Roles of DEMETER in regulating DNA methylation in vegetative tissues and pathogen resistance

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ABSTRACT

DNA methylation is an epigenetic mark important for genome stability and gene expression. In Arabidopsis thaliana, the 5-methylcytosine DNA glycosylase/demethylase DEMETER (DME) controls active DNA demethylation during the reproductive stage; however, the lethality of loss-of-function dme mutations has made it difficult to assess DME function in vegetative tissues. Here, we edited DME using clustered regularly interspaced short palindromic repeats (CRISPR) /CRISPR-associated protein 9 and created three weak dme mutants that produced a few viable seeds. We also performed central cell-specific complementation in a strong dme mutant and combined this line with mutations in the other three Arabidopsis demethylase genes to generate the dme ros1 dml2 dml3 (drdd) quadruple mutant. A DNA methylome analysis showed that DME is required for DNA demethylation at hundreds of genomic regions in vegetative tissues. A transcriptome analysis of the drdd mutant revealed that DME and the other three demethylases are important for plant responses to biotic and abiotic stresses in vegetative tissues. Despite the limited role of DME in regulating DNA methylation in vegetative tissues, the dme mutants showed increased susceptibility to bacterial and fungal pathogens. Our study highlights the important functions of DME in vegetative tissues and provides valuable genetic tools for future investigations of DNA demethylation in plants.

Keywords: active DNA demethylation, Arabidopsis, disease resistance, DME, DNA methylation

INTRODUCTION

DNA methylation at cytosine residues is an important and conserved epigenetic modification in many eukaryotes, including plants, and is associated with the suppression of transposable elements (TEs) and the regulation of gene expression (Zhu, 2009; Zhang and Zhu, 2012; Zhang et al., 2018; Liu and Lang, 2020). In plants, DNA methylation occurs in three sequence contexts: CG, CHG, and CHH (H represents A, T, or C). In Arabidopsis thaliana, CG and CHG methylation is maintained by DNA METHYLTRANSFERASE 1 (MET1) and CHROMOME-THYLASE 3 (CMT3), respectively. Depending on the chromatin

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Active DNA demethylation and pathogen resistance by DME context, CHH methylation is maintained by CMT2 or by DOMAIN REARRANGED METHYLTRANSFERASES (DRM1 and DRM2) through the RNA-directed DNA methylation pathway, which is also responsible for de novo DNA methylation.

DNA methylation levels are determined through the balanced regulation of establishment, maintenance, and removal activities. DNA methylation can be passively lost due to defective maintenance or can be actively removed through enzymatic reactions. In plants, active DNA demethylation is initiated by the 5-methylcytosine DNA glycosylase enzymes. There are four 5-methylcytosine DNA glycosylase genes in Arabidopsis: REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DEMETER-Like 2 (DML2), and DML3 (Penterman et al., 2007). The roles of ROS1, DML2, and DML3 in vegetative tissues have been characterized; in contrast, not much is known about DME function in vegetative tissues due to the seed-aborton phenotype of the dme loss-of-function mutants (Choi et al., 2002). DME is expressed in the central cell of the female gametophyte and is required for gene imprinting in the endosperm (Choi et al., 2002). A recent study indicated that DME is expressed not only in the central cell but also in vegetative tissues (Park et al., 2017; Schumann et al., 2019). During the preparation of this manuscript, Schumann et al. (2019) reported using a β-glucorinidase (GUS) reporter gene and quantitative real-time polymerase chain reaction (qRT-PCR) to show that DME is expressed in vegetative tissues, and found that Arabidopsis plants with a RNA interference (RNAi)‐mediated knockdown of DME expression showed increased susceptibility to the fungal pathogen Fusarium oxysporum. However, the lack of stable and viable genetic dme mutants in that study hindered the analysis of the genetic function of DME in regulating the DNA methylome, gene expression, and responses to the environment.

In this study, we generated stable genetic materials for studying DME function in vegetative tissues. These genetic materials included three weak dme mutants and central cell-specific DME complementation mutant lines from which quadruple DNA demethylase mutants were subsequently obtained. The analyses of the transcriptomes and DNA methylomes in the dme mutants indicated important roles for DME in regulating genomic DNA methylation in vegetative tissues, particularly in genes related to biotic stress responses. We show that DME plays a key role in resistance against the fungal pathogen Verticillium dahliae, a finding consistent with Schumann et al. (2019), and against the bacterial pathogen Pseudomonas syringae pv. tomato (Pst).

RESULTS

DME expression in vegetative tissues
DME was originally thought to be specifically expressed in the central cell to control gene imprinting (Choi et al., 2002); however, public Arabidopsis transcriptome databases (AtGenExpress Visualization Tool, eFP Browser, and TraVA) indicate that DME is also constitutively expressed in Arabidopsis vegetative tissues (Figure S1A) (Schmid et al., 2005; Mathieu et al., 2007; Kleipkova et al., 2016). Using a GUS reporter gene and qRT-PCR, two groups have found that the DME promoter has transcriptional activity in somatic cells (Park et al., 2017; Schumann et al., 2019). We also performed qRT-PCR to assess the expression of all DNA demethylases including DME in leaf tissues of wild-type Arabidopsis (Col-0 accession), as well as some DNA demethylase and methyltransferase mutants (Figure S1B). Our results provide further evidence that DME is expressed in the leaf tissues (Figure S1B). As previously reported, ROS1 expression was downregulated in the DNA methyltransferase mutants (Huettel et al., 2007; Mathieu et al., 2007; Martinez-Macias Maria et al., 2012; Lei et al., 2015). Although DME expression was previously reported to also be suppressed in met1 and drm2 mutants (Mathieu et al., 2007), we found that the DME expression level was not altered in these mutant backgrounds under our experimental conditions (Figure S1B). These results support that DME is expressed in vegetative tissues, and suggest that unlike ROS1, DME expression is not responsive to genomic DNA methylation status.

Generation of dme mutants
A complete loss of DME function causes maternal mortality, which has hindered investigations of DME functions (Choi et al., 2002; Tsuzuki et al., 2014; Yang et al., 2016). To analyze the function of this 5-methylcytosine DNA glycosylase in vegetative tissues, we first generated weak dme mutants using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9). In the three weak dme mutants generated, the mutations were located in the last exon to mitigate nonsense-mediated messenger RNA decay (Figure 1A). The first mutant allele, named dme-A-Del, contains a single adenine deletion that results in a premature stop codon (Figure 1B). The second allele, dme-T-In, contains a thymine insertion that generates extra peptide sequences (Figure 1B). The third mutant allele, dme-3-In, contains a single-nucleotide deletion and a four-base insertion that cause a one-amino-acid substitution and a one-amino-acid insertion (Figure 1B). Although these three weak-allele mutants showed a strong seed-aborton phenotype, a few seeds survived and could germinate (Figure 1C, D). The dme-A-Del mutant showed the strongest seed-aborton phenotype, producing almost no seeds (Figure 1D). The dme-3-In mutant showed the weakest seed-aborton phenotype (Figure 1D). In these dme weak-allele mutants, there were no morphological phenotypes other than seed aborton. An analysis of the gene expression of the other DNA demethylases in these mutants revealed that the expression of DML2 and DML3 was not changed, whereas ROS1 expression was slightly elevated in the strongest dme-A Del mutant (Figure S2).

According to a previous report, the DNA demethylases ROS1, DML2, and DML3 are functionally redundant in Arabidopsis (Penterman et al., 2007). To analyze DME function, we attempted to generate a dme ros1 dml2 dml3 (drdd) quadruple mutation in the ros1 dml2 dml3 (rdd-2) triple mutant background but failed because of embryo lethality.
This result may indicate that, in addition to DME, the other DNA demethylases are required for normal embryo development. We therefore altered our strategy and generated central cell-specific complementation lines in the drdd mutant background, which harbors a strong dme mutant allele in addition to the rdd-2 triple mutations in the Col-0 background (Yamamuro et al., 2014). This rdd-2 mutant is different from the previously reported rdd triple mutant (Penterman et al., 2007), which harbored six-times-backcrossed ros1-3 and dml2-1 alleles in the Ws-0 background.

First, we generated a strong dme mutant using CRISPR/Cas9 to target the first exon of DME (Figure 1A). The generated mutant contained one T insertion, which resulted in a premature stop codon (Figure 1E). This mutant had a strong seed- abortion phenotype, and no homozygous mutant was obtained, consistent with it being a strong dme allele. The seed-abortation phenotype in the dme mutants is caused by a loss-of-function in the gametogenesis stage, resulting in abnormal embryo development (Choi et al., 2002). To generate a viable dme mutant for studying DME in vegetative tissues, we therefore specifically...
expressed DME in the central cell using the DD7 and DME + 395 promoters. The DD7 promoter controls gene expression in a central cell-specific manner (Steven et al., 2007), and the +395 to +1967 sequence of the DME 5’ untranslated region (UTR) contributes to the strong expression of DME in the central cell (Park et al., 2017). These two promoters were independently fused with the full-length genomic sequence of DME (ATG to TAA) (Figure 1F), and the constructs were transformed into the heterozygous strong dme mutant. We then screened for homoygous strong mutant lines with the DME functional complementation in a central cell-specific manner. Transgenic DME expression was analyzed in T1 generation leaves using qRT-PCR, and transgenic lines with an undetectable level of DME transcripts in the vegetative tissues were then crossed with rdd-2 to obtain dme<sup>DD7 pro</sup> and dme<sup>dmeDD7 pro</sup>, which are quadruple mutants with central cell-specific DME complementation. Morphological defects and seed-abortion phenotypes were not observed in the dme<sup>DD7 pro</sup> single mutant or in the dme<sup>DD7 pro</sup> or dme<sup>dmeDD7 pro</sup> quadruple mutants.

**Pruning of DNA methylation by DME in vegetative tissues**

To determine the function of DME in vegetative tissues, we performed whole-genome bisulfite sequencing of 10-d old seedlings of the three dme weak-allele and dme<sup>DD7 pro</sup> single mutants. Most samples were represented by three replicates, and the average depth of coverage for each replicate was >10. We also used the rdd-2 triple mutant as a control. Using a method based on Fisher’s exact test, we identified 612, 870, 561, and 12,210 hypermethylated differentially methylated regions (hyper-DMRs) in dme<sup>DD7 pro</sup> single and rdd-2 mutants, respectively (Figure 2A). As a comparison, 6,902 hyper-DMRs and 495 hypo-DMRs were previously reported in the ros1 mutation (Tang et al., 2016). These results indicate that DME plays a role in DNA demethylation in vegetative tissues, although this role is minor compared with that of ROS1. Approximately 26.0%–52.4% of the hyper-DMRs in dme weak-allele mutants overlapped with those in rdd-2 (Figure 2A). Further examination of the methylation levels in CG, CHG, and CHH contexts revealed that, at these hyper-DMRs in the dme single mutants, the hypomethylation mainly occurs in CG and CHG contexts, with less change in CHH methylation (Figures 2B, S3A). A heatmap of DNA methylation also indicated that some hyper-DMRs in the dme single mutants overlapped with those in rdd-2, while some may be specific to each mutant (Figure S3B).
Figure 3. Continued

A

B

C

drgd\textsuperscript{207} pro hyper

C

D

H3K18Ac

H3K27me1

H3K27me3

H3K36me2

H3K36me3

H3K4me2

H3K4me3

H3K9Ac

H3K9me2

Figure 3. Continued
Active DNA demethylation and pathogen resistance by DME

To determine which histone marks are associated with DME targets, we used the publicly available data from the Gene Expression Omnibus (Accession No. GSE28398) (Luo et al., 2013), and analyzed histone features as previously described (Tang et al., 2016). The DME targets showed a statistically significant negative association with most of the active histone marks, such as H3K36 trimethylation (H3K36me3), H3K4me2/3, and H3K9 acetylation (H3K9ac), compared with the control regions (randomly selected genomic regions with the same length distribution as the DMRs). The negative correlation was greater in drm weak-allele mutants, especially in dme-T-In and dme-A-De1, than in rdd-2 (Figure 2C).

Using the same analysis pipeline, we identified 11 803 hyper-DMRs in drdD^395 pro and 13 948 in drdD^D7 pro. More than 60% of these hyper-DMRs (64.6% for drdD^395 pro and 62.3% for drdD^D7 pro) overlapped with the hyper-DMRs in rdd-2 (Figure 3A), while 35.4%–37.7% of the hyper-DMRs appeared to be specific to the drdd quadruple mutants (Figure 3A). The distribution of hyper-DMRs in the drdd quadruple mutants was similar to that in rdd-2, although the distribution ratio was slightly decreased in the gene body in both drdd quadruple mutants (Figure 3B). At the hyper-DMRs of drdD^D7 pro mutant, an increase in DNA methylation in all cytosine contexts was also observed in rdd-2, and a higher increase was observed in the other drdd quadruple mutant (Figures 3C, S4A). A heatmap analysis showed that DNA methylation levels at some genetic loci were increased in the drdd quadruple mutants but not in rdd-2 (Figure S4B). Because ROS1-targeted TEs tend to be located near genes (Tang et al., 2016), we also analyzed the distance between the TEs and genes, and found that both dme- and drdd-affected TEs were closer to genes compared with the unaffected TEs (Figure S4C). Together, these results indicate that the four DNA demethylases have functional redundancy, and that DME has a role in regulating DNA methylation in Arabidopsis vegetative tissues.

We next determined which histone modifications may be associated with hyper-DMRs in drdd quadruple mutants. The drdd and rdd-2 hyper-DMRs were positively associated with the repressive histone mark H3K27me3 and the active histone mark H3K18ac, but were negatively associated with the active histone marks H3K36me3, H3K36me2, H3K4me2/3, and H3K9ac, in contrast to the control regions (Figure 3D). In addition, the drdd and rdd-2 hyper-DMRs were negatively associated with the repressive histone modifications H3K9me2 and H3K27me1, unlike the control regions (Figure 3D). In these associations with histone modifications, the hyper-DMR peaks in drdD quadruple mutants largely behaved like those in rdd-2 (Figure 3D), which is consistent with the predominant role of ROS1 in regulating DNA methylation in vegetative tissues. By contrast, the positive association between hyper-DMRs and H3K27me3 and the negative associations between hyper-DMRs and H3K36me3, H3K4me2, and H3K36me2 are enhanced in the drdd quadruple mutants compared with the rdd-2 mutant (Figure 3D). These results indicate that DME has distinct genomic targets in vegetative tissues.

**DME regulates gene expression in vegetative tissues**

The distribution of hyper-DMRs in drdD^D7 pro is shown in Figure 4A, with approximately 21.6% mapped to promoter regions. Both DME- and DRDD-targeted TEs are located near genes (Figure S4C). In the central cell, DME regulates the expression of genes, such as MEA and FWA, through the DNA demethylation of their regulatory regions (Choi et al., 2002; Gehring et al., 2006). Researchers have also found that the expression of many genes was altered in ros1 and rdd mutant seedlings (Zhu et al., 2007; Lister et al., 2008; Stroud et al., 2013). Those results indicate that DME might also participate in the regulation of gene expression in vegetative tissues. We therefore performed a transcriptome analysis of the dmeD^D7 pro, drdD^395 pro, and drdD^D7 pro mutants, including rdd-2 as a control, to determine whether DME regulates gene expression in vegetative tissues. The expression of many genes was decreased in the quadruple mutants, while the single mutant also displayed a decreased expression of some genes. The drdD^395 pro and drdD^D7 pro mutants shared 208 downregulated differentially expressed genes (DEGs), which represented 38.4% of the down-regulated DEGs in drdD^395 pro and 35.6% of the down-regulated DEGs in drdD^D7 pro (Figure S5A). A total of 245 genes were downregulated in rdd-2, a number lower than in the quadruple mutants. There were a large number of genes with decreased expression in the drdd quadruple mutants and in the dmeD^D7 pro single mutant, but their expression was not substantially altered in rdd-2 (Figure S5B). These results indicate that DME is required for the expression of a large number of genes in vegetative tissues.

To analyze the relationship between DNA methylation and gene expression, we first identified 6 943 genes with hyper-DMRs in the promoter region in drdd, as revealed from the hyper-methylation data. We then analyzed the expression levels of these genes, and found that while the expression of
Figure 4. Continued
the majority of these genes with promoter hyper-DMRs (6,654) was not changed, the expression levels of 84 of these genes were increased, while 205 had decreased expression levels. Of the 205 downregulated genes, 111 were mainly regulated by DME since their expression decreased in dmeDD7 pro but not rdd-2, and 42 were redundantly regulated by the demethylases because they displayed a very strong downregulation in the quadruple mutants only (Figure 4B).

The DNA methylation levels of the promoter regions of the 205 downregulated DEGs in the drddDD7 pro mutant were elevated relative to Col-0, especially in the CG context (Figure 4C). There appeared to be a decrease in CHG methylation in these downregulated DEGs, especially at their promoters and downstream regions, even though the total cytosine methylation was increased (Figure 4C). These results show a strong correlation between DNA hypermethylation at the promoter regions and the downregulation of gene expression in the drdd mutants, indicating that active DNA demethylation is critical for the expression of these genes.

We performed a Gene Ontology (GO) analysis of the 205 downregulated DEGs associated with hyper-DMRs in drddDD7 pro. These downregulated genes were highly enriched in the GO terms “response to stress,” “defense response,” “response to stimulus,” and “response to bacterium” (Figure 4D). Our analysis therefore suggests that demethylase-dependent DNA demethylation may contribute to biotic and abiotic stress responses in vegetative tissues.

**Susceptibility of dme mutants to the bacterial pathogen Pst DC3000**

The methylome and transcriptome analyses suggested that the DNA demethylases, including DME, might be involved in biotic
stress responses (Figure 4D). We therefore determined the effects of the bacterial pathogen Pst DC3000 on the dme weak-allele mutant and the central cell-specific complementation dme and drdd mutants. In response to Pst DC3000 inoculation, all dme and drdd mutants showed a hypersensitive phenotype, including severe water-soaked lesions and chlorosis, compared with that of rdd-2 and Col-0 (Figures 5A, S6A). A hypersensitive phenotype was also observed in a plate assay using Pst DC3000 and detached leaves of the dme mutants (Figure S6B). Measurements of the bacterial titer in infected plants confirmed that the drdd quadruple mutants were more susceptible than the control Col-0 or rdd-2, and the bacterial count was also greater in the dme single mutant than in the rdd-2 mutant at 4 d post-infection (dpi) (Figures 5B, S6C). The expression of a disease-response marker gene, PATHOGENESIS-RELATED PROTEIN 5 (PR5), was suppressed in the dme and drdd mutants (Figures 5C, S6D), although the methylation level of the PR5 locus was not altered in the mutants. Further, a strong correlation was observed between the increased bacterial count and reduced PR5 expression in the dme and drdd mutants (Figures 5B, C, S6C, D). Although ROS1 and RDD have been reported to be important for plant defense responses in Arabidopsis (Yu et al., 2013; Le et al., 2014; López Sánchez et al., 2016), rdd-2 did not show a great sensitivity to Pst DC3000 infection under our experimental conditions, even though PR5 expression was lower in rdd-2 than in Col-0 (Figures 5C, S6D). This result indicates that the four DNA demethylases redundantly regulate plant resistance to bacterial infection in vegetative tissues, and that DME is more important than the other three DNA demethylases for disease resistance.

The treatment of Arabidopsis with the bacterial flagellin-derived peptide flg22 was shown to induce the down-regulation of ROS1 expression in the early stages of infection (Yu et al., 2013); therefore, we explored whether DME expression was altered by a flg22 treatment in Col-0. Under our experimental conditions, the expression levels of DME and ROS1 in Col-0 were not greatly affected by the flg22 treatment (Figure S6E, F).
Figure 7. Expression analysis for defense-related genes in *Pst* DC3000-infected *dme<sup>DD7</sup> pro* and *drdd* quadruple mutants

(A–C) The expression levels of three hyper-differentially methylated region (hyper-DMR)-associated downregulated defense-response genes (At1g17600 (SOC3); A), encoding a Toll-interleukin-1 receptor (TIR)-NB-leucine-rich repeat (TNL) protein; At1g72890 (B), encoding a TIR-NBS-LRR class disease resistance protein; and At3g49120 (C), encoding the class III peroxidase *PERX34*) were analyzed in *rdd*<sup>-2</sup>, the central cell-specific complementation *dme* single mutant, and *drdd* quadruple mutants with Col-0 as a control. The data shown are means ± SEM from three experiments (**P < 0.01, one-tailed Student's t-test). 

(D–F) Integrative Genomics Viewer (IGV) images showing the methylation status for three defense-related genes in *dme* single and *drdd* quadruple mutants. DNA methylation levels of cytosines are indicated by the heights of the vertical bars on each track. Biological replicates are shown in the same color. 

(G–I) Quantitative Chop polymerase chain reaction for the promoters of three defense-related genes in *dme* single and *drdd* quadruple mutants. DNA digested using the cytosine methylation-sensitive restriction enzymes *Hpy*99I, *AciI*, and *Hpy*188III was used as the template. The data shown are means ± SEM from three replicates (**P < 0.01, one-tailed Student’s t-test).
Active DNA demethylation and pathogen resistance by DME (Figure 7A–C) and in the dme weak-allele mutants (Figure S8) when their leaves were infected with Pst DC3000. In plants without pathogen infection, the DNA methylation at these three loci was increased in not only the quadruple mutants but also the dme single mutant, although the increases were not large (Figure 7D–F). We performed a real-time quantitative Chop-PCR (qChop-PCR) to analyze whether the DNA methylation levels at the promoter regions of the defense-related genes may be increased in the mutants when the plants are infected by Pst DC3000. The results showed that DNA methylation levels at these loci were significantly increased in pathogen-infected demethylase mutants, especially in the dme single and quadruple mutants, compared with the wild type (Figure 7G, H). These results clearly show that DME plays an important role in the regulation of the expression of defense-related genes and in pathogen resistance (Figure S9).

**DISCUSSION**

DME was originally thought to be specifically expressed in the central cell and the progenitor of the endosperm, where it controls the imprinting of genes such as FWA and MEA (Choi et al., 2002). However, public gene expression databases and recent publications now indicate that DME is ubiquitously expressed in various Arabidopsis organs and tissues (Schmidt et al., 2005; Mathieu et al., 2007; Klepkova et al., 2016; Park et al., 2017; Schumann et al., 2019). Using RNAi-mediated knockdown, Schumann et al. (2019) recently showed that DME expression in vegetative tissues contributes to the resistance against the fungal pathogen F. oxysporum. We also attempted to generate DME RNAi transgenic plants using a strong constitutive 35 S promoter to drive the expression of trigger hairpin double-stranded RNA (dsRNA), but we could not obtain any RNAi-mediated knockdown plants. Perhaps the 35 S promoter was active in the central cell and/or the vegetative cell of the pollen. Schumann et al. (2019) used a green tissue-specific Arabidopsis Rubisco small subunit (SSU) gene promoter to drive the expression of the trigger hairpin dsRNA. They obtained fully fertile but strongly downregulated DME RNAi lines in Col-0 and rdd backgrounds, on which they performed fungal infection assays. Our dme weak-allele mutants, but not our central cell-specific complementation lines, displayed a strong seed-abortion phenotype. Schumann et al. (2019) found that DME RNAi lines in the Col-0 background had greater resistance against F. oxysporum and less suppression of defense-related genes compared with the rdd mutant. In our study, both dme weak-allele mutants and central cell-specific complementation single mutants showed a hypersensitive phenotype against a bacterial pathogen and a fungal pathogen, with a stronger suppression of defense-related gene expression compared with rdd-2. These differences in phenotype between the current study and that of Schumann et al. (2019) are likely due to differences in the severity of the functional defects in DME between the two experimental systems.

DME is known to function in the central cell and the vegetative cell of the male gametophyte (Choi et al., 2002;
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Schoft et al., 2011; Park et al., 2017). In the vegetative nucleus of the pollen, DME is required for pollen germination and for the growth of the pollen tube. A complementation line expressing a DME native promoter-driven DME catalytic domain sequence rescued the dme-2 pollen defects (Zhang et al., 2019). Surprisingly, central cell-specific complementation lines in the current study did not show a seed-abortion phenotype, even though the lines had a strong mutant allele background. The two promoters that were used for complementation, DD7 and DME + 395, might also allow expression in the vegetative nucleus of the pollen. Additional research will be required to determine the molecular mechanisms underlying DME-dependent pollen growth.

An important finding of the current study is that DME is required for disease resistance against both bacterial and fungal pathogens. Although we identified several down-regulated defense-response genes and hypermethylation at their promoter regions in the dme mutants under normal growth conditions, we did not perform methylene and transcriptome analyses of pathogen-infected tissues. According to our transcriptomic analysis of the uninfected plants, the basal level of the defense machinery is suppressed in the dme mutants.

Higher plants possess two immune systems to discriminate and defend against potential invading pathogens. One defense response is known as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), and the other is termed effector-triggered immunity (ETI) (Jones and Dangl, 2006). Pathogen-associated molecular patterns and microbe-associated molecular patterns (MAMPs) are recognized by plant receptors called pattern recognition receptors (PRRs), which activate the PTI response, such as a ROS burst, the production of salicylic acid, and the activation of PR genes (Figure S9). Meanwhile, pathogen effector proteins are recognized by resistance (R) genes, which are also known as NLR receptors, to activate the ETI response. Effector-triggered immunity is often associated with programmed cell death known as the HR (Figure S9). Our results strongly indicated that DME is required for the expression of AtPRX34, SOC3, and a gene encoding a TIR-NBS-LRR protein. AtPRX33/34 catalyze the generation of ROS, which are important for PTI (Bindschedler et al., 2006; Daudí et al., 2012). SOC3 and the TIR-NBS-LRR protein play important roles in recognizing pathogen effectors to activate the ETI pathway (Monteiro and Nishimura, 2018). It appears that DME is required for both PTI and ETI. Mutants of the DNA methyltransferase and RNA-directed DNA methylation pathways showed a decreased susceptibility to pathogen attack (Dowen et al., 2012; Zhang and Zhu, 2012; Deleris et al., 2016; López Sánchez et al., 2016); thus, these pathways play antagonistic roles in disease resistance (López Sánchez et al., 2016). DME may prevent the transcriptional silencing of genes important for these defense responses (Figure S9). The transcriptomic analysis of the dme single and drdd quadruple mutants suggested that DME plays an important role in regulating gene expression in vegetative tissues. The direct linkage of hyper-DMRs in the promoter region and a downregulation of expression was only found in 205 genes, whereas more than half of the downregulated DEGs did not show hypermethylation in their promoter regions. One possibility is that DME regulates the DNA demethylation of some key regulatory genes, which in turn control the expression of these DEGs without promoter hypermethylation, such as PR5 in the current study. Another possibility is that the regulatory elements of these DEGs lacking promoter hypermethylation are located distantly from the coding region, and that these regulatory elements may be directly targeted by DME for demethylation. The booster1 (b1) locus paramutation phenotype in maize (Zea mays) is modulated by a hepta-repeat sequence located 100 kbp upstream (Stam et al., 2002), so it is possible that distant regulatory sequences could be targeted by DME. It is also possible that DME and perhaps also the other three DNA demethylases may have functions in regulating gene expression independently of their activities in DNA demethylation. Future investigations will determine whether and how the plant DNA demethylases may regulate gene expression in a DNA methylation-independent manner.

Our DNA methylome analyses of the dme single mutants and drdd quadruple mutants suggested that DME plays a role in regulating DNA demethylation in vegetative tissues, although this role is minor compared with that of ROS1. Despite this, our pathogen response assays showed that DME plays a more critical role than ROS1 in controlling pathogen resistance. DME may preferentially target the critical regulatory elements on the defense-response genes. It is also possible that some of the effect of DME in disease resistance may not depend on its DNA demethylation activity.

MATERIALS AND METHODS

Gene accession numbers

DME, At5g04560; ROS1, At2g36490; DML2, At3g10010; DML3, At4g34060; DD7, At2g20595; PR5, At1g75040. SALK T-DNA lines for rdd:2- ros1-4, SALK_045303; dml2-2, SALK_015854; and dml3-2, SALK_056440.

Plant materials and growth conditions

Arabidopsis thaliana accession Col-0 was used for all experiments. All plants were grown at 22 °C on half-strength Murashige and Skoog (MS) medium with 1% sucrose or in soil in a 16 h light/8 h dark photoperiod.

Plasmid construction

For the CRISPR/Cas9 constructs for DME mutagenesis, AtU6 promoter-driven single guide RNAs were constructed in a previously reported Cas9 vector (Feng et al., 2018). For complementation constructs, the DD7 and DME + 395 promoters and the full-length DME genomic sequence were amplified and cloned into pCambia1300. All primers used for amplifying the corresponding sequences are listed in Table S1. All transformants were generated using the flower-dipping method.
Whole-genome bisulfite sequencing and data analysis
Genomic DNA was extracted from 10-d-old seedlings grown on half-strength MS medium. The Genomics Core Facility at the Shanghai Center for Plant Stress Biology, China, performed the library construction, bisulfite treatment, and high-throughput sequencing. For the data analysis, the adapters were trimmed using cutadapt (Martin, 2011), and low-quality sequences (q < 20) were trimmed using Trimmomatic (Bolger et al., 2014). The clean reads were then mapped to the Arabidopsis thaliana TAIR 10 genome (10th release of the Arabidopsis thaliana genome sequence from the Arabidopsis Information Resource) using the Bisulfite Sequence Mapping Program (BSMAP) (Xi and Li, 2009) with a 0.08 mismatch rate. The methratio.py script was used to determine the methylation ratio from the BSMAP mapping results, with the option --r used to remove potential PCR duplicates, and --z used to report loci with zero methylation ratios.

Differential methylation analysis
Differentially methylated regions were identified as previously described with minor modifications (Zhang et al., 2013). In brief, only cytosines with a depth of at least four in the library were considered for further analysis. A sliding-window approach with a 200 bp window and a 50 bp step size was used to identify DMRs. Fisher’s exact test was used to compare the methylated and unmethylated cytosines within each window, with a P-value cutoff of 0.05. False discovery rates (FDRs) were then estimated using a Benjamini–Hochberg adjustment of Fisher’s P-value in the R environment. Windows with an FDR < 0.01 and a fold change >1.5 in the DNA methylation level and with at least five DMCs (defined as a dmC with P < 0.01 in Fisher’s exact test) were used for further analysis. Windows within 100 bp of each other were merged into a larger region, which was then adjusted to shrink to the first and last DMC.

Whole-genome transcriptomic analysis
Total RNA was isolated from 10-d-old seedlings using RNeasy Plant (Qiagen, Hilden, Germany). Transcriptome libraries were prepared from extracted total RNA samples by the Genomics Core Facility at the Shanghai Center for Plant Stress Biology. Adapters and low-quality sequences (q < 20) were trimmed using cutadapt (Martin, 2011) and Trimmomatic (Bolger et al., 2014), respectively, and the trimmed reads were aligned to the TAIR 10 genome using STAR (Dobin et al., 2013). The tool htsq-cound from the Python package HTSeq (Anders et al., 2015) was used to count the mapped fragments for each gene. The output count table was used as the input for DESeq (Anders and Huber, 2010) to compute the DEGs between pairs of samples, according to a FDR threshold of <0.01 and a fold change value of ≥1.5. Differentially methylated region-related genes were defined as genes with DMRs within their 2 kb promoter regions.

Bacterial infection assay with syringe inoculation
Plant resistance against the bacterial pathogen Pst DC3000 was measured as described previously (Li et al., 2015; Yang et al., 2015). Pst DC3000 was grown at 28°C on lysogeny broth (LB) solid medium with 50 mg/L rifampicin. After 2 d, single colonies were transferred to liquid LB medium supplemented with 50 mg/L rifampicin at 28°C. Arabidopsis seedlings were grown under short-day conditions (8 h light/16 h dark photo-period) until they bolted. A syringe was used to inoculate the fully opened leaves of 4-week-old plants with a Pst DC3000 suspension at an optical density of 0.002 at 600 nm. For the mock treatment, leaves were inoculated with 10 mmol/L MgCl2. Disks from the inoculated areas of the leaves were collected at 0 and 4 d to determine the number of colony-forming units (CFUs) per unit of leaf tissue, as described in the “Assessment of bacterial biomass” section.

Bacterial infection assay on plates
Pst DC3000 was prepared as described in the previous section. The bacterial suspension was diluted with sterile distilled water to an optical density of 0.02 at 600 nm, then Silwet L-77 was added to a final concentration of 0.025%. A 50 mL volume of the bacterial suspension was added to a plate on which an Arabidopsis seeding had grown for 2 weeks (Ishiga et al., 2011; Liu et al., 2015). After a 3 min incubation, the bacterial suspension was poured off the plates, which were returned to the growth chamber. The bacterial biomass was detected at 0–4 dpi, as described in the next section.

Assessment of bacterial biomass
The leaves of the inoculated and control plants were collected, weighed, surface-sterilized in 5% H2O2 for 3 min, and then rinsed with sterile distilled water. The leaves of each replicate were homogenized, and 1 mL of sterile distilled water was added to the homogenate. The suspension was diluted to the proper concentration and plated on LB medium containing rifampicin (50 mg/L). After 4 d, the colonies were counted and expressed as CFUs/mg of tissue.

Fungal infection assay
Verticillium dahlia strain V592 (Broekaert et al., 1990; Ellendorff et al., 2009) was grown on potato dextrose agar at 28 °C for 2 d before several colonies were transferred to Czapek–Dox liquid medium at 28 °C. A spore suspension of V. dahlia in Czapek–Dox liquid medium was obtained. The roots of 2-week-old plate-grown Arabidopsis seedlings were dipped for 5 min into the V. dahlia spore suspension or into Czapek–Dox liquid medium as a mock treatment. The seedlings were then transplanted into soil and the trays were covered with clear lids to maintain the humidity for the experiments. At 25–30 d after inoculation (at which time the symptoms were visible), the leaves were collected for the assessment of fungal biomass and the plant defense gene expression levels. DNA extraction for V. dahlia biomass assessment by qPCR with fungus-specific ITS1-F primer (AAAGTTTTAATGGTTCGCTAAGA) in combination with the V. dahlia-special reverse primer (CTTGGTCPATTTAG AGGAAGTAA). And for normalization, we used RubiCO, the Arabidopsis large subunit, for primer set.
ACKNOWLEDGEMENTS

This work was supported by a grant from the Chinese Academy of Sciences to J.K.Z., and by a grant from the National Natural Science Foundation of China (NSFC 31900482) to H.H. We thank the Genomics Core Facility at the Shanghai Center for Plant Stress Biology for assistance with the whole-genome methylome and transcriptome sequencing.

AUTHOR CONTRIBUTIONS

D.M., W.J.Z., and J.K.Z. designed the research; W.J.Z. and D.M. performed the experiments with support from X.Q.L., C. Z., K.I.K., C.F.H., and C.G.D.; H.H. performed the informatics analysis; D.M. and J.K.Z. supervised the project; D.M., W.J.Z., H.H., H.M.Z., and J.K.Z. wrote the manuscript. All authors read and approved of its content.

Edited by: Yijun Qi, Tsinghua University, China

Received Jun. 13, 2020; Accepted Nov. 3, 2020; Published Nov. 25, 2020

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SUPPORTING INFORMATION

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Figure S1. Expression of the 5-methylcytosine DNA glycosylase family genes

(A) Expression of the 5-methylcytosine DNA glycosylase genes in different tissues. The data were obtained from the public Arabidopsis transcriptome database ATGenExpress. (B) Expression of the 5-methylcytosine DNA glycosylase genes in seedlings of Col-0 and the rdd-1, rdd-2, met1-3, dm2, dm3, cm3 (ddc), and ddml1 mutants as determined using quantitative real-time polymerase chain reaction (qRT-PCR). Data shown are means ± SEM from three experiments.

Figure S2. Expression of the 5-methylcytosine DNA glycosylase genes in the weak dme mutants

(A) ROS1 expression. (B) DML2 expression. (C) DML3 expression. Data shown are means ± SEM from three experiments.

Figure S3. Characterization of the methylation of the dme single mutants

(A) Individual boxplot analysis of the DNA methylation levels (relative to Col-0 of dme-A.del, dme-T.in, dme-3.in, and dmeD07pro mutant-specific hyper-differentially methylated regions (hyper-DMRs). Methylation levels of mC, mCG, mCHG, and mCHH contexts are shown for Col-0, rdd-2, and four dme weak-allele mutants with replicates (with the same color) (P < 10−5 compared with Col-0, one-tailed Wilcoxon tests). (B) Heatmap analysis of the DNA methylation level of the hyper-DMRs of the dme single mutants in Col-0, rdd-2, and dme single mutants with biological repeats.

Figure S4. Characterization of the central cell-specific complementation drdd quadruple mutants

(A) Box plots of hyper-differentially methylated regions (hyper-DMRs) specific to the drrd−d05pro mutant. The mC, mCG, mCH3, and mCHH contexts are shown for Col-0, rdd-2, drrd−d05pro mutants, and drrd−d05pro mutants with replicates in the same color. The analysis was performed relative to Col-0 (P < 10−5 compared with Col-0, one-tailed Wilcoxon tests). (B) Heatmap analysis of the DNA methylation levels of the hyper-DMRs of the dme single mutants in Col-0, rdd-2, and dme single mutants with biological repeats.

Figure S5. Transcriptome analysis of the drdd quadruple mutants

(A) Overlap of downregulated differentially expressed genes (DEGs) among rdd-2, drrd−d05pro, and drrd−d07pro. (B) Heatmap analysis of the specific downregulated DEGs in rdd-2, drrd−d07pro, drrd−d05pro, and drrd−d05pro (P < 10−5 compared with Col-0, one-tailed Wilcoxon tests).

Figure S6. Pst DC3000 infection phenotype in the dme weak-allele mutants

(A) Leaf symptoms of dme weak-allele mutants that were syringe-inoculated with Pst DC3000 at 4 d post-inoculation (dpi). (B) Symptoms of drdd quadruple mutants that were exposed to Pst DC3000 on agar plates. (C) Bacterial biomass in dme weak-allele mutants at 4 dpi. Data shown are means ± SEM from three experiments (P < 0.05, *P < 0.01, one-tailed Student’s t-test). (D) Expression of PRS in dme weak-allele mutants at 4 dpi.
dpi, as determined using quantitative real-time polymerase chain reaction (qRT-PCR). Data shown are means ± SEM from three experiments (**P < 0.01, one-tailed Student's t-test). (E,F) Expression of DME (E) and ROS1 (F) in Col-0 at 3, 6, and 9 h after the fig22 treatment, as determined using qRT-PCR (NS; not significant, one-tailed Student's t-test).

Figure S7. Verticillium dahliae infection phenotype in the dme weak-allele mutants
(A) Symptoms of V. dahliae-inoculated dme weak-allele mutants at 28 d post-inoculation (dpi). (B) V. dahliae biomass in the leaves of inoculated dme weak-allele mutants at 28 dpi. Data shown are means ± SEM from three experiments (**P < 0.01, one-tailed Student's t-test). (C) Expression of PR5 in inoculated dme weak-allele mutants, as determined using a quantitative real-time polymerase chain reaction (qRT-PCR). Data shown are means ± SEM from three experiments (**P < 0.01, one-tailed Student's t-test).

Figure S8. Expression analysis of defense-related genes in Pst DC3000-infected dme weak-allele mutants, with Col-0 as a control
Data shown are means ± SEM from three experiments. (A) Expression of At1g72890, a TIR-NBS-LRR class disease resistance protein. (B) Expression of At2g02100, PDF1.2. (C) Expression of At3g49120, the class III peroxidase PERX34 (**P < 0.01, one-tailed Student's t-test).

Figure S9. A working model of DME function in the disease resistance response
Pathogen attack activates the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) defense responses in Arabidopsis. Pattern recognition receptors (PRRs), which are cell surface receptors, recognize the PAMPs or microbe-associated molecular patterns (MAMPs) to activate PTI. The PTI response pathway includes APRX33/34-dependent and -independent reactive oxygen species (ROS) production, followed by salicylic acid biosynthesis and pathogenesis-related (PR) gene expression. Pathogens can deliver effector proteins into host cells to suppress the PTI. As a counter measure, the effector proteins can be recognized by the intracellular receptors (R proteins), which in turn can activate the ETI response to induce the hypersensitive response (HR) cell death. DME is required for the expression of APRX34, SOC3, and a TIR-NBS-LRR-encoding gene, which encode key components of the PTI and ETI response pathways.