIKE SERINE/THREONINE-PROTEIN KINASE ERECTA family genes that encode receptors of EPF2, leading to similar defects in stomatal pattern formation as those seen in *ros1* plants[175]. The abnormal epigenetic regulation of stomatal pattern formation can be rescued by mutations of RdDM factors in *ros1* plants or by mutations in the H3K9 methyltransferase SUVH4 or in CMT3 in *ibm1* plants[174,175], thereby representing two DNA methylation-mediated mechanisms for regulating leaf epidermal cell patterning in *A. thaliana* (FIG. 5c).

Fruit ripening. About 1% of the DNA methylome in the fruit pericarp is altered during tomato fruit development. Active DNA demethylation occurs at many fruit-ripening genes whose promoter regions contain binding sites for RIPENING-INHIBITOR (RIN), which is a major ripening transcription factor[3,176]. The binding of RIN to target promoters was confirmed in most of the known ripening genes whose expression negatively correlated with promoter DNA methylation levels. Treatment with a chemical inhibitor of DNA methylation induced promoter hypomethylation and expression of the gene encoding COLOURLESS NON-RIPENING (CNR), which is a key RIN-targeted gene for fruit ripening, and also induced premature ripening of tomato fruits[176]. The *Solanum lycopersicum* DNA demethylase DME-LIKE 2 (DML2), whose expression increases dramatically in ripening fruits, mediates the progressive DNA demethylation that occurs during fruit ripening[3,119] (FIG. 5d). *S. lycopersicum* DML2 targets not only ripening-induced genes but also ripening-repressed genes, indicating that active DNA demethylation is required for both the activation of ripening-induced genes and the inhibition of ripening-repressed genes[3]. The inhibition of hundreds of ripening-repressed genes by DML2-mediated demethylation suggests that the activation of gene transcription by promoter DNA methylation is an important mechanism of gene regulation in tomato fruits. In loss-of-function *dml2* tomato mutants, fruits cannot ripen[3]. DNA methylation changes may be involved in the growth and ripening of other fruits. Skin anthocyanin accumulation in apple fruits was negatively correlated with DNA methylation levels in the *Malus domestica MYB10* gene promoter[177,178]. On a whole-genome level, developing apple fruits show CHH hypermethylation compared with leaves, and comparison between isogenic fruits also suggested a linkage between lower DNA methylation levels and smaller fruit size[179].

Epialleles and plant development. Isogenic plants may be distinguished by epialleles, which are alleles with different epigenetic modifications that are heritable through generations. Natural epialleles have been isolated in various crop species, such as tomato, rice and cotton, and affect important traits[180–182]. In the tomato *cnr* epimutant, the *CNR* gene is transcriptionally repressed by DNA hypermethylation in the promoter, resulting in colourless, non-ripening fruits[180] (FIG. 5d). In rice with the *rav6* epiallele, promoter DNA hypomethylation causes ectopic expression of *RELATED TO ABSCISIC ACID INSENSITIVE3/VIVIPAROUS1 6*, which alters leaf angle by modulating brassinosteroid homeostasis[181]. In domesticated allotetraploid cotton, *CONSTANS-LIKE 2D* is hypomethylated compared with its epiallele in wild cotton, and flowering is consequently promoted[182].

Spontaneous epialleles are rare in *A. thaliana*[183]. However, in the mutants *met1* and *ddm1*, the massive loss of DNA methylation genome wide can only be partially restored after re-introduction of the corresponding wild-type genes, thereby disclosing epialleles that can be induced and stably inherited[184–186]. Genetic crossing of isogenic plants with contrasting epigenomes generates epigenetic recombinant inbred lines (epiRILs), which display both stable inheritance of epialleles and interspersed nonparental methylation polymorphisms in their progenies[184,187]. In addition to involvement in hybrid incompatibility and paramutation[188,189], epialleles can also contribute to heterosis, as shown in *A. thaliana* epiRILs[190,191].

Responses to environmental stimuli

DNA methylation functions in the response of plants to various biotic and abiotic environmental stimuli. There is considerable interest in whether the physical environment may change DNA methylation, partly owing to a fascination about possible plant memory of past environments. Studies of *A. thaliana* population

Allotetraploid
A polyploid with four sets of chromosomes derived from two or more diverged taxa.

Heterosis
The increase in characteristics, such as size and yield, of a hybrid organism over those of its parents.

epigenomes have revealed not only methylation-based quantitative epigenetic trait loci[192] but also a correlation between DNA methylation and local adaptation, and these are highlighted by the observation that geographic origin is linked with genome-wide DNA methylation levels and with differential gene expression caused by epialleles[126,193]. In addition, accumulating evidence has demonstrated that plant DNA methylation can be altered at individual loci or across the entire genome under environmental stress conditions, although it remains unclear whether any of the changes in response to abiotic stress are adaptive (FIG. 6a).

Biotic stress. Plants display genome-wide DNA methylation changes in response to infection by pathogens and colonization by symbiotic microorganisms. Nodulation in *Medicago truncatula* requires the demethylase DME[194]. During nodule development, several hundred genomic regions are differentially methylated, including a small subset of nodule-specific symbiosis genes[194,195]. Widespread DNA hypomethylation was observed in soybean and *A. thaliana* roots infected by cyst nematodes[196,197]. In *A. thaliana* leaves, exposure to the bacterial pathogen *Pseudomonas syringae* pv. tomato str. DC3000 (Pst DC3000) causes mild but widespread differential DNA methylation; the differentially methylated cytosines are found mainly in CG and CHH contexts in gene-rich regions, especially at the 5′ and 3′ ends of protein-coding genes. Moreover, Pst DC3000-responsive DNA methylation negatively correlates with the expression levels of nearby genes across the genome[198], indicating that DNA methylation at gene boundaries is under dynamic regulation and possibly contributes to differential gene expression in response to pathogens.

Viroids, which are plant pathogenic ncRNAs, induce DNA demethylation in promoter regions and transcription activation of some ribosomal RNA (rRNA) genes in cucumber leaves and pollen grains, resulting in accumulation of abundant small RNAs derived from rRNA[199,200]. Exogenous application of salicylic acid, which is a phytohormone crucial for plant resistance to pathogens, led to megabase-scale DNA hypomethylation in pericentromeric regions in *A. thaliana*, which was accompanied by increased levels of 21-nucleotide siRNAs derived from hypomethylated transposons[198]. Genes encoding nucleotide-binding and oligomerization domain-like receptors are the major sites of methylation variation among over 1,000 worldwide *A. thaliana* accessions[126], suggesting that biotic environmental factors are the major force shaping plant epigenomes.

Mutations in regulators of DNA methylation or demethylation can alter plant susceptibility to certain pathogens[201–206]. Infiltration of *A. thaliana* leaves with the bacterial flagellin-derived peptide flg22 triggers gene suppression of RdDM factors, which is concomitant with DNA demethylation and transcription activation of some RdDM targets, including promoter regions of some immune-responsive genes[203]. The *ros1* mutant and the RdDM mutants display increased and reduced, respectively, multiplication and vascular propagation of Pst DC3000 in leaves compared with wild-type plants[203]. Similarly, plant resistance to the biotrophic pathogen *Hyaloperonospora arabidopsidis* is increased in DNA hypomethylation mutants such as *nrpe1* and is decreased in DNA hypermethylation mutants such as *ros1* (REF.[205]). In addition to increasing resistance to biotrophic pathogens, POL V mutations also decrease resistance to the fungal necrotrophic pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina*. Unlike POL V mutants, an *nrpd1* (POL IV) mutant does not have altered resistance to either Pst DC3000 or the fungal pathogens[202], indicating that POL V can regulate plant immune responses independently of canonical RdDM. However, increased susceptibility to Pst DC3000 was observed in plants with the AGO4 mutant alleles *ago4-1* and *ago4-2*, which suggests that AGO4 has a unique function in plant disease resistance compared with other RdDM factors[201,202].

Comparison of the DNA demethylase triple mutant *ros1–dml2–dml3* and wild-type *A. thaliana* revealed that DNA hypermethylation in the mutant preferentially occurs at regions flanking gene bodies, including upstream promoter regions and 3′ untranslated regions. Over 200 genes are repressed in the *ros1–dml2–dml3* plants, a substantial portion of which have known or putative functions in biotic stress response and are enriched with small transposons in their promoters. Consistent with this, the *ros1–dml2–dml3* mutant exhibits increased susceptibility to the fungal pathogen *Fusarium oxysporum*[204] (FIG. 6b). It is therefore apparent that plants dynamically regulate DNA methylation to regulate the expression of defence genes.

Abiotic stress. Researchers have intensively explored the potential roles of DNA methylation in plant responses to a wide range of abiotic environmental stress conditions, including heat, cold, drought, high salinity, hyperosmotic stress, ultraviolet radiation stress, soil nutrient deficiency, laser irradiation, anoxia and re-oxygenation, pesticides and climate change. This research has involved a variety of plants including *A. thaliana*, maize, rice, winter wheat, *Brassica rapa*, *Brassica napus*, barley, *Populus trichocarpa* and *Quercus lobata*[207–227]. Similar to the studies on plant responses to biotic stress, many early studies of abiotic stress showed stress-induced DNA methylation and/or demethylation patterns either genome wide or at specific loci. In some cases, these changes in DNA methylation may be associated with transcriptional regulation of genes involved in plant stress responses[210–212,228], suggesting that DNA methylation is important in mediating plant responses to abiotic environmental stimuli. Recent studies have highlighted the potential importance of persistent stress for establishing DNA methylation-dependent stress memory in plants[209,219,224].

Inorganic phosphate (P_i) starvation in rice plants generates over 100 differentially methylated regions (DMRs), which are mostly CHH hypermethylated and almost exclusively overlap with transposons near stress-responsive genes known as Pi-starvation-induced (PSI) genes[207]. Time course analyses revealed that PSI-gene transcription occurred before the local DNA methylation change, indicating that these DMRs may be consequences of PSI-gene activation and may not affect the stress responses. Consistent with this possibility, after the plants had been resupplied with inorganic

Nucleotide-binding and oligomerization domain-like receptors
Receptors that mediate recognition of pathogen avirulence effectors and activate immune responses.

Biotrophic pathogen
A pathogen that feeds on only live host cells.

Necrotrophic pathogens
Pathogens that feed on nutrients released from dead cells.

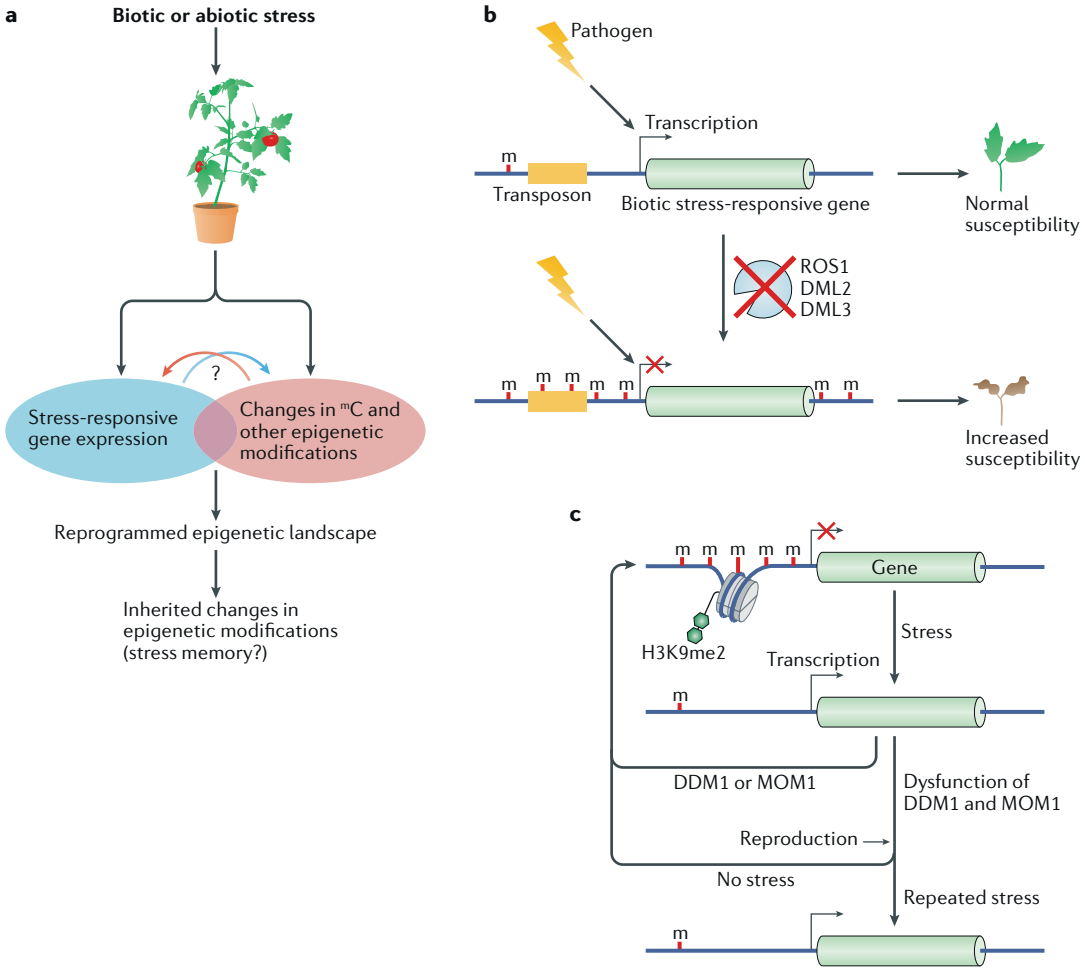

Fig. 6 | **Stress-responsive changes in epigenetic modifications and possible stress memory. a** | Biotic or abiotic stress conditions can cause changes in 5-methylcytosine (ᵐC) DNA methylation in plants, some of which are correlated with altered expression of stress-responsive genes. Conversely, stress-responsive gene expression may lead to changes in DNA methylation and other epigenetic modifications. In stressed plants with a reprogrammed epigenetic landscape, some changes in epigenetic modifications may be inherited. **b** | In *Arabidopsis thaliana*, the DNA demethylase REPRESSOR OF SILENCING 1 (ROS1) and its homologues TRANSCRIPTIONAL ACTIVATOR DEMETER (DME)-LIKE 2 (DML2) and DML3 collectively regulate many biotic stress-responsive genes by removing DNA methylation (m) in their vicinity. Plants defective in all three demethylases show increased susceptibility to the fungal pathogen *Fusarium oxysporum*[204]. **c** | *A. thaliana* DECREASED DNA METHYLATION 1 (DDM1) and MORPHEUS MOLECULE 1 (MOM1) redundantly mediate the erasure of stress-induced epigenetic memory during plant recovery from heat stress. DDM1 is a chromatin remodeller whose mutation alleviates transcriptional silencing, with a massive loss of DNA methylation. MOM1 mediates transcriptional silencing through an unclear mechanism without affecting DNA methylation. Inheritance of heat stress-induced gene de-silencing can be observed in plants exposed to repeated stress when DDM1 and MOM1 are simultaneously dysfunctional[232]. H3K9me2, demethylated histone H3 lysine 9.

phosphate, an action which would shut down PSI-gene expression, DNA methylation levels at most PSI DMRs gradually returned towards inorganic-phosphate-sufficient levels. Meiotic transmission of PSI DMRs was not observed, thus demonstrating the transient nature of PSI DMRs in rice. Similarly, non-CG hypermethylation in the wheat *VERNALIZATION-A1* gene can be induced by cold treatment and is transmitted through mitosis but not meiosis. In tomato fruits, cold treatment downregulates the expression of the DNA demethylase DML2, thereby causing promoter hypermethylation and silencing of genes responsible for the biosynthesis of flavour volatiles[228]. This explains why tomato fruits lose flavour during cold storage.

In *A. thaliana* stressed by high salinity, the induced changes in DNA methylation can be partially transmitted to the next generation, which preferentially occurs through the female germ line[209,219]; the inherited epigenetic status is gradually reset, however, if the progeny are not continuously stressed[219]. The fact that the persistence of stress-induced epigenetic memory in plants requires continuous stress was also observed in the *SDC* gene, which encodes SUPPRESSOR OF DRM1 DRM2 CMT3 and is silenced through promoter DNA methylation in vegetative tissues[224]. Following heat-induced activation of *SDC*, repeated heat treatments are required to prevent *SDC* re-silencing. Additionally, in *A. thaliana* exposed to ultraviolet light and temperature stress, epigenetic-stress

memory characterized by reduced histone H3 occupancy and increased H3K9 or H3K14 acetylation rather than by changes in DNA methylation can be transmitted to non-stressed progeny but only for a few generations[226], which is consistent with the inference that epigenetic memory in plants does not last.

A. thaliana DDM1 and MORPHEUS MOLECULE 1 (MOM1) are known to mediate the erasure of stress-induced epigenetic memory during plant recovery from heat stress. Unlike DDM1, whose mutation alleviates transcriptional silencing through a massive loss of DNA methylation, MOM1 mediates transcriptional silencing without affecting DNA methylation through an unclear mechanism[229–231]. In the *ddm1–mom1* double mutant, heat stress-induced release of epigenetic silencing is transmitted to the progeny, whereas in the *ddm1* and *mom1* single mutants, stress-induced release of silencing cannot be inherited, indicating that DDM1 and MOM1 function redundantly in preventing transgenerational epigenetic inheritance of stress-induced de-silencing[232]. On the other hand, DDM1 and MOM1 would probably not prevent stress-induced silencing, as they are positive regulators of epigenetic silencing. The *ddm1* mutation causes ectopic DNA hypermethylation that can be inherited in the presence of MOM1 (REF.[233]). Thus, erasure of stress-induced epigenetic memory in the form of silencing would likely require mechanisms independent of DDM1 and MOM1. Alternatively, the loss of stress-induced epigenetic patterns may be a passive process resulting from the lack of a persistent stress stimulus (FIG. 6c).

Future perspective

Recent research on the regulation and function of plant DNA methylation has led to a number of important discoveries, such as the identity of the initial ncRNAs that trigger de novo DNA methylation, the IDM protein complex that guides the targeting of DNA demethylase, the MEMS methylstat element that controls the balance between DNA methylation and demethylation, the ASI1–AIPP1–EDM2 protein complex that recognizes intronic heterochromatin and promotes mRNA distal polyadenylation, DNA methylome interactions in genetic hybrids and the epigenomes and transcriptomes from the 1001 Genomes collection of natural accessions of *A. thaliana*. These and other discoveries not only expand our knowledge of DNA methylation dynamics in plants but may also illuminate how DNA methylation patterns are generated in non-plant organisms, including mammals.

The recent discoveries have also produced many new questions. For instance, in DCL-independent RdDM, are the short P4 RNAs loaded onto AGOs to guide DNA methylation? The reciprocal enhancement of DNA methylation between two alleles at many *trans*-chromosomal DNA methylation loci in *A. thaliana* hybrids suggested that RdDM involves allelic interactions[234] (Supplementary Box 1). These allelic interactions cannot be explained by existing models of RdDM, implying that radical changes to these models may be needed. SHH1 recruits POL IV to only a subset of canonical RdDM target loci; similarly, the IDM complex recruits ROS1 to only a subset of ROS1-dependent demethylation target loci. It follows that the alternative mechanisms for the initial recruitment in both the RdDM pathway and ROS1-mediated demethylation remain to be determined. *A. thaliana* has served and continues to serve as an excellent model system to study the basic mechanisms of DNA methylation and demethylation, partly because the role of DNA methylation is limited in this plant and thus DNA methylation and demethylation mutants are generally not lethal. It is exciting that DNA methylation appears to regulate many more important genes for growth and development and for stress responses in plants with more complex genomes than *A. thaliana*. Future research will undoubtedly reveal new roles for DNA methylation in these plants, new mechanisms of targeting DNA methylases and demethylases and the mechanisms by which DNA methylation epialleles are generated, maintained, converted and erased.

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