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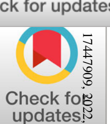

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Active DNA demethylation in plants: 20 years of discovery and beyond[FA]

Heng Zhang[1]*, Zhizhong Gong[2,3]* and Jian-Kang Zhu[4]*

1. State Key Laboratory of Molecular Plant Genetics, Shanghai Centre for Plant Stress Biology, Centre for Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai 200032, China
2. State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100193, China
3. School of Life Sciences, Institute of Life Science and Green Development, Hebei University, Baoding 071002, China
4. School of Life Sciences, Institute of Advanced Biotechnology, Southern University of Science and Technology, Shenzhen 518055, China
*Correspondences: Heng Zhang (hengzhang@psc.ac.cn); Zhizhong Gong (gongzz@cau.edu.cn); Jian-Kang Zhu (zhujk@sustech.edu.cn, Dr. Zhu is responsible for the distribution of the materials associated with this article)

Heng Zhang

Jian-Kang Zhu

ABSTRACT

Maintaining proper DNA methylation levels in the genome requires active demethylation of DNA. However, removing the methyl group from a modified cytosine is chemically difficult and therefore, the underlying mechanism of demethylation had remained unclear for many years. The discovery of the first eukaryotic DNA demethylase, *Arabidopsis thaliana* REPRESSOR OF SILENCING 1 (ROS1), led to elucidation of the 5-methylcytosine base excision repair mechanism of active DNA demethylation. In the 20 years since ROS1 was discovered, our understanding of this active DNA demethylation pathway, as well as its regulation and biological functions in plants, has greatly expanded. These exciting developments have laid the groundwork for further dissecting the regulatory mechanisms of active DNA demethylation, with potential applications in epigenome editing to facilitate crop breeding and gene therapy.

Keywords: abiotic stress response, base excision repair, DNA demethylation, DNA methylation, histone modification, plant growth and development, plant microbe interaction

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INTRODUCTION

5-Methylcytosine (5mC) is the most abundant modified nucleotide in eukaryotic genomes. Plants typically have higher 5mC contents compared to animals: in plant genomes with high repetitive sequences, up to 50% of cytosines are methylated (Matassi et al., 1992; Montero et al., 1992). Indeed, 5mC is sometimes called the fifth base, although the methyl group is added after DNA replication and does not interfere with C:G base pairing (Figure 1). In plants, DNA methylation occurs at symmetrical CG and CHG sites and asymmetrical CHH sites (where H is A, C, or T) and 5mC is targeted to repetitive sequences, gene bodies, and intergenic regions. However, its distribution varies greatly in different evolutionary lineages and organisms (Suzuki and Bird, 2008; Zemach and Zilberman, 2010; Schmitz et al., 2019).

DNA methylation plays critical roles in mammals and plants, including defending the genome against transposable elements (TEs) or viral sequences and the transcriptional regulation of developmental and stress-responsive genes (Zhang et al., 2018). The loss of DNA methylation due to mutations in the major DNA methyltransferases leads to early embryo lethality in mammals (Li et al., 1992; Okano et al., 1999). By contrast, *Arabidopsis thaliana* DNA methyltransferase mutants are viable, although their growth and development are affected (Finnegan et al., 1996; Ronemus et al., 1996; Ramsey et al., 1997). Thus, *Arabidopsis* serves as an excellent model system for studying DNA methylation dynamics in multicellular eukaryotes.

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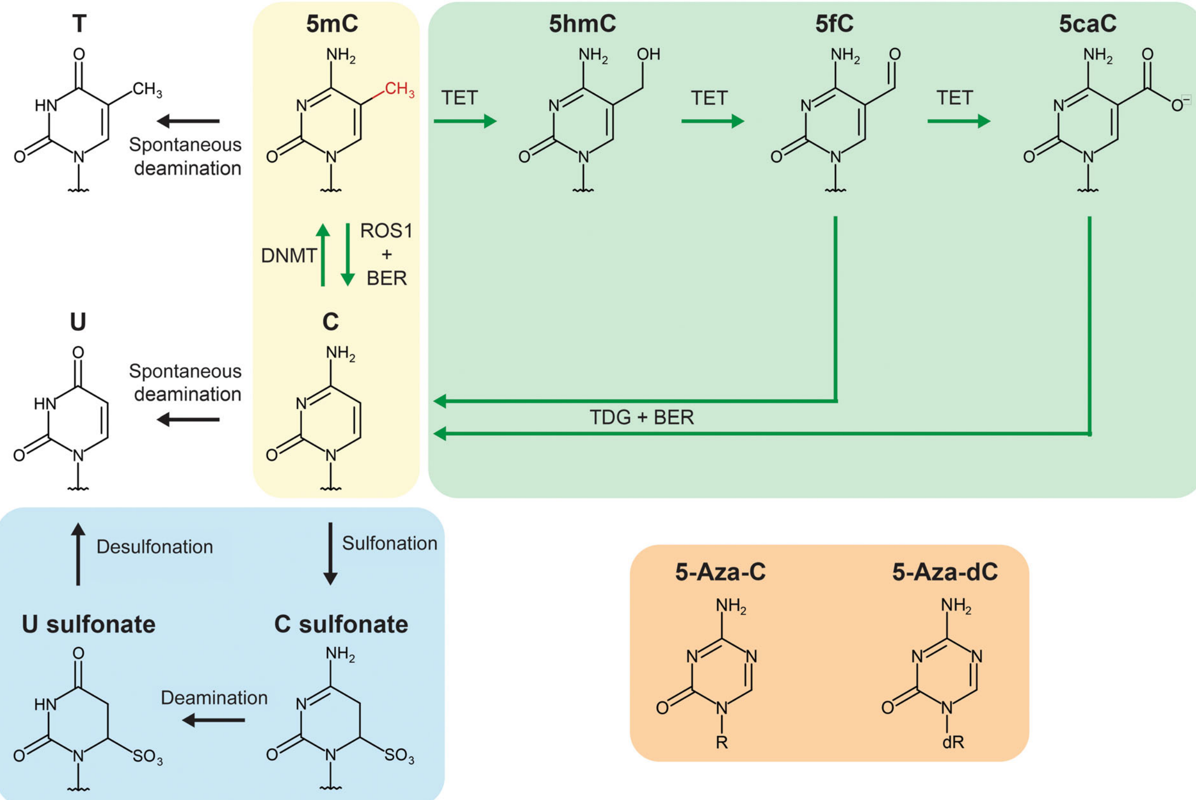

Figure 1. Structures of 5-methylcytosine and related chemicals

The abbreviated name of each chemical is indicated above each structure. Conversion among 5mC and related molecules is indicated by arrows: green arrows indicate enzyme-catalyzed reactions. Conversion between 5mC and C in plants is highlighted in yellow; the active DNA methylation pathway in mammals is highlighted in green; the bisulfite conversion reaction used in DNA methylome profiling is highlighted in blue; DNA methyltransferase inhibitors are highlighted in orange. 5mC, 5-methylcytosine; BER, base excision repair pathway; DNMT, DNA methyltransferase.

The activities of DNA methyltransferases and demethylases determine the steady-state DNA methylation level in the cell. One key activity of DNA methyltransferases is maintaining DNA methylation following DNA replication. In transmission of CG methylation, METHYLTRANSFERASE 1 (MET1), the plant ortholog of mammalian DNA methyltransferase 1 (DNMT1), interacts with PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) and UBIQUITIN-LIKE PHD AND RING FINGER DOMAIN 1 (UHRF1) at DNA replication foci. The SET- and RING associated (SRA) domain of UHRF1 binds to hemimethylated CG sites in the parental DNA strand (Bostick et al., 2007; Sharif et al., 2007), while DNMT1 catalyzes DNA methylation on the newly synthesized strand. This semi-conserved mechanism is critical for the faithful transmission of CG methylation via cell division.

The maintenance of CHG methylation requires a positive feedback loop involving histone H3 with methylated lysine 9 (H3K9me) and two types of methyltransferases. The DNA methyltransferase CHROMOMETHYLASE 3 (CMT3) binds to histone H3 with mono- or di-methylated lysine 9 (H3K9me1/2) through its bromo adjacent homology (BAH) and chromo domains and catalyzes CHG methylation (Du et al., 2012). KRYPTONITE/SU(VAR)3-9 HOMOLOG 4 (KYP/SUVH4) and its homologs SUVH5 and SUVH6 function as H3K9 methyltransferases (Ebbs and Bender, 2006). KYP binds to methylated CHG (mCHG) through its SRA domain and catalyzes H3K9 methylation (Du et al., 2014).

The maintenance of CHH methylation requires the DNA methyltransferases CHROMOMETHYLASE 2 (CMT2) and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2). Like CMT3, CMT2 binds to methylated H3K9 but shows a preference for H3K9me2 over H3K9me1 (Stroud et al., 2014). Thus, the histone modification marks H3K9me1/2 are important for the maintenance of non-CG methylation. CMT2 and DRM2 target complementary regions of heterochromatin. CMT2 mainly catalyzes CHH methylation at histone H1-containing heterochromatin, most of which is pericentromeric, while DRM2 affects small interfering RNAs (siRNAs) generating loci, which are usually found at non-pericentromeric regions and/or at the edges of long TEs (Zemach et al., 2013).

In addition to maintaining DNA methylation, DNA methyltransferases also function in *de novo* DNA methylation. In plants, the establishment of DNA methylation requires the RNA-directed DNA methylation (RdDM) pathway, in which two classes of non-coding RNAs together with an array of proteins direct DRM2 activity to specific genomic regions

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(Zhang et al., 2018). The production of non-coding RNAs, including 24-nt siRNAs and scaffold RNAs, is initiated by the plant-specific DNA-dependent RNA polymerases Pol IV and Pol V, respectively (Onodera et al., 2005). SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1)/DNA-BINDING TRANSCRIPTION FACTOR 1 (DTF1), which harbors a plant-specific SAWADEE domain that recognizes H3K9me2, is responsible for targeting Pol IV to a fraction of RdDM loci (Law et al., 2013; Zhang et al., 2013). The Pol IV transcripts are converted to 24-nt siRNAs via the sequential actions of RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) and DICER-LIKE (DCL) proteins. One strand of the mature siRNA is loaded into ARGONAUTE 4 (AGO4) or AGO6 and subsequently pairs with complementary scaffold RNAs and recruits DRM2, which methylates DNA in all sequence contexts (Wierzbicki et al., 2009).

DNA demethylation can be conceptually divided into passive demethylation and active demethylation. Passive demethylation refers to the dilution of DNA methylation through DNA replication when the cellular DNA methylation machinery is suppressed. A recent study in *Arabidopsis* found that the Dimerization partner, Retinoblastoma-like protein, E2F and Multivulval B core (DREAM) complex directly binds to and represses the transcription of DNA methylation genes including *MET1*, *CMT3*, and *KYP* (Ning et al., 2020). The DREAM complex plays a conserved role in transcriptionally repressing cell cycle genes and initiating the quiescent cell state in both animals and plants (Fischer and Muller, 2017).

Active demethylation refers to enzyme-catalyzed demethylation processes. Because DNA methylation levels were found to decrease at rates faster than passive demethylation during the differentiation of certain mammalian cell lines (Bestor et al., 1984; Razin et al., 1984; Young and Tilghman, 1984), it was long assumed that DNA demethylases exist. Since the C-C bond connecting the 5-methyl group is energetically difficult to break, multiple alternative mechanisms for active DNA demethylation were proposed (Ooi and Bestor, 2008; Gehring et al., 2009b; Zhu, 2009; Wu and Zhang, 2010). The first gene responsible for active DNA demethylation was cloned from plants 20 years ago (Gong et al., 2002). Subsequent studies revealed that members of the REPRESSOR OF SILENCING 1 (ROS1)/DEMETER-LIKE (DML) family of DNA glycosylases/lyases catalyze the first step of a series of enzymatic reactions that replace the methylated cytosine with an unmodified cytosine base (Zhu, 2009; Li et al., 2018b; Parrilla-Doblas et al., 2019). These reactions involve specific enzymes in the DNA repair pathway known as the base excision repair (BER) pathway.

Mammalian cells also use the BER pathway for active DNA demethylation. Animals do not have ROS1 homologs, but they utilize the Ten-Eleven translocation (TET) family of methylcytosine dioxygenases to sequentially convert 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). The 5fC and 5caC can then be excised by thymine DNA glycosylase (TDG) and replaced by an unmethylated cytosine via the BER pathway (reviewed in Wu and Zhang, 2017).

The discovery of ROS1/DML proteins in *Arabidopsis* 20 years ago opened the door to the identification and characterization of their downstream BER enzymes as well as upstream regulatory components in this model plant. In addition, genetic manipulation of ROS1/DML enzymes and base-resolution DNA methylation profiling in other plant species, including many crops, revealed many diverse functions of active DNA demethylation (Liu and Lang, 2020). Here, we review these exciting discoveries and propose directions for future research on active DNA demethylation, particularly in the context of genome editing.

ACTIVE DNA DEMETHYLATION BY BASE EXCISION REPAIR

Identification of DNA glycosylases as DNA demethylases

Although it was long known that DNA demethylation activity exists in mammalian cells (Jost, 1993; Zhu et al., 2000), the candidate DNA demethylases (i.e., the first enzyme in the demethylation pathway) and the possible demethylation mechanisms remained a matter of debate due to the lack of strong biochemical and genetic evidence. In plants, a breakthrough occurred in 2002 with the identification of the *ROS1* gene, which encodes a 5mC DNA glycosylase that functions in preventing DNA hypermethylation (Gong et al., 2002).

ROS1 was identified in a forward genetics screen originally designed to search for components of abiotic stress signaling in *Arabidopsis*. *RESPONSE TO DESICCATION 29A* (*RD29A*), also known as *COLD REGULATED 78* or *LOW-TEMPERATURE INDUCED 78*, is a stress-responsive gene whose expression is strongly induced by dehydration, cold, or high salt treatment or the phytohormone abscisic acid (ABA) (Yamaguchi-Shinozaki and Shinozaki, 1994). A genetic screen was designed to search for stress signaling components that were required for the expression of the luciferase (*LUC*) reporter gene driven by the *RD29A* promoter (*pRD29A*::*LUC*) (Ishitani et al., 1997; Zhu, 2002). This system successfully identified a series of mutants with defects in abiotic stress responses, such as *fiery1*, which shows enhanced cold-, osmotic stress-, and ABA-responsive gene expression (Xiong et al., 2001b); *high expression of osmotically responsive genes 1* (*hos1*), which shows enhanced cold-responsive gene expression (Lee et al., 2001); *low expression of osmotically responsive genes* 4 (*los4*), which shows reduced cold-responsive gene expression (Gong et al., 2005); and *los5* and *los6*, which show reduced osmotic stress-responsive gene expression (Xiong et al., 2001a; Xiong et al., 2002).

The same screen also identified a group of *repressor of silencing* (*ros*) mutants. In these mutants, both *pRD29A*::*LUC* and the endogenous *RD29A* gene were no longer responsive

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to any of these stress treatments, while the expression of other stress-responsive genes was unaffected. Map-based cloning revealed that *ROS1* encodes a plant-specific protein with an atypical DNA glycosylase domain (Gong et al., 2002). In *ros1* mutants, the DNA methylation level of the core promoter region of *RD29A* was significantly higher than in the wild type. The lack of *pRD29A*::*LUC* expression in the mutants was reversed by treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5aza-dC) or by introducing *decreased dna methylation 1* (*ddm1*) mutations (Gong et al., 2002), which cause a global decrease in DNA methylation levels. Recombinant ROS1 proteins exhibited DNA glycosylase activity against methylated but not unmethylated plasmid DNA in *vitro*, indicating that ROS1 is a 5mC DNA glycosylase, i.e., a DNA demethylase (Gong et al., 2002).

Another study showed that the maternal allele of *DEMETER* (*DME*) is essential for seed viability (Choi et al., 2002). DME is required for the expression of the maternal allele of *MEDEA* (*MEA*), which is also necessary for embryogenesis in *Arabidopsis* (Grossniklaus et al., 1998). DME belongs to the same protein family as ROS1; hence, ROS1 is also known as DEMETER-LIKE 1 (DML1) (Choi et al., 2002). *DME* is preferentially expressed in the central cells of female gametophytes (Choi et al., 2002). When *DME* was ectopically expressed in leaves, *MEA* expression was activated, indicating that DME is both necessary and sufficient to prevent the silencing of *MEA* (Choi et al., 2002). Consistent with the notion that *DME* encodes a DNA glycosylase domain protein, DME induced nicks in the promoter region of *MEA* (Choi et al., 2002). However, DNA methylation was not detected in the promoter region of *MEA* in wild-type or *dme* plants, leading to the conclusion that DME does not function via DNA demethylation (Choi et al., 2002). A later study demonstrated that the maternal allele of *MEA* is hypermethylated in the endosperm of *dme* mutants and that like ROS1, the recombinant DME protein has 5mC DNA glycosylase activity (Gehring et al., 2006).

ROS1/DML proteins are plant-specific 5mC DNA glycosylases

ROS1 and DME belong to a small family of plant-specific DNA glycosylases that also include DML2 and DML3 in *Arabidopsis*. Sequence analysis indicated that these proteins belong to the HhH-GPD superfamily of DNA glycosylases, which are named after their hallmark helix-hairpin-helix and Gly/Pro-rich loop, followed by a conserved Asp(D) (Nash et al., 1996). Similar to other members of this superfamily, DMLs use an invariant aspartate residue (Asp971 for ROS1) for nucleophile activation (Gehring et al., 2006; Morales-Ruiz et al., 2006). DML proteins are bifunctional DNA glycosylases (Gong et al., 2002) that have both DNA glycosylase and apurinic/apyrimidinic (AP) lyase activity (Agius et al., 2006; Gehring et al., 2006). They use an Asp-activated lysine (Lys953 in ROS1), instead of a water molecule like monofunctional glycosylases, for nucleophilic attack at the C1′ carbon of the deoxyribose ring (Nash et al., 1996; Scharer and Jiricny, 2001) (Figure 2). This leads to the generation of an animal product that rearranges into a Schiff's base intermediate, ultimately resulting in β- or β, δ-elimination and strand scission (Scharer and Jiricny, 2001) (Figure 2). Mutating any of the above residues or other residues involved in substrate binding eliminated the DNA glycosylase activity of these proteins (Agius et al., 2006; Gehring et al., 2006; Morales-Ruiz et al., 2006; Ortega-Galisteo et al., 2008; Mok et al., 2010; Ponferrada-Marin et al., 2011).

Plant DML proteins range from 900 to 2,000 amino acids (aa) long, making them unusually large compared to typical DNA glycosylases. Multiple sequence alignment indicated that the three conserved regions are located in the C-terminal regions of most DMLs, all of which are indispensable for their glycosylase activities (Gehring et al., 2006). The DNA glycosylase domain is composed of two conserved regions (aa 567–626 and aa 866–1057 in ROS1) interrupted by a variable sequence (Ponferrada-Marin et al., 2011). A conserved iron-sulfur cluster motif located at the very C-terminus of the DNA glycosylase domain (aa 1037-1057 in ROS1) is essential for the glycosylase activity of the protein (Mok et al., 2010).

ROS1 directly interacts with MET18, a component of the cytosolic iron-sulfur assembly (CIA) complex; this observation is consistent with the finding that mutants of the CIA pathway exhibit elevated DNA methylation levels at thousands of genomic loci that overlap with those of *ros1* or *ros1 dml2 dml3* mutants (Duan et al., 2015). The redox state of the iron-sulfur cluster motif is thought to serve as a mechanism to detect the substrate nucleotide and regulate the binding affinity of the glycosylase with DNA (Buzas, 2016). The conserved C-terminal domain (aa 1250–1381 in ROS1) contains a permutated CXXC motif and an RNA recognition motif (RRM) fold, which were uniquely identified in DMLs, although this domain is more variable than the DNA glycosylase domain. The C-terminal domain is required for the glycosylase activity of DME and ROS1 (Mok et al., 2010; Hong et al., 2014). Adding the purified C-terminal domain to the DNA glycosylase domain *in vitro* restored the DNA glycosylase activity, indicating that the two domains cooperate to perform their enzymatic function (Hong et al., 2014).

Whereas some DNA methyltransferases show sequence context-dependent activities, DMLs can demethylate 5mC in any sequence context (Agius et al., 2006; Morales-Ruiz et al., 2006; Ortega-Galisteo et al., 2008). Purified ROS1 excises 5mC more efficiently when the 5mC is mispaired, suggesting that base flipping is a rate-limiting step of this reaction (Ponferrada-Marin et al., 2009). Based on homology modeling, two conserved residues (Gln607 and Asn608 in ROS1) were proposed to fill in the space left by the flipping of 5mC, but mutational analyses indicated that their relative importance for 5mC excision activity varies among DML proteins (Ponferrada-Marin et al., 2011; Parrilla-Doblas et al., 2013; Brooks et al., 2014). When 5mC is within the CG context, ROS1 binds tightly to the excised product

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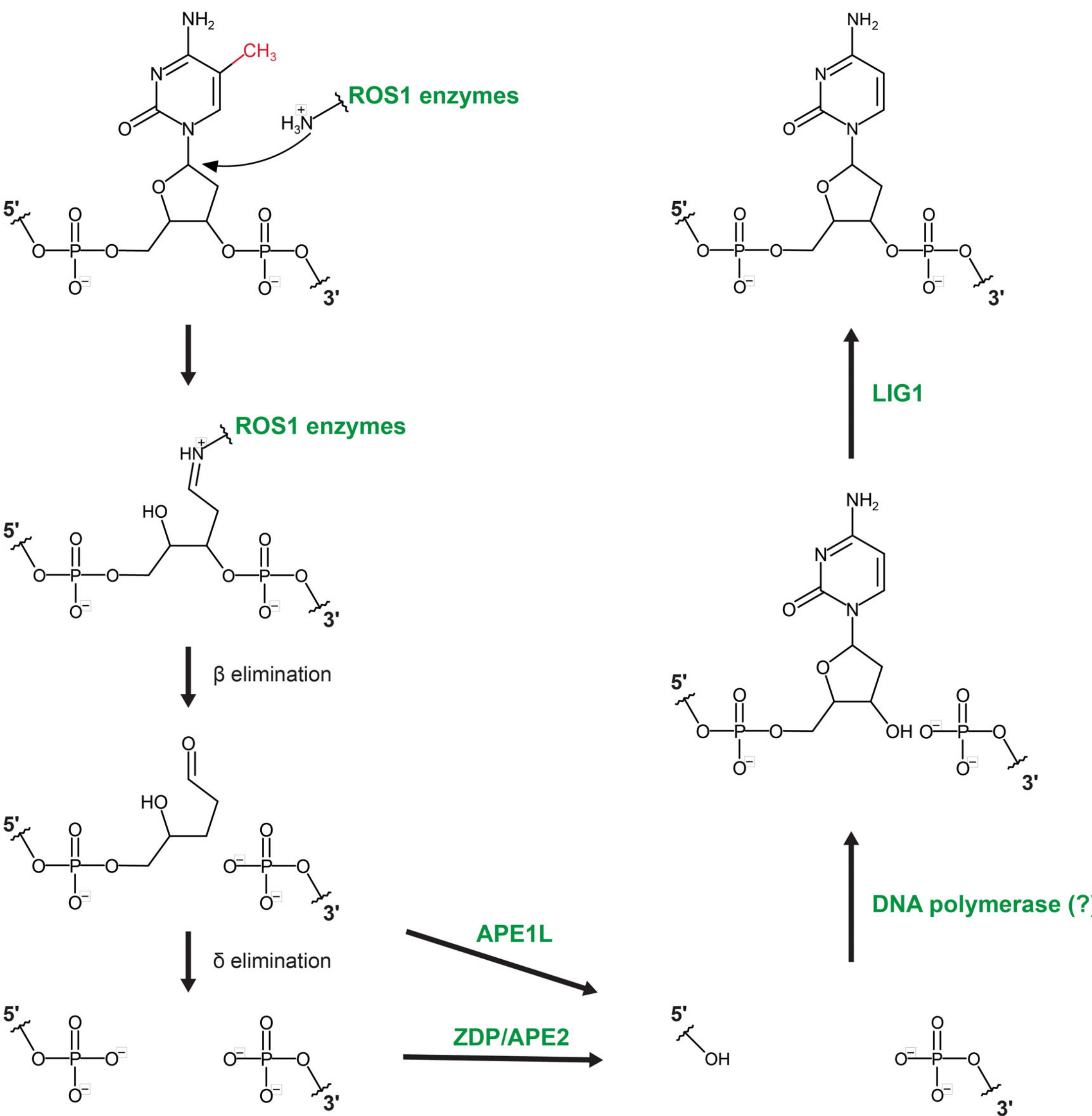

Figure 2. DNA demethylation via the base excision repair pathway
The chemical structures of parts of the single-stranded DNA (5′ and 3′ ends marked) undergoing the demethylation reaction are shown. Enzymes are indicated in bold green text.

(Ponferrada-Marin et al., 2009; Ponferrada-Marin et al., 2010), and the presence of an abasic site on the symmetric C position of the complementary strand inhibits 5mC excision (Gehring et al., 2006). This could reflect a mechanism by which DMLs avoid producing more deleterious double-strand breaks when acting on symmetric mCG or mCHG substrates. Recently, molecular structures the catalytic domain (residues 510–1393) of ROS1 in complex with substrate DNA and reaction intermediate were solved through cryo-electron microscopy (Du et al., 2022). The structure indeed revealed a flipping-out mechanism of 5mC, which is then recognized by a small hydrophobic pocket (Du et al., 2022).

ROS1/DML proteins can also excise T, but only when the T is mispaired with G. Although this thymine glycosylase activity operates with much lower efficiency compared to 5mC excision, it suggests a possible role for DMLs in repairing T:G mismatches (Agius et al., 2006; Morales-Ruiz et al., 2006; Ortega-Galisteo et al., 2008). METHYL-CPG-BINDING PROTEIN 4 LIKE (MBD4L) is the only other DNA glycosylase in *Arabidopsis* known to exhibit thymine excision activity (Ramiro-Merina et al., 2013). Given the relatively high mutation rate of 5mC to T due to spontaneous deamination (Ehrlich et al., 1986), this activity might be necessary to maintain a low mutation rate at methylated sequences (Figure 1).

Active DNA demethylation through DNA repair

The AP lyase activity of ROS1/DML proteins generates single-strand breaks in DNA that must be repaired (Figure 2). The product generated after ROS1 catalysis is a single

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nucleotide gap with either 3′-phospor-α, β-unsaturated aldehyde (3′-PUA, product of β-elimination), or 3′-phosphate (3′-P, product of β, δ-elimination) ends (Figure 2). Both 3′-PUA and 3′-P must be converted to 3′-hydroxy (3′-OH) before the gap can be filled in by DNA polymerase and ligase enzymes (Robertson et al., 2009). *Arabidopsis* contains three homologs of mammalian AP endonucleases: APEX1-LIKE (APE1L), APURINIC/APYRIMIDINIC ENDONUCLEASE 2 (APE2), and APURINIC ENDONUCLEASE-REDOX PROTEIN (ARP) (Murphy et al., 2009). Only APE1L and ARP can process DNA with 3′-PUA ends *in vitro* (Lee et al., 2014), with APE1L exhibiting higher activity than ARP (Li et al., 2015b). The *ape1l* mutant also contains an order of magnitude more hyper-differentially methylated regions (hyper-DMRs) that show significant increases in DNA methylation levels compared with *arp* mutants at the seedling stage (Li et al., 2015b), indicating that APE1L is the major endonuclease that processes 3′-PUA ends (Figure 2).

ZINC FINGER DNA 3′ PHOSPHATASE (*ZDP*) encodes a 3′ DNA phosphatase that repairs DNA double-strand and single-strand breaks (Petrucco et al., 2002). Purified ZDP proteins exhibit potent phosphatase activity against the β, δ-elimination product of ROS1 (Martinez-Macias et al., 2012). A loss-of-function mutation of *ZDP* led to increased DNA methylation levels at over 1,500 genomic regions (Li et al., 2015b). APE2 has weak DNA 3′ phosphatase activity and strong DNA 3′-5′ exonuclease activity and can process 3′-P ends *in vitro* (Li et al., 2018a). The *ape2* mutants contain over 900 hyper-DMRs that partially overlap with those of *zdp* mutants, while the *zdp ape2* double mutant contains >2100 hyper-DMRs (Li et al., 2018a). Therefore, both ZDP and APE2 are involved in processing 3′-P ends into 3′-OH, with ZDP playing a major role in this process (Figure 2). Consistent with their primary roles in processing 3′-PUA and 3′-P ends, respectively, the *zdp ape1l* double mutant is embryonic lethal, with reduced expression of DME-regulated genes including *FLOWERING WAGENINGEN* (*FWA*) and *MEA*, suggesting that ZDP and APE1L are also required for DME-catalyzed DNA demethylation (Li et al., 2015a).

The DNA polymerase involved in DNA demethylation is yet to be identified (Figure 2). Among the three DNA ligases encoded in the *Arabidopsis* genome, DNA LIGASE 1 (LIG1) is specifically required for DNA demethylation (Li et al., 2015c) (Figure 2). Similar to *dme*, loss-of-function *lig1* mutants are maternally lethal (Andreuzza et al., 2010), whereas *lig1* knockdown mutants show elevated DNA methylation levels and reduced transcription of imprinted genes, such as *FWA* and *MEA* (Li et al., 2015c).

In addition to these enzymes, the scaffold protein XRCC1, whose mammalian homolog promotes BER by forming a complex with multiple DNA repair proteins (London, 2015), interacts with ROS1 and ZDP and promotes their enzymatic activities *in vitro* (Martinez-Macias et al., 2013). Both ZDP and APE1L colocalize and physically interact with ROS1 in plant cells (Martinez-Macias et al., 2012; Li et al., 2015b). LIG1 also colocalizes with ROS1, ZDP, and APE1L in the nucleus (Li et al., 2015c). Thus, these DNA repair proteins may form a complex with ROS1 at the DNA lesion site to ensure that the ROS1-generated single-strand break is rapidly fixed.

REGULATION OF ACTIVE DNA DEMETHYLATION

DNA methylation of the DNA methylation monitoring sequence promotes *ROS1* expression

ROS1 is downregulated in mutants defective in DNA methylation or in wild-type plants treated with the DNA methyltransferase inhibitor 5Aza-dC (Huettel et al., 2006; Mathieu et al., 2007; Gao et al., 2010), suggesting that some DNA methylation-sensitive *cis*- or *trans*-regulatory elements control the expression of *ROS1*. Downregulation of *ROS1* homologs was also observed in maize (*Zea mays*) RdDM mutants (Jia et al., 2009; Erhard et al., 2015). The methylation level of the 200-bp promoter region immediately upstream of *ROS1* varies in DNA methylation mutants (Figure 3A) (Lei et al., 2015; Williams et al., 2015). This region partially overlaps with a Helitron-type TE (AT2TE6823) that negatively regulates *ROS1* expression (Lei et al., 2015). A 39-bp sequence within this region, methylation of which leads to increased *ROS1* transcript levels, antagonizes the role of the TE in repressing *ROS1* expression (Lei et al., 2015). This sequence is located at -40 to -2 bp relative to the annotated *ROS1* transcriptional start site and was termed the DNA methylation monitoring sequence (MEMS) (Lei et al., 2015). The DNA methylation and demethylation machinery are recruited to the MEMS region, as chromatin immunoprecipitation experiments detected significant signals of AGO4, the RNA polymerase Pol V subunit NRPE1, and ROS1 (Lei et al., 2015; Williams et al., 2015; Schalk et al., 2016).

Thus, the DNA methylation and demethylation pathways converge at the MEMS to regulate *ROS1* expression. Restoring MEMS methylation and *ROS1* expression in RdDM mutants resulted in the cumulative loss of DNA methylation at thousands of genomic regions and progressively worsened their growth phenotypes (Williams and Gehring, 2017). Therefore, maintaining the balance between methylation and demethylation activities is critical for plant growth and development.

SUVH1/3/7/8 (SU(VAR)3-9 HOMOLOG 1/3/7/8) are a group of SRA-domain containing proteins that bind to the MEMS and promote *ROS1* expression (Xiao et al., 2019) (Figure 3A). SRA domains are methyl-DNA binding modules (Du et al., 2015). The SRA domain of SUVH3 binds to the methylated MEMS *in vitro*, and the binding of SUVH3 to the MEMS *in vivo* is positively correlated with the DNA methylation level of the MEMS (Xiao et al., 2019). SUVH1 and SUVH3 form a complex with three DNAJ domain-containing proteins (Harris et al., 2018; Xiao et al., 2019), and this complex acts as a DNA methylation reader to promote

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Figure 3. Regulation of the ROS1 family of 5mC DNA glycosylases
(A) *ROS1* transcription is positively regulated by the DNA methylation level of the MEMS element located immediately upstream of the transcriptional start site. The RdDM pathway increases and the RWD40 complex decreases the DNA methylation level of the MEMS. The SUVH complex binds to methylated MEMS and promotes *ROS1* expression. **(B)** At some target loci of ROS1, the sequential action of the IDM complex and the PIE1-containing SWR complex is needed to achieve efficient DNA demethylation. **(C)** The four-component RWD40 complex binds to some ROS1 targets and demethylates DNA in an IDM complex-independent manner. Please see the main text for details. 5mC, 5-methylcytosine.

transcriptional activation (Harris et al., 2018; Zhao et al., 2019). Mutations of *SUVH1* and *SUVH3* resulted in increased DNA methylation levels at >800 genomic regions and altered the expression of >200 genes (Xiao et al., 2019; Zhao et al., 2019).

In contrast to the SUVH1–SUVH3–DNAJ protein complex, the RWD40 complex restricts *ROS1* expression (Liu et al., 2021) (Figure 3A). This four-component complex contains ROS1, the methyl-DNA binding protein RMB1, the homeodomain-containing protein RHD1, and the WD40 protein RWD40, and is required for the demethylation of the MEMS and the repression of *ROS1* expression (Liu et al., 2021) (see the next section for details). In addition, the DNA lesion binding protein DNA DAMAGE-BINDING PROTEIN 2 (DDB2) binds to the *ROS1* promoter and directly interacts with ROS1 and AGO4 (Cordoba-Canero et al., 2017). Whether DDB2 functions before or after ROS1 catalysis remains unknown.

Cis-elements controlling *DME* expression in gametophytes

DME is transiently expressed in the central cell of the female gametophyte (Choi et al., 2002) and at the bicellular stage of the vegetative cell of the male gametophyte (Park et al., 2017). The major *cis*-elements controlling this specific expression pattern are located downstream of the transcriptional start site (Figure 3). The introduction of *DME* cDNA driven by a minimal promoter approximately 1.9 kb long and including parts of the 1st and 2nd exons and the 1st intron of *DME* was sufficient to rescue the seed abortion phenotype and DNA methylation defects of the *dme* mutant (Park et al., 2017). Promoter analysis using the β-glucuronidase (*GUS*) reporter gene identified overlapping 15-bp and 47-bp regions in the 1st intron that are necessary for *DME* expression in the central cell and vegetative cell, respectively, whereas a 13-bp sequence located in the 1st exon is required for its sporophytic expression (Park et al., 2017).

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Targeting of DNA demethylation activity

Although ROS1/DML proteins demethylate thousands of genomic loci, how they are targeted to specific regions remains an important question. Whole-genome profiling indicated that ROS1 preferentially targets intergenic regions and TEs close to protein-coding genes (Tang et al., 2016). The hyper-DMRs of *ros1* mutants are enriched in H3K18Ac and H3K27me3 and depleted of H3K27me1 and H3K9me2 (Tang et al., 2016). In addition, the RNA-binding protein ROS3 is required for DNA demethylation at some ROS1 targets, implying a role for non-coding RNAs in targeting ROS1 (Zheng et al., 2008). In the central cell, DME targets small euchromatic TEs that are AT-rich and nucleosome-depleted (Ibarra et al., 2012) and heterochromatic TEs that are GC-rich with high nucleosome occupancy (Frost et al., 2018). In the sporophyte, DME also targets hundreds of genomic loci that are distinct from those of its homologs (Zeng et al., 2021; Williams et al., 2022). During the past decade, several chromatin-associating protein complexes that regulate ROS1 activity have been identified (Figure 3). Analysis of these complexes indicated that no single type or combination of chromatin features determines the specificity of ROS1 targets.

The INCREASED DNA METHYLATION (IDM) complex is a histone acetyltransferase complex required for the targeting of ROS1 (Qian et al., 2012; Qian et al., 2014; Lang et al., 2015; Duan et al., 2017). This complex has an estimated molecular weight of approximately 339 kD and contains six components: IDM1 (Li et al., 2012; Qian et al., 2012), IDM2/ROS5 (Qian et al., 2014; Zhao et al., 2014), IDM3 (Lang et al., 2015), METHYL-CPG-BINDING DOMAIN 7 (MBD7) (Lang et al., 2015; Li et al., 2015a; Wang et al., 2015), HARBINGER TRANSPOSON-DERIVED PROTEIN 1 (HDP1), and HDP2 (Duan et al., 2017) (Figure 3B). IDM1 uses its PHD finger and MBD domain to bind to H3K9me1/2 and methylated CG, respectively (Qian et al., 2012). MBD7 preferentially binds to highly methylated, CG-dense regions (Lang et al., 2015) and the SANT/Myb/trihelix domain of HDP2 has DNA binding activity that is required for its function (Duan et al., 2017). Thus, the target specificity of this complex is jointly determined by multiple chromatin-binding modules.

In addition, IDM2 and IDM3 contain α-crystallin domains that are typically found in small heat shock proteins (sHSPs), suggesting that these sHSP-like proteins might function as chaperones required for IDM complex activity, particularly the *in vivo* histone acetyltransferase activity of IDM1 (Qian et al., 2014; Lang et al., 2015). IDM1 is the core histone acetyltransferase that catalyzes the acetylation of K14, K18, and K23 of histone H3 (Qian et al., 2012). In the absence of IDM1, the binding of ROS1 to chromatin was abolished at several tested loci, indicating that histone acetylation is required for ROS1 activity (Qian et al., 2012). Mutants of enzymes in the fatty acid β-oxidation pathway, including *acyl-CoA oxidase* (*acx4*), *multifunctional protein 2* (*mfp2*), and *3-ketoacyl-CoA thiolase-2* (*kat2*) exhibit DNA hypermethylation at many ROS1 target loci, whereas overexpressing *ACLA1* or *ACLB2* (encoding subunits of ATP-citrate lyase (ACL)) rescued the hypermethylation at selected ROS1 targets in *acs4* (Wang et al., 2019). The *acs4* mutant exhibits reduced acetyl-CoA and histone acetylation levels, while overexpressing ACL subunit genes increased global acetyl-CoA levels (Wang et al., 2019). Thus, primary metabolism might also affect DNA demethylation via histone acetylation.

The interpretation of histone acetylation established by the IDM complex requires the SWR1 complex (Nie et al., 2019), a chromatin remodeling complex that catalyzes the deposition of the histone variant H2A.Z (March-Diaz and Reyes, 2009) (Figure 3B). Two bromodomain-containing proteins of the SWR1 complex, MBD9 and NUCLEAR PROTEIN X1 (NPX1), bind to histone H3 acetylated at K14, K18, and K23 and are required for the deposition of H2A.Z at IDM1 target loci (Nie et al., 2019). Mutants of genes encoding the core components of the SWR1 complex, including *PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1* (*PIE1*) and *ACTIN-RELATED PROTEIN 6*- (*ARP6*), and mutants of the three major *H2A.Z* genes showed strikingly similar DNA methylation profiles to the *idm1* mutant (Nie et al., 2019). Importantly, ROS1 interacts with H2A.Z *in vitro* and *in vivo* (Nie et al., 2019). These observations support a model in which IDM-catalyzed histone acetylation promotes SWR1-dependent deposition of H2A.Z, which in turn recruits ROS1 to its target loci.

Overall, the IDM and SWR1 complexes affect approximately one-third of ROS1-regulated loci, as estimated by whole-genome bisulfite sequencing (Nie et al., 2019). In addition to SWR1, a protein complex composed of BROMODOMAIN AND ATPASE DOMAIN-CONTAINING PROTEIN 1 (BRAT1) and BRAT1 PARTNER 1 (BRP1) is also required for ROS1-dependent DNA demethylation at hundreds of genomic loci (Zhang et al., 2016). The bromodomain of BRAT1 binds to acetylated histone H4 *in vitro*; this binding capacity is required to maintain low DNA methylation levels at various loci (Zhang et al., 2016). Whether the BRAT1 complex functions downstream of IDM1 remains to be determined.

A ROS1-containing complex was recently found to regulate DNA demethylation in an IDM1-independent manner (Liu et al., 2021). Besides ROS1, the approximately 350-kD RWD40 complex contains three additional proteins: ROS1-ASSOCIATED WD40 DOMAIN-CONTAINING PROTEIN (RWD40), ROS1-ASSOCIATED METHYL-DNA BINDING PROTEIN 1 (RMB1), and ROS1-ASSOCIATED HOMEODOMAIN PROTEIN 1 (RHD1) (Liu et al., 2021) (Figure 3C). These three proteins interact with each other, but only RWD40 directly binds to ROS1 (Liu et al., 2021). The MBD domain of RBM1 binds to methylated DNA with micromolar affinity without notable sequence preferences (Liu et al., 2021). Loss-of-function mutations in *RWD40*, *RMB1*, or *RHD1* lead to impaired ROS1 binding and increased DNA methylation at the MEMS of the *ROS1* promoter and multiple other endogenous loci, independently of IDM1 (Liu et al., 2021). Thus, the targets of this complex represent another class of ROS1-regulated loci.

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The histone chaperone complex FACILITATES CHROMATIN TRANSACTION (FACT) is required for DME-mediated demethylation at over half of its target loci in *Arabidopsis* endosperm (Frost et al., 2018). FACT, which is conserved in eukaryotes, facilitates nucleosome assembly and disassembly by interacting with the histone dimers H2A-H2B and H3-H4 as well as its target DNA (Grasser, 2020). Both subunits of the complex, SSRP1 and SPT16, interact with DME in the nucleus and facilitate DME activity at GC-rich heterochromatic regions with high nucleosome density (Frost et al., 2018). Loss of SSRP1 or SPT16 resulted in increased DNA methylation levels and reduced expression of some imprinted genes (Ikeda et al., 2011; Frost et al., 2018). The requirement for FACT at a small fraction of DME target loci was partially relieved by mutations in histone H1 (Frost et al., 2018), which was also shown to physically interact with DME in yeast two-hybrid assays (Rea et al., 2012). Overall, the FACT complex may be required to provide DME with access to heterochromatic targets, whereas histone H1 negatively regulates this process.

Active DNA demethylation during plant growth and development

The role of DNA demethylation in transcriptional regulation

ROS1 and DME were discovered based on their function in preventing transcriptional silencing. ROS1 is required to prevent DNA methylation at the transgenic and endogenous *RD29A* promoters in *pRD29A::LUC* transgenic plants (Gong et al., 2002). DME is required to prevent silencing of the maternal alleles of paternally imprinted genes including *MEA* and *FIS2*, whose silencing leads to seed abortion (Choi et al., 2002). In general, DNA methylation in the promoter region is associated with transcriptional silencing, although the exact mechanism by which DNA methylation affects transcription remains unclear. DNA methylation, together with other repressive epigenetic modifications, is thought to create a compact chromatin conformation that restricts the access of transcription factors and RNA polymerase II. In addition, DNA methylation directly affects the DNA binding of many transcription factors. Systematic profiling of the binding sites of 327 transcription factor in *Arabidopsis* indicated that the DNA binding activity of over 75% of these transcription factors are sensitive to DNA methylation (O'Malley et al., 2016). The methylation of specific *cis* elements may negatively or positively regulate transcription, depending on whether a transcription factor functions as an activator or repressor.

Among the four ROS1/*DML* genes in *Arabidopsis*, only mutants of *DME* exhibit severe developmental defects (Choi et al., 2002). The seed abortion phenotype of *dme* is attributed to the transient expression of *DME* in the central cell of the female gametophyte (Choi et al., 2002; Park et al., 2016). *DME* is also transiently expressed in the vegetative cell of the male gametophyte, and mutations of *DME* result in decreased pollen viability and germination rates in certain *Arabidopsis* ecotypes (Schoft et al., 2011; Khouider et al., 2021). Thus, DME plays important roles in gametophyte development.

In sporophytic tissues of *Arabidopsis*, *ROS1* is expressed at significantly higher levels compared to *DML2*, *DML3*, and *DME*; *DML2* and *DME* are expressed at moderate levels in most sporophytic tissues but with different expression patterns; *DML3* is minimally expressed in most tissues except for developing anthers (Figure 4A). Higher-order mutants of *ROS1*, *DML2*, and *DML3* exhibit no obvious defects under normal growth conditions. However, ROS1, DML2, and DML3 were shown to regulate the development of some terminally differentiated cell types. For example, the density of stomatal stem cells is 3–4 fold higher in the *ros1* or *ros1 dml2 dml3* (*rdd*) mutants than in the wild type (Yamamuro et al., 2014). This phenotype is very similar to that observed in mutants of *EPIDERMAL PATTERNING FACTOR 2* (*EPF2*), which encodes a cysteine-rich peptide that negatively regulates stomatal development. The promoter region of *EPF2* is hypermethylated in the *ros1* and *rdd* mutants, leading to the silencing of *EPF2* expression (Yamamuro et al., 2014).

ROS1 also plays a role in the differentiation of tracheary elements, which together with parenchyma cells and fibers constitute the xylem tissue. Changes in the DNA methylome during tracheary element differentiation were profiled using an *in vitro* system (Lin et al., 2020). ROS1, DML2, and DML3 are responsible for demethylating thousands of genomic loci, and the hyper-DMRs identified in *rdd* mutants are associated with genes involved in tracheary element differentiation (Lin et al., 2020). The *ros1* and *rdd* mutants exhibited lower tracheary element differentiation rates *in vitro* and impaired xylem development *in vivo* in young roots compared to the wild type (Lin et al., 2020).

Viable quadruple *dml* mutants were created in *Arabidopsis* by restoring *DME* expression in the central cell of the *drdd* (*dme ros1 dml2 dml3*) mutant in two recent studies (Zeng et al., 2021; Williams et al., 2022). *drdd pAGL61::DME* plants exhibited early flowering, which was correlated with hyper-methylation and the reduced expression of the flowering repressor gene *FLOWERING LOCUS C* (*FLC*) (Williams et al., 2022). However, in the other study, early flowering was not observed in the *drdd pDD7::DME* plants (Zeng et al., 2021). The *dme-2* homozygous mutant, which has a 97.1% seed abortion rate but still produces some seeds, also exhibits early flowering (Kim et al., 2021), suggesting that DME is responsible for *FLC* demethylation. When and where DME acts on the FLC promoter remains to be determined.

Depending on the mutant alleles tested and the threshold used for statistical analysis, loss-of-function *ros1* mutants contain approximately 6,000 hyper-DMRs, while *rdd* mutants contain 9,000 to 12,000 hyper-DMRs, and central cell-complemented *drdd* (*dme ros1 dml2 dml3*) mutants contain approximately 14,000 DMRs (Zeng et al., 2021). Among the 6,943 genes with hyper-DMRs in the 2-kb promoter regions, 205 were downregulated and 84 were upregulated in the *drdd pDD7::DME* mutant, indicating that variations in DNA

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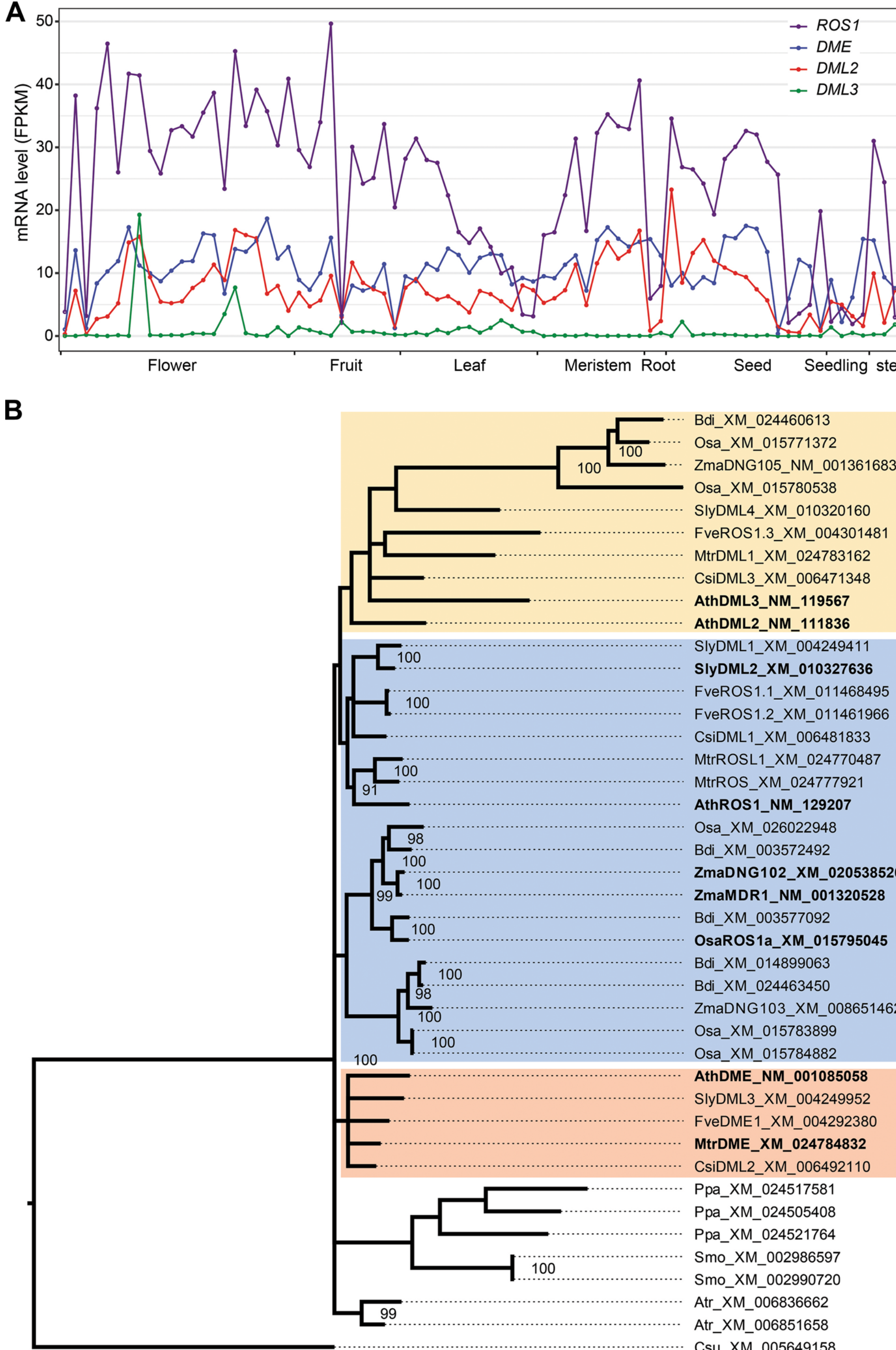

Figure 4. Gene expression patterns and phylogenetic tree of the ROS1 family of DNA glycosylase domain proteins
(A) Expression levels of four *ROS1/DML* genes in various tissues of *Arabidopsis thaliana*. The FPKM (fragments per kilobase per million) values were extracted from (Klepikova et al., 2016). **(B)** Phylogenetic tree of *ROS1/DML* genes. The coding sequences were retrieved from the NCBI RefSeq database, and codon alignment was performed using MUSCLE. Parts of the alignment corresponding to the DNA glycosylase domain were extracted and used to construct the phylogenetic tree using MrBayes. The lineages of ROS1, DML2/3, and DME (Zemach et al., 2010) are respectively highlighted in blue, yellow, and red. The first three letters of sequence names indicate abbreviated species names: Ath, *Arabidopsis thaliana*; Atr, *Amborella trichopoda*; Bdi, *Brachypodium distachyon*; Csi, *Citrus sinensis*; Csu, *Coccomyxa subellipsoidea*; Fve, *Fragaria vesca*; Mtr, *Medicago truncatula*; Osa, *Oryza sativa*; Ppa, *Physcometrilla patens*; Sly, *Solanum lycopersicum*; Smo, *Selaginella moellendorffii*; Zma, *Zea mays*.

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methylation do not necessarily alter the expression of nearby genes (Zeng et al., 2021).

DNA demethylation as a primary driver of imprinting

Genome imprinting is an epigenetic phenomenon in sexually reproducing organisms in which genes exhibit a parent-of-origin-specific expression pattern. Expression of the imprinted allele is repressed. In angiosperms, imprinting mostly occurs in the endosperm (Montgomery and Berger, 2021). During double fertilization, the fusion between the haploid nucleus of a sperm cell with the diploid central cell gives rise to the endosperm, while another sperm cell and the egg form the zygote, which develops into an embryo. Maternal and paternal alleles of most genes in the endosperm are expressed at a 2-to-1 ratio, while the expression of alleles from imprinted genes strongly deviates from this ratio. Transcriptome analysis of dissected endosperm indicated that both *Arabidopsis* and maize contain approximately 100 imprinted genes (Schon and Nodine, 2017; Wyder et al., 2019).

DNA methylation and H3K27me3 are the two main epigenetic marks (imprints) associated with imprinted genes (Batista and Kohler, 2020). Global DNA demethylation is initiated in the central cell in *Arabidopsis* and rice (*Oryza sativa*), resulting in global loss of DNA methylation in the maternal genome before fertilization (Park et al., 2016), while DNA methylation is maintained in the sperm genome (Calarco et al., 2012). The hypomethylated state of the maternal genome is maintained in the endosperm (Hsieh et al., 2009; Gehring et al., 2009a). A significant portion of maternally expressed genes in Arabidopsis, such as *FWA*, *FERTILIZATION INDEPENDENT SEED 2* (*FIS2*), and *MEA*, are flanked by sporophytically methylated regions, whose demethylation in the central cell is required for their activation (Kinoshita et al., 2004; Gehring et al., 2006; Jullien et al., 2006; Kinoshita et al., 2007; Batista and Kohler, 2020). MEA and FIS2, together with (FERTILIZATION INDEPENDENT ENDOSPERM (FIE, also termed FIS3) and MULTICOPY SUPPRESSOR OF IRA 1 (MSI1), form a PRC2 complex that catalyzes the trimethylation of H3K27 in the central cell and endosperm (Luo et al., 2000; Jullien et al., 2008; Mozgova and Hennig, 2015). On the other hand, the sperm undergoes global demethylation of H3K27me3 (Borg et al., 2020). As a result, paternally expressed genes are strongly correlated with asymmetric deposition of H3K27me3 at the maternal alleles (Batista and Kohler, 2020). A significant portion of paternally expressed genes are associated with both DME-dependent DMRs and H3K27me3, and the silencing of their maternal alleles requires DME, suggesting that DNA demethylation of maternal alleles is required for PRC2-mediated H3K27me3 deposition and silencing at these loci (Moreno-Romero et al., 2016; Gehring and Satyaki, 2017; Batista and Kohler, 2020).

Interestingly, ROS1 was found to demethylate the paternally imprinted allele of *DELAY OF GERMINATION1-LIKE 4* (*DOGL4*). The maternal allele of *DOGL4* is preferentially expressed in wild-type endosperm, whereas the paternal allele is partially methylated and its expression is suppressed (Zhu et al., 2018). Loss-of-function *ros1* mutants show an increase in DNA methylation and further silencing of the paternal allele (Zhu et al., 2018). *ros1* seeds exhibit increased dormancy and sensitivity to ABA; these phenotypes are at least partially due to reduced *DOGL4* expression (Zhu et al., 2018). Although when and where ROS1 demethylates the *DOGL4* gene remain to be elucidated, this finding demonstrates that active DNA demethylation can also negatively regulate imprinting.

Many cases of imprinting have been reported in maize, which, like *Arabidopsis*, contains four *ROS1* homologs (Figure 4B). One of them, named *maternal derepression of r1* (*MDR1*), was recently cloned as the causal gene of a mutant exhibiting defects in maternal expression of the imprinted gene *r1*, which gives the kernel a red color (Gent et al., 2022). Among the four *ROS1*-like genes, *MDR1* and its close homolog *DNG102* likely function redundantly and are required for male and female fertility, indicating that they function during gametophyte development (Gent et al., 2022). *MDR1* and *DNG102* are also highly expressed in the endosperm. *MDR1* is responsible for approximately one-third of hypo-DMRs identified in the endosperm compared to the embryo, and maternally expressed genes preferentially overlap with these hypo-DMRs (Gent et al., 2022). Surprisingly, these hypo-DMRs are associated with increased 24-nt siRNA accumulation in the *mdr1* mutant, suggesting that MDR1 is responsible for repressing siRNA expression at its target loci.

Maintenance of genome integrity

DNA methylation-mediated silencing of TEs is critical for the maintenance of genome stability. Global DNA demethylation was observed in the companion cells of male and female gametophytes in *Arabidopsis* and rice (Ibarra et al., 2012; Kim et al., 2019b). This may represent an indirect mechanism to enhance TE silencing of gametes in flowering plants. *DME* was the main 5mC DNA glycosylase gene expressed in vegetative cells of pollen, although weak expression of *ROS1*, *DML2*, and *DML3* was also detected (Schoft et al., 2011). The mutation of *DME* affects pollen germination and paternal transmission of the mutant allele in certain *Arabidopsis* ecotypes (Schoft et al., 2011). Similar to central cells, the vegetative cell undergoes DME-dependent global DNA demethylation (Calarco et al., 2012; Ibarra et al., 2012). Similar genomic regions are targeted by DME in central cells and vegetative cells (Ibarra et al., 2012), and hypomethylation results in the derepression of similar sets of genes and TEs (Gehring et al., 2006; Schoft et al., 2011; Calarco et al., 2012; Ibarra et al., 2012). Approximately 10,000 DME-dependent DMRs are present in companion cells, which is approximately two orders of magnitude higher than the number of imprinted genes, suggesting that establishing genome imprinting is not the main function of demethylation in gametophytes (Ibarra et al., 2012).

The finding that 21-nt siRNAs accumulate in sperm led to the hypothesis that the released silencing of TEs in vegetative cells generates mobile small RNAs that travel to the gamete

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to reinforce TE silencing (Slotkin et al., 2009). Indeed, siRNAs produced from vegetative cell transcripts repressed the expression of reporter genes in sperm cells (Martinez et al., 2016). The production of these 21-nt siRNAs in the pollen grain requires an RNAi pathway that includes AGO1, AGO2, and DCL4 (Martinez et al., 2016). The function of active DNA demethylation in gametophyte development appears to be conserved in rice, which contains six ROS1/DML glycosylases, including four in the ROS1 lineage (Figure 4B). OsROS1a is indispensable for the development of male and female gametophytes in rice (Ono et al., 2012). OsROS1a is required for local hypomethylation in gamete companion cells and indirectly promotes non-CG methylation in the sperm (Kim et al., 2019b), suggesting that it is functionally equivalent to DME in *Arabidopsis*.

DNA demethylation during fruit ripening

Ripening, the process by which fruits mature, is associated with changes in fruit flavor, color, texture, and other properties. Studies during the past decade have revealed extensive epigenetic reprogramming in fleshy fruits during the ripening process, in which DNA methylation-mediated transcriptional regulation plays a central role (Tang et al., 2020). Large-scale epigenome profiling of 11 plant species during fruit ripening found that the DNA methylation level is often negatively correlated with DNA accessibility (Lu et al., 2018).

Whole-genome bisulfite sequencing of tomato (*Solanum lycopersicum*) fruits at different developmental stages indicated that extensive DNA hypomethylation correlates with fruit development in this model fruit (Zhong et al., 2013). Treating tomatoes with the DNA methyltransferase inhibitor 5-Azacytidine (5-Aza-C) resulted in early ripening (Zhong et al., 2013). Among the four ROS1/DML genes in tomato, *SlDML2* is the main gene expressed in the fruit pericarp during ripening (Liu et al., 2015b) (Figure 4B). Reducing the expression or knockout of *SlDML2* was sufficient to inhibit tomato ripening (Liu et al., 2015b; Lang et al., 2017). Over 13,000 hypo-DMRs were identified during tomato fruit ripening, and *SlDML2* is required for DNA hypomethylation in almost all these regions, indicating that SlDML2 is the main enzyme mediating DNA demethylation during the ripening process (Lang et al., 2017). SlDML2-dependent promoter demethylation is strongly associated with the expression of many ripening genes, indicating that active DNA demethylation coordinates their expression (Lang et al., 2017).

Studies from other fleshy fruit species also indicated that DNA methylation is critical for fruit ripening, but how DNA methylation is regulated varies among species. Like tomato, strawberry (*Fragaria vesca*) ripening also correlates with global DNA hypomethylation, and 5-Aza-C treatment caused an early ripening phenotype (Cheng et al., 2018). However, none of the *ROS1* homologs or other genes encoding regulators of demethylation show increased expression during strawberry fruit ripening (Cheng et al., 2018). Instead, *CMT3* homologs and components of the canonical RdDM pathway are downregulated during this process (Cheng et al., 2018). Thus, passive DNA demethylation caused by decreased RdDM activity plays a major role in strawberry ripening.

In contrast to tomato and strawberry, fruit development and ripening in sweet orange (*Citrus sinensis*) is associated with steady increases in global DNA methylation (Huang et al., 2019). Treating sweet orange fruits with 5-Aza-C delayed ripening (Huang et al., 2019). Ripe fruits (210 d after bloom) contained over 30,000 hyper-DMRs across the genome compared to immature fruits (90 d after bloom) (Huang et al., 2019). While all *CsDML* genes were expressed at low levels in fruit during ripening, *CsDRM1* and *CsDRM2* were expressed at higher levels than the other DNA methyltransferase genes by an order of magnitude (Huang et al., 2019), suggesting that constant RdDM and reduced active DNA demethylation contribute to DNA hypermethylation during sweet orange fruit development and ripening.

ACTIVE DNA DEMETHYLATION IN RESPONSE TO EXTERNAL STIMULI

Abiotic stress response and adaptation

Many reviews describe the roles of DNA methylation or epigenetic regulation in plant responses to abiotic stress (Crisp et al., 2016; Chang et al., 2020; Oberkofler et al., 2021; Arora et al., 2022; Liu et al., 2022), including drought (Sadhukhan et al., 2022), high salinity (Singroha et al., 2022), high temperature (Liu et al., 2015a; Perrella et al., 2022), low temperature (Luo and He, 2020; Hereme et al., 2021), and nutrient deficiency (Secco et al., 2017; Fan et al., 2022). Here, we briefly review changes in DNA methylation associated with abiotic stress responses with a focus on the possible roles of active DNA demethylation in these processes.

Early studies examining individual genes found a correlation between DNA methylation and the expression of certain key genes related to abiotic stress responses. For example, the expression of *AtHKT1*, which encodes a sodium transporter located in xylem parenchyma cells, is repressed by a methylated region approximately 2.6 kb upstream of the ATG start codon (Baek et al., 2011). Low relative humidity led to reduced stomatal density, which was correlated with increased DNA methylation levels and reduced expression of two stomatal lineage genes, *SPEECHLESS* (*SPCH*) and *FAMA* (Tricker et al., 2012). DNA methylome profiling of drought- or high salinity-treated plants revealed an increase in overall DNA methylation levels compared to control plants (Colaneri and Jones, 2013; Rutowicz et al., 2015; Wibowo et al., 2016; Ganguly et al., 2017).

Whether abiotic stresses induce consistent changes in DNA methylation remains controversial. While some studies identified good correlations between changes of DNA methylation and changes in the expression levels of specific genes (Tricker et al., 2012; Wibowo et al., 2016), other studies found most DMRs to be stochastic (i.e., the DNA methylation levels of DMRs varied between biological replicates) and thus

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poorly correlated with the expression of stress-responsive genes (Eichten and Springer, 2015; Ganguly et al., 2017). A significant portion of abiotic stress-induced DMRs overlaps with spontaneous variations in DNA methylation identified in the mutation accumulation (MA) lines (Becker et al., 2011; Schmitz et al., 2011; Ganguly et al., 2017), indicating that multiple biological replications must be performed in future methylome sequencing studies. In all these studies, only a small fraction of DMRs were detected in the vicinity of stress-induced differentially expressed genes. Therefore, the functional consequences of most changes in DNA methylation remain unclear. Most drought-induced DMRs in *Arabidopsis* are in the CHH context and correlate with siRNA accumulation (Ganguly et al., 2017; Van Dooren et al., 2020), suggesting that RdDM is responsible for the increased DNA methylation in plants exposed to drought.

A time-course experiment examining the DNA methylome of *Arabidopsis* seedlings under heat treatment (42°C for 24 h) followed by recovery (up to 48 h) found progressively decreased DNA methylation in all sequence contexts (Korotko et al., 2021). Multiple heat stress-inducible genes, such as *HEAT SHOCK PROTEIN 70*, *CBL-INTERACTING SERINE/THREONINE-PROTEIN KINASE 6*, and *OXYGEN-EVOLVING ENHANCER PROTEIN 1-1* exhibited decreased DNA methylation in the gene body (Korotko et al., 2021) (Figure 5). Heat stress significantly increases protein SUMOylation (Yoo et al., 2006). The DNA glycosylase domain of ROS1 can be SUMOylated *in vitro* and directly interacts with SUMO1 *in vivo* (Kong et al., 2020). ROS1 protein levels are significantly reduced in the SUMOylation E3 ligase mutant *siz1*, which contains over 1,000 hyper-DMRs that strongly overlap with those of *ros1* (Kong et al., 2020). Whether heat stress-induced DNA demethylation depends on ROS1 is currently unknown.

Loss-of-function mutants of *ROS1* were examined under various stress conditions. Cold-treated *ros1* mutants exhibited more severely deformed leaves and increased anthocyanin accumulation compared to the wild type (Yang et al., 2022). This phenotype was correlated with cold-induced and ROS1-dependent promoter demethylation in a few stress-responsive

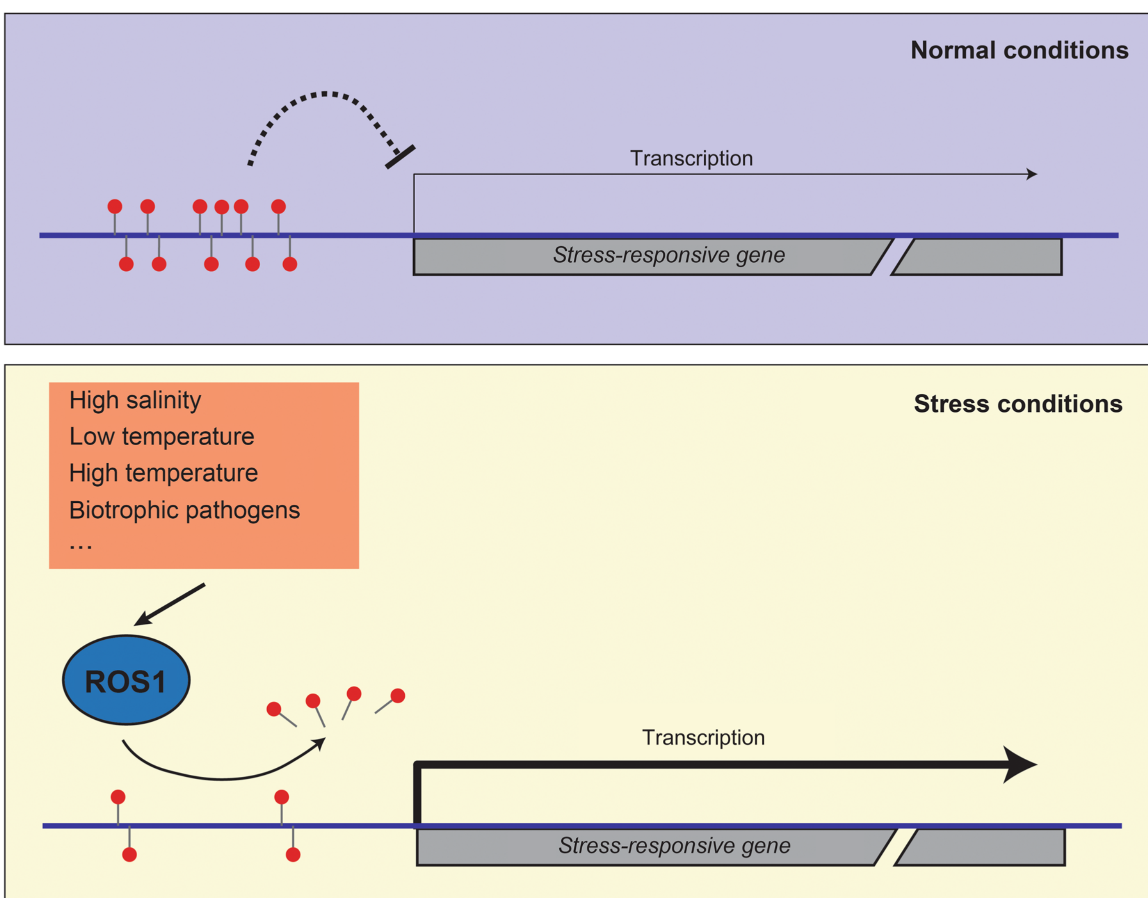

Figure 5. The roles of 5mC DNA glycosylases in plant stress responses
Under most stress conditions, ROS1 and its homologs are recruited to the promoter regions of specific stress-responsive genes to reduce their 5mC levels, which usually leads to increased gene expression. For simplicity, nucleosomes are not illustrated. DNA methylation of the promoter can sometimes result in increased gene expression (e.g., the rice *Pib* gene); certain stresses (e.g., cadmium stress) lead to decreased ROS1 activity. Please see the main text for details. 5mC, 5-methylcytosine.

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genes, including *ACCELERATED CELL DEATH 6*, *ACONITATE HYDRATASE 3*, and *GLUTATHIONE S-TRANSFERASE 14* (Yang et al., 2022) (Figure 5). Heat stress mildly upregulated *ROS1* and genes involved in DNA methylation (*MET1*, *CMT3*, *DRM2*, *NRPD1*, and *NRPE1*) (Naydenov et al., 2015). Cadmium stress significantly reduced the expression of three *ROS1/DML* genes (*ROS1*, *DML2*, and *DML3*) (Fan et al., 2020). Cadmium treatment induced a global increase in DNA methylation in wild-type plants resembling that of non-treated *rdd* mutants; these mutants exhibit reduced cadmium accumulation in roots and enhanced cadmium tolerance compared to the wild type (Fan et al., 2020). During seed germination and seedling growth, the *ros1* mutants exhibit hypersensitivity to ABA (Zhu et al., 2018; Kim et al., 2019a), the main phytohormone that mediates abiotic stress responses in plants.

Despite these associations between changes in the DNA methylome and/or *ROS1/DML* expression levels and abiotic stress treatment, the cause-effect relationship remains largely unclear. Studies on how phosphate starvation affects DNA methylation provided an interesting clue. Low phosphate treatment (5 μM) led to a progressive increase in DNA methylation in both the shoots and roots of *Arabidopsis* seedlings (Yong-Villalobos et al., 2015). This increase correlated with the elevated expression levels of *MET1* and *DRM2* (Yong-Villalobos et al., 2015). Approximately 1,200 and 2,000 DMRs were identified at 7 and 16 days post low-phosphate treatment, respectively, whereas 317 and 421 low-phosphate-related differentially expressed genes were differentially methylated in shoot and root tissues, respectively, including the representative phosphate starvation-responsive gene *SPX DOMAIN-CONTAINING PROTEIN 2* and the microRNA miR827 (Yong-Villalobos et al., 2015). In a separate study, following 10 days of phosphate starvation, no DMR was identified in *Arabidopsis* roots, which was attributed to the different statistical algorithms used (Secco et al., 2015). Using a similar approach, however, DNA methylome analysis of rice roots under long-term phosphate starvation (21 days) identified 175 DMRs, most of which showed increased DNA methylation in the CHH context (Secco et al., 2015). More than half of these DMRs were associated with phosphate-induced genes, whose changes in expression were positively correlated with changes in DNA methylation (Secco et al., 2015). The transcript levels of DMR-associated differentially expressed genes plateaued at 7 days after phosphate starvation, whereas the DNA methylation levels did not reach a plateau until 21 days after treatment, suggesting that variations in DNA methylation were not the reason for the changes in expression of these genes (Secco et al., 2015).

Plants do not set aside a germ cell lineage or experience active DNA demethylation during early embryogenesis as mammals do. Hence it can be hypothesized that DNA methylation being part of a mechanism mediating stress priming and transgenerational memory. Priming refers to a widely observed phenomenon in which exposing plants to mild stress prepares them for improved resistance to subsequent severe stress (Pieterse et al., 2014; Conrath et al., 2015; Hilker and Schmulling, 2019). The nature of stress memory remains an enigma, but epigenetics is one mechanism that is often evoked (Crisp et al., 2016). For example, short-term memory of heat stress (on the order of days) was associated with hyperaccumulation of H3K4me2/3 and altered nucleosome occupancy at genes exhibiting memory behavior (Brzezinka et al., 2016; Lamke et al., 2016). However, no clear-cut examples of transgenerational inheritance of stress-induced epialleles have been reported. Subjecting parental *Arabidopsis* plants to mild drought stress (30% soil water content) failed to confer better resistance to the same stress in filial generations, as determined by measuring relative growth rates (Van Dooren et al., 2020). In another study, repeated drought exposure in ancestral generations resulted in increased seed dormancy in the next two generations but had no effects on drought survival rates (Ganguly et al., 2017). In both studies, few consistent transgenerational changes in DNA methylation were detected (Ganguly et al., 2017; Van Dooren et al., 2020). Priming *Arabidopsis* plants with moderate salinity (25 and 75 mM NaCl) increased plant survival in the next generation under 150 mM NaCl treatment, although the memory lasted for only one generation (Wibowo et al., 2016). The priming effect required both demethylation (ROS1) and non-CG methylation machineries (RdDM and CMT3) (Wibowo et al., 2016) (Figure 5). The progenies of salt-exposed *dme* mutants exhibited higher salt stress resistance compared to the progenies of salt-treated wild-type plants (Wibowo et al., 2016). Analysis following crosses between stressed and non-stressed plants indicated that the paternal DME allele is required for this effect, suggesting that salinity memory is restricted to the male germline (Wibowo et al., 2016).

Plant–microbe interactions

Infection by viral, bacterial, or fungal pathogens can lead to widespread changes in DNA methylation in plants. The RdDM pathway was named based on its function in methylating viral DNA *de novo* (Wassenegger et al., 1994). DNA methylation-deficient *Arabidopsis* plants were hypersusceptible to geminivirus infection (Raja et al., 2008). Different viruses use various counter-defense strategies to downregulate the host DNA methylation machinery (reviewed by Jin et al., 2021). A recent study also found that certain geminiviruses can utilize the host's DNA demethylases to subvert defense. The βC1 protein encoded by the betasatellite of tomato yellow leaf curl China virus interacted with NbROS1L in *Nicotiana benthamiana* and with DME in *Arabidopsis* (Gui et al., 2022). βC1 facilitates the enzymatic activity of DME *in vitro* and *in vivo*, presumably by demethylating the viral DNA and increasing viral virulence (Gui et al., 2022).

Several studies using (hemi)biotrophic pathogens indicated that active DNA demethylation positively regulates plant basal resistance against these pathogens (Figure 5). Biotrophic pathogens take up nutrients from living plant cells during biotrophic growth. *Arabidopsis* mutants defective in

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DNA methylation exhibit hyper-resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), whereas the *ros1* mutant is more susceptible to *Pst* than the wild type (Dowen et al., 2012; Yu et al., 2013). Similar results were reported for fungal pathogens including *Fusarium oxysporum* (*Fo*) and *Hyaloperonospora arabidopsidis* (*Hpa*) (Le et al., 2014; Lopez Sanchez et al., 2016). Priming with flg22 (a peptide derived from bacterial flagellin) before pathogen inoculation improved resistance to *Pst* in wild-type plants, but this effect was compromised in *hdp2*, *idm1*, *ros1*, *dml2*, *dml3*, and *rdd* (Huang et al., 2022). *Pst* infection or treatment with the defense-related hormone salicylic acid resulted in approximately 1,500 and 4,000 DMRs in *Arabidopsis*, respectively. Many of these DMRs were located in the vicinity of differentially expressed genes and were negatively correlated with changes in mRNA levels (Dowen et al., 2012).

A time-series methylome analysis of flg22-elicited *Arabidopsis* seedlings indicated that flg22 induced hypomethylation at thousands of genomic loci within 1 h of treatment (Huang et al., 2022). This flg22-induced methylome reprogramming largely depended on *ROS1*, *DML2* and *DML3* (Huang et al., 2022). More than half of flg22-induced DMRs are mainly associated with promoter and coding regions of genes (Huang et al., 2022). Significantly fewer upregulated genes were detected in *rdd* mutants than the wild type at later time points (3 and 24 h post flg22 treatment) (Huang et al., 2022). The induction of salicylic acid signaling-related genes and phytoalexin biosynthesis related-genes, including *PR1* (*PATHOGENESIS-RELATED GENE 1*), is compromised in *ros1* and *rdd* mutants (Yu et al., 2013; Huang et al., 2022). Defense genes demethylated by ROS1 include *RESISTANCE METHYLATED GENE 1* (*RMG1*), encoding a NB-LRR disease resistance protein, and *RECEPTOR LIKE PROTEIN 43* (*RLP43*), encoding an orphan immune receptor (Yu et al., 2013; Halter et al., 2021). Artificial siRNA-directed DNA methylation at the ROS1-dependent hypomethylated regions of *RMG1* and *RLP43* was sufficient to repress flg22-induced gene expression and reduce plant basal resistance to the same level as *ros1* (Halter et al., 2021). ROS1-mediated demethylation likely opens binding sites for WRKY transcription factors, whose binding to DNA is generally sensitive to 5mC (Halter et al., 2021).

DME also plays a role in promoting defense in the somatic tissues of *Arabidopsis*. Two studies compared the pathogen resistance of the *drdd* (*dme ros1 dml2 dml3*) mutant to that of the *rdd* (*ros1 dml2 dml3*) mutant. Knocking down *DME* expression in sporophytic tissues of *rdd* mutants increased plant susceptibility to the fungal pathogen *Fo* (Schumann et al., 2019). Somatic mutants of *DME* were created by expressing *DME* under the control of the central cell-specific promoter *DD7* (Zeng et al., 2021). The *drdd pDD7::DME* plants were more susceptible to both *Pst* and the fungal pathogen *Verticillium dahliae* compared to *rdd* (Zeng et al., 2021). The *dme pDD7::DME* mutant was also more susceptible to these pathogens than *rdd*, indicating that *DME* plays a significant role in plant defense in vegetative tissues (Zeng et al., 2021).

A few cases in which DNA demethylation plays a negative role in pathogen resistance have also been reported. In rice, 5-Aza-C treatment compromised plant resistance against *Magnaporthe grisea*, the pathogen causing blast disease (Li et al., 2011). This was attributed to reduced DNA methylation at the *Pib* promoter and the reduced mRNA level of this gene, the first cloned resistance (R) gene for rice blast (Li et al., 2011). The *Arabidopsis ros1* mutant displayed enhanced resistance to the necrotrophic pathogen *Plectosphaerella cucumerina*, while the *nrpe1* mutant (defective in RdDM) was more susceptible to this pathogen than the wild type (Lopez Sanchez et al., 2016).

A few studies explored the potential function of DNA methylation in transgenerational acquired resistance. Plants inoculated with *Pst* in an earlier generation showed increased resistance to *Pst* or *Hpa* in the next generation (Luna et al., 2012). This effect appears to depend on ROS1-mediated DNA demethylation because *nrpe1* mutants, but not *ros1* mutants, exhibit transgenerational acquired resistance (Lopez Sanchez et al., 2016).

Active DNA demethylation is also important for plant interactions with avirulent or beneficial microbes. Avirulent *P. syringae* strains induced twofold more DMRs than the pathogen *Pst* (Dowen et al., 2012), suggesting that DNA methylation is generally involved in plant–microbe interactions. *Bacillus megaterium* strain YC4 is a beneficial rhizobacterium that promotes the growth of multiple plant species, including *Arabidopsis* and tomato. Root-secreted myo-inositol is required for YC4 colonization and attracts other beneficial microbes (Vilchez et al., 2020). The growth-promoting effect of YC4 is abolished in *Arabidopsis rdd* mutants and tomato *sldml2* mutants (Vilchez et al., 2020). ROS1-mediated DNA demethylation counteracts RdDM at genes involved in *myo*-inositol homeostasis such as *FAR-RED IMPAIRED RESPONSE1* and *FAR-RED ELONGATED HYPOCOTYL3* (Vilchez et al., 2020). As a result, exudates of *rdd* roots contained 16-fold less *myo*-inositol than the wild type (Vilchez et al., 2020). The plant growth-promoting bacteria PGP5 (*Bacillus* sp.) and PGP41 (*Arthrobacter* sp.) also promote plant growth in a DNA methylation-dependent manner (Chen et al., 2022). Inoculation with either bacterium resulted in the increased expression of DNA methyltransferase and DNA demethylase genes, suggesting that both enzymes are involved in methylome reprogramming (Chen et al., 2022). DNA methylation has a long-term growth-promoting effect even after the inoculum has been removed from the microbiome (Chen et al., 2022).

In *Medicago truncatula*, laser capture microdissection followed by transcriptome analysis found that the *DEMETER* ortholog *MtrDME4* is highly expressed in the late differentiation zone of the root nodule (Satge et al., 2016). Knock-down of *MtrDME* resulted in the hypermethylation and downregulation of approximately 400 genes, most of which are involved in nodule differentiation (Satge et al., 2016). Laser capture microdissection coupled with whole genome bisulfite sequencing further revealed an increase in CHH methylation in the differentiation zone and fixation zone (Pecrix et al., 2022). While the nodule number was not significantly affected in *MtrDRM2*

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knockout plants, the mutant nodules exhibited defects in differentiation and nitrogenase activity (Pecrix et al., 2022). These findings indicate that both MtrDME-mediated active demethylation and RdDM-mediated *de novo* methylation are important for transcriptional reprogramming during nodule development in Medicago.

SUMMARY AND PERSPECTIVES

Since the discovery of *Arabidopsis* ROS1 20 years ago (Gong et al., 2002), our understanding of active DNA demethylation in plants has greatly improved. Except for the DNA polymerase, all BER enzymes involved in active DNA demethylation have been identified. Mammals also utilize the BER pathway for 5mC demethylation, representing a case of convergent evolution, but they utilize TET dioxygenases to oxidize 5mC before a monofunctional DNA glycosylase initiates BER (Wu and Zhang, 2017). The discovery of the MEMS uncovered a simple yet important mechanism that adjusts demethylation activity in response to methylation levels (Lei et al., 2015; Williams et al., 2015). Multiple protein complexes that act upstream of or together with ROS1 or DME have been identified. Besides methyl-DNA binding modules, protein components of these complexes also harbor histone-binding domains and transcription factor-like domains, suggesting that a combination of chromatin features determine the targeting of DNA demethylase proteins. Despite these advances, our understanding of the targeting mechanisms of DNA demethylases during growth and development and in response to endogenous and external stimuli is still far from clear.

During the past decade, we also saw a burst of reports on novel biological functions of active DNA demethylation, primarily in crops. Different plant genomes contain varying contents and distributions of repetitive sequences, which are typically methylated and are preferential targets of DNA demethylases. TE proliferation is a major driving force in plant genome evolution and many crops have TE-rich genomes. TEs and repetitive sequences surrounding genes provide sources for epigenetic regulation of gene expression. As genome editing technology is utilized in more plant species, additional DNA demethylase mutants will be created. We expect that more examples of the diverse functions of active DNA demethylation in plants will be provided in the near future.

The combination of CRISPR/Cas technology and DNA demethylases provides a valuable tool for epigenome editing (Zhan et al., 2021). By fusing catalytically inactive Cas proteins (e.g., dCas9) with the DNA glycosylase domain of ROS1, DNA demethylase activity can be targeted to demethylate specific sequences in cells including mammalian cells (Devesa-Guerra et al., 2020). This tool will benefit not only basic research in epigenetics, but also crop breeding and human health. Specific methylated regions of the genome can be targeted for demethylation and the inheritance of demethylated states examined in different mutant backgrounds, thus providing opportunities to interrogate mechanisms underlying the establishment and stability of specific epialleles (Li et al., 2020). In plants, stably inherited natural epialleles have been shown to control important agronomic traits (Luo et al., 1996; Manning et al., 2006; Ong-Abdullah et al., 2015). This tool can be used to create variations in DNA methylation in the genomes of various crops, providing an additional layer of genetic diversity (Gallusci et al., 2017).

In humans, the DNA methylation status of specific genomic regions is tightly correlated with aging and the development of various cancers (Hannum et al., 2013; Hao et al., 2017); indeed, chemical inhibitors of DNA methyltransferases, such as 5-Aza-C, are widely used for cancer treatment. Besides 5mC, plant ROS1/DML proteins also exhibit excision activity against the 5mC oxidation products 5-hydroxymethylcytosine and 5-carboxylcytosine (Brooks et al., 2014). Unlike TET-TDG proteins, plant 5mC DNA glycosylase does not generate these intermediate products, hence minimizing the chances of unintentional gene regulation. This tool could hence be used to demethylate abnormally methylated regions in aging and cancer cells to achieve a more targeted loss of methylation compared to commonly used hypomethylating chemicals, potentially revolutionizing the field of cancer treatment and aging intervention.

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CONFLICTS OF INTEREST

The authors declare no competing interests.

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