



# Critical roles of DNA demethylation in the activation of ripening-induced genes and inhibition of ripening-repressed genes in tomato fruit

Zhaobo Lang<sup>a,b,1</sup>, Yihai Wang<sup>b</sup>, Kai Tang<sup>b</sup>, Dengguo Tang<sup>b</sup>, Tatsiana Datsenka<sup>b</sup>, Jingfei Cheng<sup>c</sup>, Yijing Zhang<sup>c</sup>, Avtar K. Handa<sup>b</sup>, and Jian-Kang Zhu<sup>a,b,1</sup>

<sup>a</sup>Shanghai Center for Plant Stress Biology and Center of Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai 200032, China; <sup>b</sup>Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907; and <sup>c</sup>Shanghai Institute of Plant Physiology and Ecology and Center of Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai 200032, China

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**DNA methylation is a conserved epigenetic mark important for genome integrity, development, and environmental responses in plants and mammals. Active DNA demethylation in plants is initiated by a family of 5-mC DNA glycosylases/lyases (i.e., DNA demethylases). Recent reports suggested a role of active DNA demethylation in fruit ripening in tomato. In this study, we generated loss-of-function mutant alleles of a tomato gene, *SIDML2*, which is a close homolog of the *Arabidopsis* DNA demethylase gene *ROS1*. In the fruits of the tomato mutants, increased DNA methylation was found in thousands of genes. These genes included not only hundreds of ripening-induced genes but also many ripening-repressed genes. Our results show that *SIDML2* is critical for tomato fruit ripening and suggest that active DNA demethylation is required for both the activation of ripening-induced genes and the inhibition of ripening-repressed genes.**

DNA demethylase | 5-mC DNA glycosylase | DNA methylation | epigenetic regulation | gene silencing

**D**NA methylation is a conserved epigenetic modification that is generally associated with inactive transcription in plants and mammals. As such, DNA methylation plays important roles in many biological processes, such as genome stability, gene imprinting, development, and response to the environment (1–3). In contrast to mammals, in which DNA methylation predominantly occurs at cytosines in the symmetric CG sequence context, plants commonly have methylation in the asymmetrical CHH sequence context (H = A, C, or T), as well as in the symmetrical CG and CHG contexts (1, 2). In plants, cytosines in all sequence contexts can be de novo methylated through the well-known RNA-directed DNA methylation pathway (RdDM), in which 24-nt siRNAs guide the DNA methyltransferase domains rearranged methyltransferase 2 (DRM2) to methylate target loci (4). DNA methylation can be maintained during replication; mCG and mCHG are maintained by the DNA methyltransferases DNA methyltransferase 1 (MET1) and chromomethylase 3 (CMT3), respectively, whereas mCHH is maintained by CMT2 and RdDM (4, 5).

Cytosine methylation levels are dynamically regulated by DNA methylation and demethylation reactions (3, 6). DNA methylation can be lost either because of failure in maintaining methylation after replication (i.e., passive DNA demethylation) or because of active removal by enzymes (i.e., active DNA demethylation). Previous studies have identified and characterized several enzymes important for active DNA demethylation in *Arabidopsis* (7–11). The ROS1 family of bifunctional 5-methylcytosine DNA glycosylases/lyases, often referred to as DNA demethylases, initiate active DNA demethylation by removing the methylcytosine base from the DNA backbone, resulting in a single nucleotide gap that can be filled with an unmethylated cytosine through a base excision repair pathway (7, 8, 12, 13). Several enzymes acting downstream of ROS1, such as the 3' DNA phosphatase

ZDP, AP endonuclease-like protein APEIL, and DNA ligase I (AtLIG1), have also been identified (9–11). Because the ROS1 family of enzymes function in the first step of the DNA demethylation pathway, their targeting to specific genomic sequences determines the patterns of DNA methylation removal. Recent studies revealed a histone acetyltransferase complex that regulates DNA demethylation by facilitating the targeting of ROS1 (14–17).

Because they prevent DNA hypermethylation, enzymes and regulatory factors involved in DNA demethylation are regarded as antisilencing factors. Expression of some transgenes, endogenous genes, and transposable elements (TEs) is reduced in *ros1* mutants because of DNA hypermethylation (14, 16, 18, 19). In *Arabidopsis*, ROS1 preferentially targets TEs (20). When ROS1-targeted TEs are located in the promoter regions of genes, ROS1 can protect gene expression by demethylating the adjacent TEs. In *Arabidopsis*, *ros1* mutants have an abnormal epidermal cell organization due to hypermethylation of a TE located in the promoter region of EPIDERMAL PATTERNING FACTOR 2, which encodes a negative regulator of stomata formation (21). In addition, *Arabidopsis* DNA demethylase mutants show enhanced susceptibility to fungal infection due to hypermethylation of TEs

## Significance

**DNA methylation is generally considered an epigenetic mark for transcriptional gene silencing. In this work, we generated loss-of-function mutant alleles of *SIDML2*. We characterized the mutant fruits that failed to ripen and discovered that *SIDML2* is required for the demethylation and activation of genes important for fruit ripening, including genes involved in fruit pigment and flavor synthesis, ethylene synthesis and signaling, and cell wall hydrolysis. Unexpectedly, we found that *SIDML2*-mediated DNA demethylation is also necessary for fruit ripening-induced repression of hundreds of genes involved in photosynthesis and cell wall synthesis and organization. Our study has therefore revealed a broad and critical role of DNA methylation as an activation mark for the expression of many genes in a eukaryotic organism.**

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<sup>1</sup>To whom correspondence may be addressed. Email: jkzhu@purdue.edu or zblang@sibs.ac.cn.

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located in pathogen-responsive genes (22). These studies have demonstrated that, by demethylating nearby TEs, active DNA demethylation is critical for gene activation or for the prevention of silencing.

To date, active DNA demethylation in plants has been studied mostly in *Arabidopsis*. *Arabidopsis* mutants defective in DNA demethylation have been important in understanding the mechanism and functions of active DNA demethylation. Despite the many advantages of *Arabidopsis*, the use of this model plant is limited by its lack of some agronomically important processes, such as fiber growth in cotton and ripening of fleshy fruits, such as tomato. Tomato (*Solanum lycopersicum*) is an economically important crop and a model for studying fleshy fruit ripening. Fruit ripening is associated with distinct physiological, biochemical, and structural changes, such as the accumulation of sugars, flavor volatiles, and pigments, and the hydrolysis of cell walls (23). These changes are driven by phytohormones and developmental factors. Ethylene is one of the most important factors promoting ripening, especially for climacteric fruits, which show a rapid increase in ethylene level during ripening (23). In addition, several transcription factors (TFs), including RIPENING INHIBITOR (RIN), NON-RIPENING (NOR), and COLORLESS NON-RIPENING (CNR), have been identified as major ripening regulators. These regulators function upstream of both ethylene-dependent and -independent ripening pathways by directly and indirectly regulating the expression of many ripening-related genes (24–26). Evidence has recently emerged that, in addition to ethylene and the TFs, DNA methylation is another key regulator of fruit ripening (27). Furthermore, a recent study revealed that DNA methylation is associated with chilling-induced flavor loss in tomato fruits (28). Tomato fruits undergo a dramatic loss in DNA methylation during ripening (27). The application of a DNA methylation inhibitor facilitated ripening, and RNAi-mediated down-regulation of putative DNA demethylases inhibited fruit ripening in tomato, suggesting that active DNA demethylation plays an important role in regulating fruit ripening (27, 29). It remains unclear, however, how active DNA demethylation contributes to ripening and ripening-associated changes in DNA methylation patterns.

Tomato contains four putative DNA demethylases (SIDML1 to -4) according to sequence homology with the *Arabidopsis* DNA demethylases, and two of them, SIDML1 and SIDML2, are most closely related to the *Arabidopsis* ROS1 (AtROS1) (29, 30). A previous study used RNAi against the conserved HhH-GPD domain to investigate the function of SIDMLs in tomato (29). The RNAi lines showed an altered expression of all four SIDMLs, with SIDML2, the most abundant DNA demethylase in fruits, showing the most dramatic reduction in expression. Fruits of the RNAi plants displayed a delayed ripening phenotype, suggesting critical roles of the SIDMLs in fruit ripening. However, because the RNAi lines were not specific to a particular SIDML, it is unclear which SIDML(s) is required for fruit ripening. In addition, the genome-wide effect of active DNA demethylation during fruit ripening has not been determined thus far.

In the present study, we generated two mutant alleles of SIDML2 in the tomato cultivar (cv.) Micro-Tom using CRISPR/Cas9-mediated gene editing. To determine the genome-wide effect of SIDML2 on DNA demethylation during fruit ripening, we compared the DNA methylomes of WT and *sdml2* mutant fruits. We found that SIDML2 is responsible for the demethylation of as many as 29,764 genomic regions, which are preferentially distributed in chromosomal arms, and is required for virtually all ripening-induced DNA demethylation. Our transcriptome analysis of *sdml2* mutant fruits suggested that SIDML2 is necessary for the activation of hundreds of ripening-related genes, such as RIN, and genes involved in ethylene and pigment synthesis and cell wall hydrolysis. Unexpectedly, our analysis also revealed that SIDML2-

mediated DNA demethylation is required for the repression of hundreds of genes during fruit ripening. These repressed genes are involved in processes such as photosynthesis and cell wall synthesis, and their repression correlates with DNA hypomethylation in their promoter regions. Thus, our study documents a broad silencing role of DNA demethylation in gene regulation.

## Results

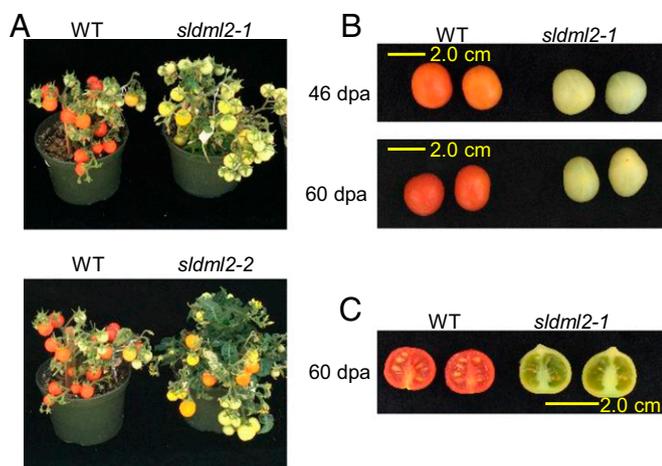
**Generation of Stable Loss-of-Function Mutant Alleles of SIDML2 Using the CRISPR/Cas9 Gene-Editing System.** During tomato fruit ripening, DNA demethylation occurs in numerous genomic regions (27). In tomato, there are four annotated 5-mC DNA glycosylase/DNA demethylase genes; one of them, *SIDML2*, is highly induced during fruit ripening (29, 30). A recent study using RNAi lines of the demethylases suggested that SIDMLs-mediated active DNA demethylation is important for fruit ripening (29). To better define the role of SIDML2 in active DNA demethylation and fruit ripening, we generated stable loss-of-function *sdml2* mutants using the CRISPR/Cas9 gene-editing system. A single guide RNA (sgRNA) was designed to specifically target the first exon of *SIDML2* (Fig. S1A). We cloned the sgRNA sequence into a binary vector that contains sgRNA and Cas9 expression cassettes (31), and the resulting construct was transformed into WT tomato cv. Micro-Tom using *Agrobacterium* infection of leaf explants.

A total of 30 transgenic plants regenerated from tissue culture were genotyped through direct sequencing of PCR products from genomic DNA flanking the target site. We found 2- to 56-bp deletion mutations in 18 plants. Three of the plants carried the same homozygous 28-bp deletion, and two of the plants had the same homozygous 2-bp deletion in the first exon of *SIDML2* (Fig. S1B). Both the 28-bp and 2-bp deletion mutations are predicted to cause premature stop codons in the first exon of *SIDML2*. We did not find any off-target editing events in any of the other three DNA demethylase genes. We refer to the mutant with the 28-bp deletion as *sdml2-1* and that with the 2-bp deletion as *sdml2-2* (Fig. S1B).

**The *sdml2* Mutations Inhibit Fruit Ripening.** To exclude the effect of the tissue culture process, we also regenerated from tissue culture the WT plants that were used as controls for the *sdml2* mutants in this study. Compared with fruit ripening in the WT plants, fruit ripening in the *sdml2-1* mutants was dramatically inhibited (Fig. 1A). We compared fruits of the WT and *sdml2* mutants at four stages: 25 d after pollination (dpa), 41 dpa, 46 dpa, and 60 dpa. Fruits of the WT turned red by 46 dpa, but fruits of the *sdml2-1* mutants remained green at all stages (Fig. 1B and Fig. S1C). The *sdml2-2* mutation had a similar inhibitory effect on fruit ripening (Fig. 1A).

The fruits of the *sdml2-1* and *sdml2-2* mutants contained seeds although they seemed to contain fewer seeds than the WT fruits (Fig. 1C and Fig. S1D). At 60 dpa, fruits of the WT and *sdml2* mutants were similar in height and in the width and thickness of the pericarp (Fig. S1E), which is consistent with the fact that the induction of *SIDML2* expression occurs after the breaker stage, when fruits have already reached full size (29). Our results are consistent with the previous study that used RNAi lines (29) and demonstrate that SIDML2 is critical for tomato fruit ripening.

**The *sdml2* Mutations Cause Genome-Wide DNA Hypermethylation.** To investigate the genetic function of SIDML2 in DNA demethylation during fruit ripening, we performed whole-genome bisulfite sequencing to generate single-base resolution maps of DNA methylation in the following fruits: WT at 25 dpa (WT-25dpa), *sdml2-1* at 25 dpa (*sdml2-1*-25dpa), WT at 46 dpa (WT-46dpa), and two biological replicates of *sdml2-1* at 46 dpa (*sdml2-1*-46dpa). Over 95% of the genomic cytosines were covered in each sample, and each methylome was sequenced



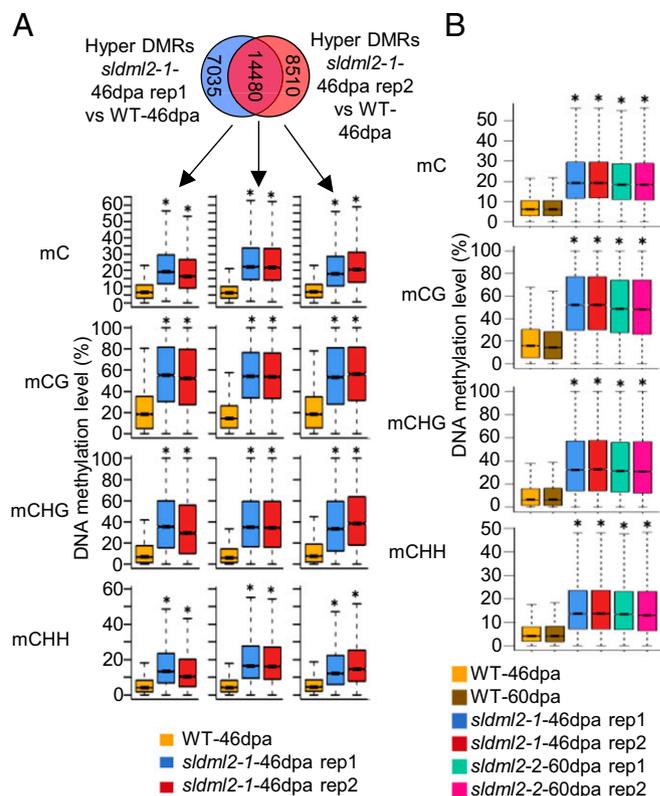
**Fig. 1.** Fruit-ripening phenotypes of tomato *sldml2* mutants. (A) Plants of the WT (cv. Micro-Tom), *sldml2-1*, and *sldml2-2* at the same stage. All plants were from the T0 generation. (B) Fruits of the WT and *sldml2-1* at 46 dpa and 60 dpa. (C) Photograph of the inside of fruits of the WT and *sldml2-1* at 60 dpa.

with >10.7-fold coverage per DNA strand (Table S1). The coverage and depth of these methylomes were higher than those of published methylomes of *Solanum lycopersicum* cv. Ailsa Craig (AC) (27).

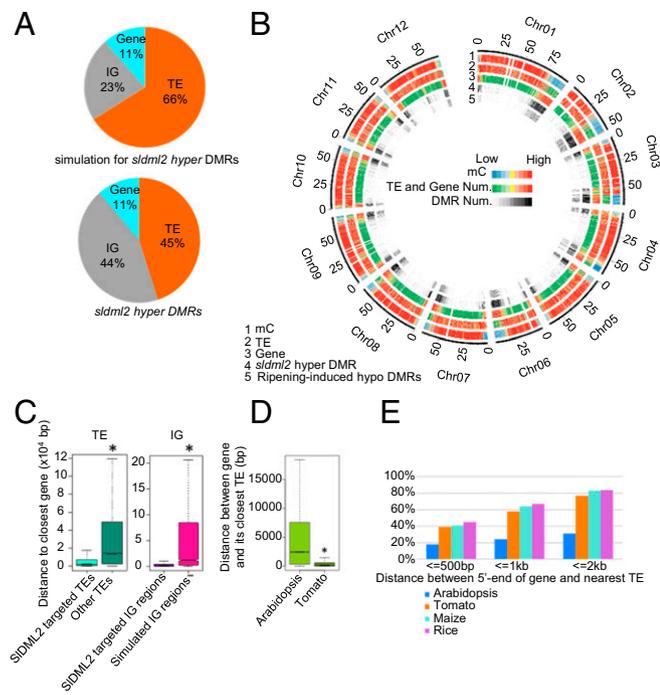
To begin to identify SIDML2 targets in fruits, we compared the methylome of WT-46dpa with those of two biological replicates of *sldml2-1-46dpa*. Our analysis identified 21,515 hyper-DMRs (Table S2) and 6,643 hypo-DMRs in *sldml2-1-46dpa* replication 1 (rep1), and 23,026 hyper-DMRs (Table S3) and 6,689 hypo-DMRs in *sldml2-1-46dpa* rep2. The overwhelmingly higher numbers of hyper-DMRs than hypo-DMRs in the *sldml2* mutant are consistent with the presumed function of SIDML2 in DNA demethylation. To investigate the role of SIDML2 in DNA demethylation, we focused on hyper-DMRs in the *sldml2* mutant. There were 14,480 hyper-DMRs shared between the two biological replicates of *sldml2-1-46dpa* (Fig. 2A). For hyper-DMRs unique to *sldml2-1-46dpa* rep1, we also observed increased DNA methylation in *sldml2-1-46dpa* rep2, and, similarly, for hyper-DMRs unique to *sldml2-1-46dpa* rep2, we also observed increased DNA methylation in *sldml2-1-46dpa* rep1 (Fig. 2A), suggesting that the number of overlapped hyper-DMRs was underestimated because of the artificial cutoff used in defining the DMRs. We therefore used the combined hyper-DMRs (29,764 hyper-DMRs, using bedtools merge) of the two biological replicates as SIDML2 targets in the following analysis.

The *sldml2* hyper-DMRs contain 1.18 million hyper-DMCs and cover a total length of 17.67 Mb of the genomic sequence (with an average length of 594 bp per DMR). As shown in Fig. 2A and consistent with features of AtROS1 targets in *Arabidopsis* (20), DNA hypermethylation in *sldml2* mutant fruits occurred in all three sequence contexts: mCG, mCHG, and mCHH. To confirm the function of SIDML2 in DNA demethylation, we examined DNA methylation levels of the other mutant allele of SIDML2, *sldml2-2*, and found that the identified SIDML2 targets in *sldml2-1* have similar increases in mCG, mCHG, and mCHH levels in *sldml2-2* at 60 dpa compared with the WT at 60 dpa (Fig. 2B). Several examples of *sldml2* hyper-DMRs are shown in Fig. S2. Even though the methylation level of *sldml2-1* was measured at 46 dpa and the methylation level of *sldml2-2* was measured at 60 dpa, the similar increases in DNA methylation in the two mutants are consistent with the inference that the hyper-DMRs of *sldml2-1* represent the genuine targets of SIDML2.

In *Arabidopsis*, ROS1 preferentially targets TEs (20). Among the simulated genomic regions in tomato, 66.1% are in TEs, 22.5% in intergenic regions (IGs), and 11.4% in genic regions. Among the SIDML2 targets, 45.0% are in TEs, 43.7% in IGs, and 11.3% in genic regions (Fig. 3A). These data suggest that SIDML2 preferentially targets IG regions and TEs in tomato. In both *Arabidopsis* and tomato, DNA methylation and TEs are mostly distributed in pericentromeric regions (27) (Fig. 3B). However, in contrast to AtROS1 targets that are concentrated around pericentromeric regions (20), *sldml2* hyper-DMRs aggregate in chromosomal arms (Fig. 3B). These results indicate that SIDML2 may preferentially target TEs located in euchromatin regions in tomato. This conclusion is also supported by analysis of the density of SIDML2-targeted TEs and total TEs across chromosomes (Fig. S3). We found that SIDML2-targeted TEs are closer to genes than are TEs that are not targeted by SIDML2 (Fig. 3C). Similarly, SIDML2-targeted IG regions are also closer to genes than are simulated IG regions (Fig. 3C). These results suggest that, during fruit ripening, SIDML2 may have a function similar to that of AtROS1 in preventing gene silencing by demethylating nearby TEs or highly methylated intergenic regions. However, in contrast to AtROS1, which targets TEs in both euchromatic and heterochromatic regions (20), SIDML2 preferentially targets TEs in euchromatic regions.



**Fig. 2.** *sldml2* mutations cause genome-wide DNA hypermethylation in ripening tomato fruits. (A) DNA methylation levels of *sldml2-1* hyper-DMRs in fruits at 46 dpa. (Upper) Hyper-DMRs in two biological replicates of *sldml2-1-46dpa* relative to WT-46dpa were identified and compared using a Venn diagram. (Lower) Boxplot analysis of mC, mCG, mCHG, and mCHH levels of different groups of the hyper-DMRs. (B) Boxplots showing DNA methylation levels at combined *sldml2-1* hyper-DMRs in WT-46dpa, WT-60dpa, two biological replicates of *sldml2-1-46dpa*, and two biological replicates of *sldml2-2-60dpa*. WT-46dpa served as control for *sldml2-1-46dpa*, and WT-60dpa served as control for *sldml2-2-60dpa* (\**P* value < 2.2e-16, one-tailed t test).

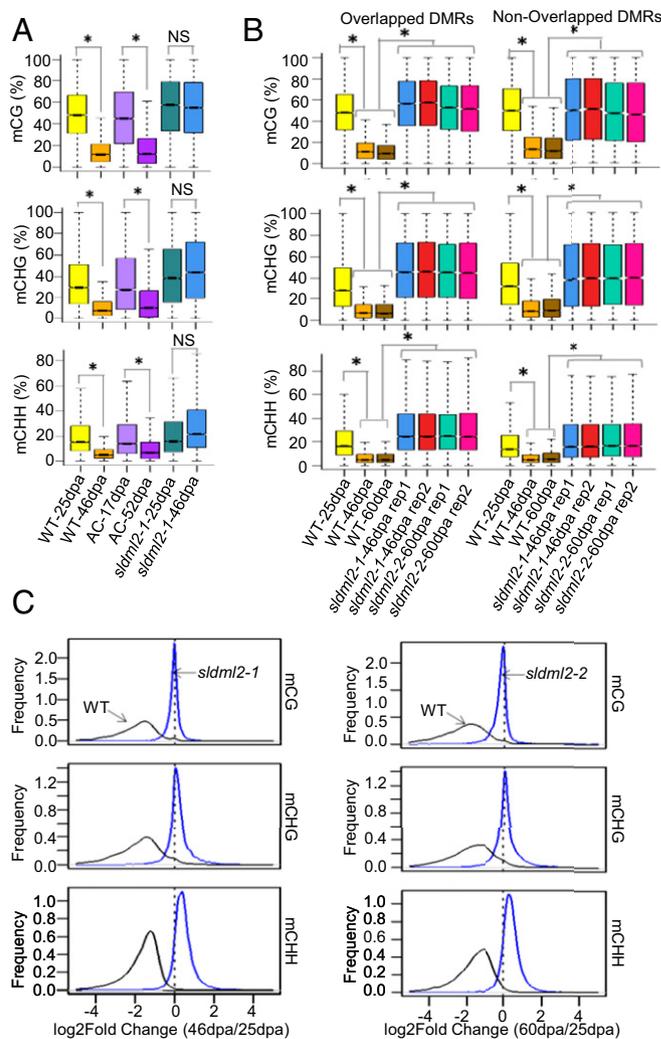


**Fig. 3.** Characterization of the DNA methylomes of ripening *sldml2* mutant fruits. (A) The compositions of hyper-DMRs (combined hyper-DMRs from two biological replicates of *sldml2-1-46dpa*) in the *sldml2-1* mutant and simulated genomic regions. (B) Density plots of DNA methylation, transposable elements (TEs), genes, *sldml2-1* hyper-DMRs, and ripening-induced hypo-DMRs. Chromosome numbers and coordinates (Mb) are indicated on the outer rim. (C) Boxplots showing distances between SIDML2-targeted or nontargeted TEs (Left) and IG regions (Right) and their nearest protein-coding genes. (D) Boxplot showing the distances between genes and their nearest TEs in *Arabidopsis* and tomato. (E) Percentages of genes that contain TEs in their 500-bp, 1,000-bp, and 2,000-bp upstream sequences in *Arabidopsis*, tomato, maize, and rice (\**P* value < 2.2e-16, one-tailed Wilcoxon rank sum test).

In both *Arabidopsis* and tomato, TEs are concentrated in pericentromeric regions, and genes are preferentially located in chromosomal arms (Fig. 3B). The content of TEs is higher in the tomato genome than in the *Arabidopsis* genome. Approximately 20.9% of the *Arabidopsis* genome but ~54.7% of the tomato genome consists of TEs. As shown in Fig. 3D, the distance between a gene and its closest TE is much less in tomato than in *Arabidopsis*. About 18%, 24%, and 31% of *Arabidopsis* genes have TEs within 500 bp, 1,000 bp, and 2,000 bp of the 5' end of genes, respectively, but these percentages are ~39%, 58%, and 77% in tomato (Fig. 3E). The percentages are 41%, 64%, and 83% in rice and 45%, 67%, and 84% in maize (Fig. 3E). These results suggest that, compared with *Arabidopsis* genes, genes in tomato and the other crops are more likely to be affected by the methylation status of nearby TEs. Consequently, the DNA demethylases likely play more important roles in regulating gene expression in these crop plants than in *Arabidopsis*.

**SIDML2 Is Required for Ripening-Induced DNA Demethylation.** We profiled DNA methylation changes during fruit ripening in tomato cv. Micro-Tom. We compared the methylomes of WT-25dpa and WT-46dpa and identified a total of 16,412 differentially methylated regions (hereafter referred to as ripening-induced DMRs), among which 3,306 are hypermethylated and 13,106 are hypomethylated in WT-46dpa relative to WT-25dpa (Table S4). The DNA methylation changes occurred in all three sequence contexts (Fig. 4A). These 13,106 hypo-DMRs are still hypomethylated in WT-60dpa, suggesting that the hypomethylation status is

maintained during later stages of ripening (Fig. 4B). In the *sldml2-1* mutant, the DNA methylation levels at these hypo-DMRs do not decrease from 25 dpa to 46 dpa, suggesting that SIDML2 is required for the fruit ripening-induced DNA demethylation (Fig. 4A). We examined the DNA methylation levels of these DMRs in tomato cv. AC (27) and found that the hypo-DMRs in cv. Micro-Tom are also hypomethylated in AC during fruit ripening (from 17 dpa to 52 dpa) (Fig. 4A), which suggests that ripening-induced DNA demethylation is conserved in the two tomato varieties. In contrast, the hyper-DMRs in cv. Micro-Tom are not hypermethylated in cv. AC during fruit ripening



**Fig. 4.** SIDML2 is required for ripening-induced DNA demethylation. (A) Boxplots showing mCG, mCHG, and mCHH levels at ripening-induced hypo-DMRs in WT-25dpa, WT-46dpa, AC-17dpa, AC-52dpa, *sldml2-1-25dpa*, and *sldml2-1-46dpa* (average of two replicates) (\**P* value < 2.2e-16, one-tailed *t* test; NS, not significant). (B) Boxplots showing mCG, mCHG, and mCHH levels at ripening-induced hypo-DMRs that are overlapped (Left) or not overlapped (Right) with *sldml2-1* hyper-DMRs in WT-25dpa, WT-46dpa, WT-60dpa, two biological replicates of *sldml2-1-46dpa*, and two biological replicates of *sldml2-2-46dpa*. For mCG, mCHG and mCHH levels, WT-25dpa, two biological replicates of *sldml2-1-46dpa*, and two biological replicates of *sldml2-2-46dpa* were significantly higher than WT-46dpa and WT-60dpa (\**P* value < 2.2e-16, one-tailed *t* test). (C) The distribution of log<sub>2</sub>fold-change (46dpa/25dpa) of mCG, mCHG, and mCHH levels in *sldml2-1* mutant and the WT (Left), and the distribution of log<sub>2</sub>fold-change (60dpa/25dpa) in the *sldml2-2* mutant and the WT (Right). A peak at 0 corresponds to no change in DNA methylation during ripening.

(Fig. S4A), suggesting that the ripening-associated increase in DNA methylation differs between the two varieties.

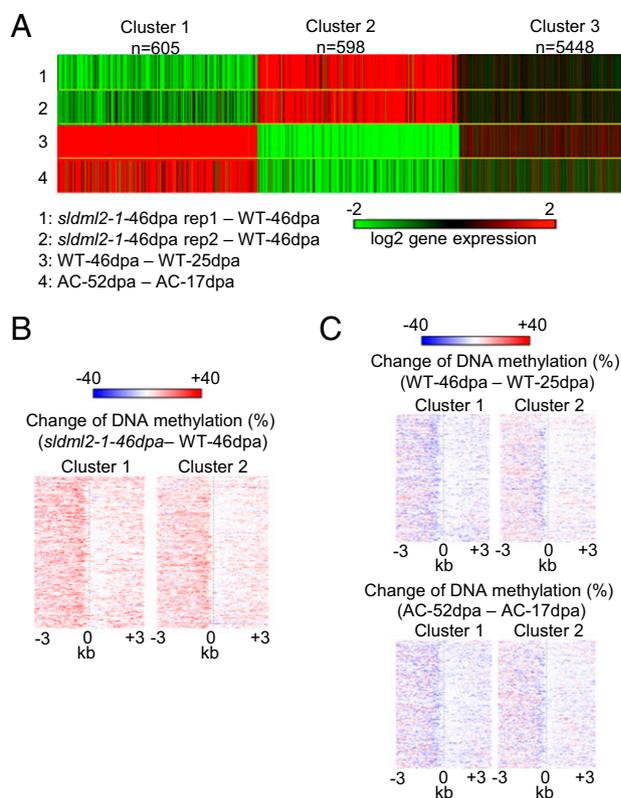
To further investigate the role of SIDML2 in ripening-induced DNA demethylation, we compared the ripening-induced hypo-DMRs with *slamdml2* hyper-DMRs and found that 8,176 of the 13,106 ripening-induced hypo-DMRs overlap with the *slamdml2* hyper-DMRs. As shown in Fig. 4B, the 8,176 shared ripening-induced hypo-DMRs have increased DNA methylation levels in *slamdml2-1* and *slamdml2-2* mutants compared with the WT at the same stage, and the data were consistent for the two biological replicates of both mutants. For the 4,930 nonoverlapped hypo-DMRs, DNA methylation was also higher in the *slamdml2-1* and *slamdml2-2* mutants than in the WT (Fig. 4B). This result suggested that, although these 4,930 ripening-induced hypo-DMRs do not overlap with *slamdml2* hyper-DMRs (perhaps because of the artificial cutoff used to define the DMRs), their methylation levels are still controlled by SIDML2 during ripening. Several examples of ripening-induced hypo-DMRs, such as those at the promoters of *RIN*, *CNR*, and *DML2*, are shown in Fig. S4B. The methylation patterns at these DMRs are consistent with our analysis above, which showed that, during ripening, they are hypomethylated in the WT but not in *slamdml2* mutants. To further assess the effect of SIDML2 dysfunction on ripening-induced DNA demethylation, we compared the DNA methylation changes in ripening-induced hypo-DMRs from the immature to the ripe stage in the WT and in the *slamdml2-1* and *slamdml2-2* mutants. During ripening, DNA methylation decreased in the WT in all three sequence contexts, as shown in Fig. 4A and also indicated by the left-skewed peaks in Fig. 4C. This decrease in DNA methylation, however, did not occur in the fruits of *slamdml2-1* (Fig. 4A). The failure in DNA demethylation in both *slamdml2-1* and *slamdml2-2* mutants is also indicated by the peak at “0” in Fig. 4C. Interestingly, the peaks of mCHG and mCHH in *slamdml2-1* and *slamdml2-2* mutants were skewed to the right of 0, indicating that non-CG methylation, particularly mCHH in *slamdml2* mutants, was slightly increased from the immature stage to 46 or 60 dpa (Fig. 4C). The increase in DNA methylation during ripening was not compromised by SIDML2 dysfunction (Fig. S4). These results demonstrate that SIDML2 is responsible for virtually all ripening-induced DNA demethylation and but not for ripening-induced hyper-DNA methylation.

**SIDML2-Mediated DNA Demethylation also Has Silencing Functions.** DNA methylation is generally associated with inactive transcription, especially when it occurs in the promoter regions of genes (1–3). Enzymes and regulators involved in active DNA demethylation are also known as antisilencing factors because of their function in preventing DNA hypermethylation and in therefore restraining transcriptional gene silencing (3, 14). In *slamdml2-1*, there are 29,764 hyper-DMRs relative to the WT, and 47.3% (14,074) of them are located within 1 kb of 12,902 genes. Expression of these hyper-DMR-associated genes is potentially regulated by SIDML2-mediated DNA demethylation.

To determine the role of SIDML2 in the regulation of gene expression during tomato fruit ripening, we generated gene expression profiles for WT-25dpa, WT-46dpa, *slamdml2-1-25dpa*, and two biological replicates of *slamdml2-1-46dpa*. Among the 12,902 hyper-DMR-associated genes, 6,651 were expressed in at least one sample and were used for the following analysis. For each gene, we calculated the expression change in WT-46dpa vs. WT-25dpa [ $\log_2$  (WT-46dpa/WT-25dpa)] and in *slamdml2-1-46dpa* vs. WT-46dpa [ $\log_2$  (*slamdml2-1-46dpa*/WT-46dpa)]. Using *k*-means clustering, we assigned the 6,651 hyper-DMR-associated genes to three clusters: cluster 1 included 605 genes that were up-regulated during ripening in the WT but not in *slamdml2* (Table S5); cluster 2 included 598 genes that were silenced during ripening in the WT but the silencing was compromised in *slamdml2* (Table S6); and cluster 3 included 5,448 genes that were not

obviously influenced by ripening or SIDML2 dysfunction (Fig. 5A). To investigate whether the hyper-DMRs that are associated with cluster 3 genes might be weaker SIDML2 targets (and therefore less able to cause expression changes) than those associated with cluster 1 and 2 genes, we examined the DNA methylation levels of cluster 1, 2, and 3 genes in *slamdml2-46dpa* and WT-46dpa. We found that the increases in DNA methylation were similar in cluster 1, 2, and 3 genes (Fig. S5A), suggesting that the DNA methylation increases at cluster 3 genes are not sufficient to cause gene expression changes in *slamdml2*.

Consistent with the general notion that DNA hypermethylation is associated with inactive transcription, the expression level of the 605 cluster 1 genes was negatively correlated with their DNA methylation level. Cluster 1 genes were hypermethylated in *slamdml2* vs. WT-46dpa (Fig. 5B and Fig. S5A) and had reduced expression levels in *slamdml2* vs. WT-46dpa (Fig. 5A). The data were consistent between the two biological replicates of *slamdml2-1-46dpa* (Fig. 5A). For both cluster 1 and cluster 2 genes, the hypermethylation mainly occurred in the genomic regions upstream of the transcription start site (Fig. 5B). Compared with mCG and mCHG changes, mCHH changes in the upstream regions were more concentrated near the transcription start site of the genes (Fig. S5B). By comparing the expression profiles of immature and ripe fruits, we found that cluster 1 genes were



**Fig. 5.** SIDML2-mediated DNA demethylation correlates with ripening-induced gene activation, as well as with ripening-induced gene repression. (A) The *k*-means clustering of *slamdml2-1* hyper-DMR-associated gene expression. Genes associated with *slamdml2-1* (46 dpa) hyper-DMRs were subjected to the clustering analysis. (B) DNA methylation changes in cluster 1 and cluster 2 genes in *slamdml2-1-46dpa* vs. WT-46dpa. The sequences flanking cluster 1 and 2 genes were aligned, and DNA methylation levels for each 100-bp interval were plotted. The dashed line marks transcription start sites. (C) DNA methylation changes in cluster 1 and cluster 2 genes in ripe fruits compared with immature fruits. (Upper) DNA methylation differences between WT-46dpa and WT-25dpa. (Lower) DNA methylation differences between AC-52dpa and AC-17dpa.

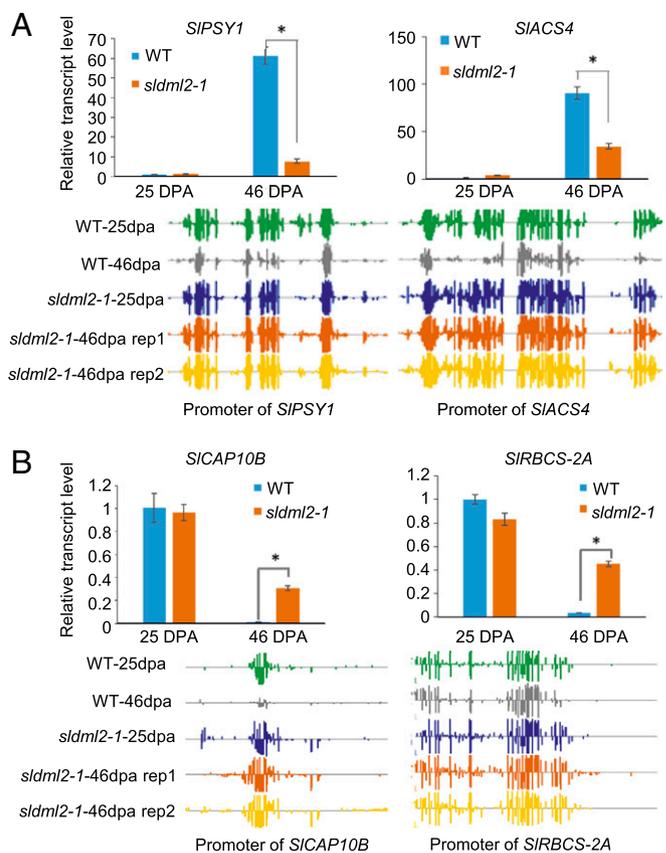
up-regulated during fruit ripening in both Micro-Tom and AC varieties (Fig. 5A) and that their promoter regions lost DNA methylation in both varieties (Fig. 5C). These results suggest that SIDML2-mediated DNA demethylation is required for active transcription of the ripening-induced genes and that SIDML2 has an antisilencing function for the genes.

In addition to cluster 1 genes, we also identified 598 genes (cluster 2) that were repressed during ripening in the WT but remained active in the *slm12* mutant (Fig. 5A). As shown in Fig. 5B, the promoters of cluster 2 genes had increased DNA methylation in *slm12* mutants, but their expression levels were consistently higher in the two biological replicates of *slm12*-46dpa than in WT-46dpa (Fig. 5A). These results demonstrate that the expression of cluster 2 genes is positively correlated with DNA methylation and that the DNA hypermethylation in *slm12* is important for these genes to remain active in the *slm12* mutants. Cluster 2 genes were repressed during fruit ripening in both cv. Micro-Tom and cv. AC (Fig. 5A), and this repression was correlated with a decrease in the DNA methylation level during ripening in both varieties (Fig. 5C). Our data demonstrate that a large number of genes are repressed during ripening and that SIDML2-mediated DNA demethylation is critical for this repression. Thus, SIDML2 has a silencing function for these ripening-repressed genes.

**Functions of SIDML2-Activated and -Repressed Genes.** To understand why SIDML2 is critical for tomato fruit ripening, we performed Gene Ontology (GO) term enrichment analysis of cluster 1 and cluster 2 genes. For cluster 1, genes involved in flavonoid biosynthesis and carotenoid biosynthesis were highly enriched (Table S7). We examined the promoter methylation and expression pattern of genes in the carotenoid biosynthesis pathway, including *PSY1*, *Z-ISO*, *ZDS*, and *CRTISO*, and found that all of them are regulated by SIDML2-mediated DNA demethylation during ripening because all of them were hypermethylated and silenced in the *slm12* mutant (Fig. S64).

Many other genes known to be important for fruit ripening were also found in cluster 1, and these included *PG2a* and *PL* (which are involved in cell wall degradation); *ACS*, *ACO*, and *ETR* (which are involved in ethylene biosynthesis or signaling); and genes encoding ripening-associated transcription factors, such as *RIN* (Fig. S64). A previous study showed that three ripening-related genes, *RIN*, *NOR*, and *PSY1*, are hypermethylated and silenced during ripening in the RNAi lines of DNA demethylases (29). In agreement with this previous finding, these three genes were found in cluster 1 (Table S5). We performed the reverse transcription quantitative PCR (RT-qPCR) to examine the expression patterns of *PSY1* and *ACS4* in fruits of *slm12*-1 mutant and the WT. Consistent with the RNA-seq results, *PSY1* and *ACS4* were highly induced in WT fruits during ripening, but their induction was significantly less in the *slm12*-1 mutant than in the WT at 46 dpa (Fig. 6A, Upper). As shown in Fig. 6A, Lower, the promoters of these two genes were demethylated in the WT but not in the *slm12* mutants during ripening. These data show that SIDML2 is required for ripening-induced demethylation and expression of genes involved in ripening-related processes, including pigment synthesis, flavonoid synthesis, and fruit softening.

CNR is an important transcription factor for tomato fruit ripening, and its expression is known to be regulated by DNA methylation (25). *RIN* binds to the *CNR* promoter in a DNA demethylation-dependent manner (32). Intriguingly, in our study, ripening-induced expression of *CNR* was not silenced by the *slm12* mutation (Fig. S64) even though the *CNR* promoter was hypermethylated in *slm12* mutants (Figs. S4B and S64). This observation suggests that promoter hypermethylation is not sufficient to cause the silencing of *CNR*.



**Fig. 6.** Expression and methylation levels of representative cluster 1 and cluster 2 genes in fruits of the WT and *slm12* mutants. RT-qPCR measurements to validate the expression of two cluster 1 genes, *SIPSY1* and *SIACS4* (A), and two cluster 2 genes, *SICAP10B* and *SIRBCS-2A* (B), in WT and *slm12*-1 mutant fruits at 25 dpa and 46 dpa. *EF1 $\alpha$*  was used as the internal control. The DNA methylation levels of promoter regions of these four genes are shown with screenshots of Integrative Genome Browser (IGB) display of whole-genome bisulfite sequencing data, where each vertical bar represents an mC and the height of the bar indicates methylation level. Error bars indicate SD,  $n = 3$  (\* $P$  value < 0.01, two-tailed  $t$  test).

Our GO enrichment analysis showed that cluster 2 genes involved in photosynthesis and cell wall organization or biogenesis are highly enriched (Table S8). We tested the expression of *CAP10B* (encoding a chlorophyll a/b-binding protein) and *RBCS-2A* (encoding a Rubisco small subunit) by RT-qPCR. The results validated the RNA-seq data in that the expression of these genes was completely inhibited in the WT-46dpa but not in the *slm12* mutant (Fig. 6B, Upper). Integrated genome browser (IGB) screenshots clearly show that the promoter regions of these genes were demethylated in the WT but not in the *slm12* mutants during fruit ripening (Fig. 6B, Lower). Similarly, the *slm12* mutation also disrupted ripening-induced promoter demethylation and silencing of *GPI* (encoding a regulator of polygalacturonase) and *EXPA5* (encoding an expansin) (Fig. S6B).

Fruit development has three stages: cell division, cell enlargement, and ripening. Fruit photosynthesis has been suggested to be important in early fruit development by contributing to fruit growth and production of starch that is turned into soluble sugars during ripening (33). As fruits ripen, however, an important transition takes place: The photosynthetically active chloroplasts differentiate into chromoplasts. Chromoplasts no longer contain chlorophylls but instead produce the lycopene and carotene necessary for fruit color. This transition is coupled with a decreased expression of both nuclear- and plastid-encoded genes

involved in photosynthesis. Our analysis showed that SIDML2 regulates at least 23 genes involved in photosynthesis during ripening, including 6 genes encoding chlorophyll a/b binding proteins and 8 genes encoding proteins of photosystem 1 and 2 reaction centers (Table S6). These genes were repressed during ripening, and this repression coincided with hypomethylation of their promoter. However, the promoter hypomethylation and ripening-induced repression were disrupted or diminished in *sltml2* mutants (Fig. 6B), suggesting a critical role of SIDML2 in the regulation of the chloroplast-to-chromoplast transition during ripening.

In addition to genes involved in photosynthesis, 22 genes involved in cell wall biogenesis were enriched in cluster 2, and these included genes encoding cellulose synthase, expansins, and xyloglucan endotransglucosylases/hyrolases (XETs) (Table S6). In the first stage of fruit development, enzymes involved in cell wall synthesis, such as cellulose synthase, are active. As fruits shift to the second stage, cell walls are loosened via expansins and XETs to allow the cells to expand. As the fruit ripens, genes involved in cell wall biogenesis and organization are no longer required for fruit development and are thus repressed. Our results revealed that these cluster 2 cell wall-related genes are silenced in ripe fruits because of promoter hypomethylation and that the silencing depends on SIDML2-mediated DNA demethylation (Fig. 5 A and B and Fig. S6B). Thus, SIDML2 is required not only for the activation of genes important for ripening but also for the silencing of genes that are needed for the early stages of fruit development, but that must be repressed for ripening to occur.

Zhong et al. (27) showed that genomic regions that are hypomethylated during ripening are enriched with RIN-binding sites. RIN has both transcription activation and transcription repression activities (34). Previous studies identified 137 RIN activation targets and 109 RIN repression targets (34). We found that previously identified RIN activation targets are enriched in cluster 1 genes whereas RIN repression targets are enriched in cluster 2 genes (Table S9).

## Discussion

Cytosine methylation is generally thought to be associated with inactive transcription (i.e., with silencing), especially when the methylation occurs in the promoter region of genes. Therefore, components involved in DNA demethylation are often referred to as antisilencing factors (3). In *Arabidopsis*, dysfunction of the 5-mC DNA glycosylase/demethylase ROS1 enhances the transcriptional silencing of some transgenes, endogenous genes, and TEs due to DNA hypermethylation (14, 16, 19). RNAi of ROS1 orthologs in cotton and tomato also reduce the expression of several genes due to promoter DNA hypermethylation (29, 35). Consistent with the expected role of the tomato ROS1 ortholog SIDML2 as an antisilencing factor, *sltml2* mutations caused the silencing of hundreds of genes (cluster 1 genes in Fig. 5A) in tomato fruits due to DNA hypermethylation. Surprisingly, we also found that SIDML2-mediated DNA demethylation is associated with the repression of hundreds of genes (cluster 2 genes in Fig. 5A) during tomato fruit ripening. Thus far, there are only a few reports of DNA demethylation causing transcriptional silencing. In *Arabidopsis*, the expression of ROS1 is regulated by both ROS1-mediated active DNA demethylation and DNA methylation pathways, including RNA-directed DNA methylation (RdDM) (36). In *met1* and RdDM mutants, *AtROS1* is silenced due to promoter hypomethylation whereas, in *ros1* mutants with point mutations, the *AtROS1* transcript level is enhanced due to promoter hypermethylation (36). Thus, ROS1 expression is promoted by DNA methylation and antagonized by DNA demethylation. In tomato, we discovered that hundreds of genes are repressed by DNA hypomethylation during ripening. The expression of these cluster 2 genes was higher in the fruits of

*sltml2* mutants than in those of the WT. These results have revealed a broad role of DNA methylation in promoting gene expression. The mechanisms underlying the antisilencing role of DNA methylation and the silencing role of DNA demethylation at the cluster 2 genes are unknown. It is possible that a transcriptional activator is recruited to the promoters of the cluster 2 genes by a methyl-DNA-binding protein. It is also possible that DNA methylation prevents the binding of a transcriptional repressor to these genes.

Like *AtROS1*, the tomato SIDML2 also regulates its own promoter DNA methylation (Fig. S4B). However, contrary to *AtROS1*, SIDML2 promoter demethylation is correlated with its transcriptional induction during ripening. It would be interesting to determine whether any of the other SIDML genes may be regulated by DNA methylation like *AtROS1* and may thus function like *AtROS1* as a “methylstat” to maintain DNA methylation homeostasis (36). *AtROS1* antagonizes RdDM in *Arabidopsis* (20). A recent study reported that RdDM targets in tomato are located in gene-rich euchromatin regions (37), which is similar to the distribution of SIDML2 targets (Fig. 3B), suggesting a potential antagonism between SIDML2 and RdDM in tomato as well. The increase in mCHH levels in *sltml2* fruits at ripening-induced hypo-DMRs (Fig. 4C) may be caused by enhanced RdDM in the mutant because ROS1 dysfunction in *Arabidopsis* causes enhanced RdDM (20). RdDM in tomato might also be important for the DNA methylation-dependent expression of cluster 2 genes in immature fruits.

Tomato fruits undergo genome-wide loss of DNA methylation during ripening (27). By characterizing the DNA methylomes of *sltml2* mutant fruits, we found that ~30,000 genomic regions require SIDML2 for demethylation during fruit ripening. In fact, SIDML2 was critical for virtually all ripening-induced demethylation (Fig. 4). Our analyses showed that, among the SIDML2-targeted genes, cluster 1 is enriched in genes involved in the synthesis of pigments and flavor compounds, as well as in ethylene biosynthesis and signaling, and cell wall hydrolysis. These genes are well-known to be required for fruit ripening. In contrast, cluster 2 is enriched with genes involved in photosynthesis and cell wall organization. These cluster 2 genes have important functions in the growth of immature fruits but are no longer needed after ripening and therefore must be repressed during ripening. Despite the genome-wide role of SIDML2 in fruit ripening, many highly methylated genomic regions remain methylated. It would be useful to investigate how SIDML2 is preferentially targeted to genes relevant to fruit ripening. Given that so many genes are affected by SIDML2-mediated demethylation, the tomato fruit would be an excellent system for studying the mechanism underlying the targeting of DNA demethylases to genes.

The mechanism and function of active DNA demethylation has been studied mostly in *Arabidopsis*. *AtROS1*, the predominant DNA demethylase in vegetative tissues in *Arabidopsis*, targets more than 6,000 genomic regions for demethylation (20). Most of these genomic regions are in TEs or intergenic regions. Only a small number of genes are hypermethylated and silenced in *Arabidopsis ros1* mutants, and the mutants do not show dramatic phenotypes in growth or development (7, 14, 19, 21). Like ROS1 in *Arabidopsis* (20), SIDML2 in tomato preferentially targets highly methylated TEs and intergenic regions near genes (Fig. 3C). However, unlike in *Arabidopsis*, the expression of thousands of genes is impaired by DNA demethylase dysfunction in tomato, and the *sltml2* mutants display dramatic phenotypes in fruit ripening. Clearly, the role of active DNA demethylation in regulating gene expression is broader in tomato than in *Arabidopsis*. One reason for this difference is that the tomato genome has a higher content of TEs and other repeats and that more tomato genes than *Arabidopsis* genes contain highly methylated TEs and other repeats in their promoters (Fig. 3 D and E). Therefore, the expression of these tomato genes is more likely to

be regulated by DNA demethylation. Rice genes tend to have mCHH methylation in their promoter regions (38). Our analysis showed that, like the tomato genome, rice and maize genomes also have higher percentages of genes with highly methylated TEs in their promoters compared with the *Arabidopsis* genome (Fig. 3E). These results suggest that active DNA demethylation has a broader effect on gene expression in crops and other plants with large genomes than in *Arabidopsis*.

Our DNA methylome analysis showed that ripening-induced demethylation is abolished at promoters of several well-known fruit-ripening genes, such as *PSY1*, *RIN*, and *CNR* in *slidml2* mutants (Fig. 6A and Figs. S4B and S6A). The *CNR* promoter is a direct RIN-binding target (27, 32). *CNR* expression is substantially reduced in the *Cnr* epiallele, which is thought to be caused by DNA hypermethylation at the *CNR* promoter region (25, 27). Interestingly, in Micro-Tom WT fruit, we observed a high level of DNA methylation at the *CNR* promoter, and the methylation was lost during ripening in a SIDML2-dependent manner (Figs. S4B and S6A). However, even though *RIN* expression was silenced and both *RIN* and *CNR* promoters were hypermethylated in *slidml2* mutant fruits (Figs. S4B and S6A), *CNR* expression was even higher in the mutant fruits than in WT fruits at the same stage (Fig. S6A). In the RNAi lines for *SIDMLs*, induction of *CNR* is also not fully suppressed but is instead delayed for several days during ripening (29). These observations suggest that DNA hypermethylation is not sufficient to silence *CNR* and that RIN binding is not required for *CNR* expression. It may be worthwhile to reexamine the role of DNA hypermethylation at the *Cnr* epiallele: e.g., by using CRISPR/dCas9 to target SIDML2 to specifically demethylate the *CNR* promoter in the epiallele.

In summary, our results demonstrate that SIDML2 is critical for tomato fruit ripening because it mediates all ripening-induced DNA demethylation. This genome-wide demethylation is important not only for activating the expression of ripening factors but also for silencing hundreds of genes that are no longer needed and must be repressed when the fruit is ripe.

## Materials and Methods

**Plant Materials.** All plant materials used in this study were first generation (T0) plants regenerated from callus. Tomato cv. Micro-Tom was obtained from Ball. Tomato plants were grown in a greenhouse at Purdue University, West Lafayette campus. Tomato tissue culture was performed according to the established protocol (39). All tissue culture steps were conducted at room temperature with a photoperiod of 16 h light and 8 h dark.

**CRISPR/Cas9 Gene Editing.** A 19-bp sgRNA oligo (5'-GAACAAAGTCTGAAATGTG-3') targeting the first exon of *SLDML2* was cloned into the psgR-Cas9-At vector (31), and the cassette including chimeric RNA driven by the AtU6 promoter and Cas9 driven by the AtUBQ1 promoter was subcloned into the pCambia1300 binary vector. The construct was transformed into tomato cv. Micro-Tom using *Agrobacterium* infection of leaf explants.

**Whole-Genome Bisulfite Sequencing and Analysis.** Genomic DNA was extracted from tomato fruits using a DNeasy Plant Maxi Kit (Qiagen) and then was used for library construction using Illumina's standard DNA methylation analysis

protocol and the NEB Ultra II DNA Library Prep Kit. The samples were sequenced at the Genomics Core Facility of the Shanghai Centre for Plant Stress Biology, Chinese Academy of Sciences with Illumina HiSeq2500.

For data analysis, low-quality sequences ( $q < 20$ ) were trimmed using trim in BRAT-BW (40), and clean reads were mapped to the reference genome using BRAT-BW, allowing two mismatches. The reference genome version is SL2.50 ([http://ftp.solgenomics.net/tomato\\_genome/assembly/build\\_2.50/](http://ftp.solgenomics.net/tomato_genome/assembly/build_2.50/)) (41).

To remove potential PCR duplicates, the remove-dupl command of BRAT-BW was used. DMRs were identified using the same method as in ref. 20. In brief, only cytosines with at least 4 $\times$  coverage in all libraries were considered. A sliding-window approach with a 200-bp window sliding at 50-bp intervals was used to identify DMRs. Fisher's exact test was performed for methylated vs. unmethylated cytosines for each context within each window, with false discovery rates (FDRs) estimated using a Benjamini-Hochberg adjustment of Fisher's *P* values calculated in the R environment. Windows with an FDR  $\leq 0.05$  were considered for further analysis, and windows within 100 bp of each other were merged into larger regions. Regions were then adjusted to shrink to the first and last differentially methylated cytosines (DMCs). A cytosine was considered a DMC if it showed at least a twofold change in methylation percentage in the mutant. The regions were then filtered to include only those with at least 10 DMCs and with at least a twofold change in the arithmetic mean of methylation percentage of all cytosines.

**RNA Analysis.** Total RNA was extracted with TRIzol reagent (Ambion) from pericarps of fruits.

For reverse transcription, 1  $\mu$ g of RNA and oligo dT primers were used to synthesize cDNA in a 20- $\mu$ l reaction using the qScript cDNA SuperMix kit (Quanta). Real-time PCR was then carried out on a CFX96 Real-Time PCR Detection System (Bio-Rad) with PerfeCTa SYBR Green FastMix (Quanta). *EF1 $\alpha$*  was used as an internal control. Primers used in q-PCR reactions are listed in Table S10.

For RNA-seq, the libraries were constructed and sequenced at the Genomics Core Facility of the Shanghai Centre for Plant Stress Biology, Chinese Academy of Sciences, with an Illumina HiSeq2500.

For RNA-seq data processing, quality control was checked using FastQC ([www.bioinformatics.babraham.ac.uk/projects/fastqc](http://www.bioinformatics.babraham.ac.uk/projects/fastqc)). RNA-Seq reads were trimmed using the fastx\_trimmer command FASTX-Toolkit ([hannonlab.cshl.edu/fastx\\_toolkit/index.html](http://hannonlab.cshl.edu/fastx_toolkit/index.html)) with parameter "-f 14 -l 80" before alignment. The trimmed reads were aligned to the tomato genome using TopHat2 (42). The ITAG2.4 annotation gff3 file was supplied as the "-G" option to TopHat2. The program featureCounts (43) was used to count the mapped fragments for each gene. The output count table was used as the input for edgeR (44) to compute the log<sub>2</sub>wfold change of each gene.

DMR-associated genes were defined as genes with DMRs within 1 kb. For gene clustering, we used only DMR-associated genes that had transcripts per kilobase million (TPM)  $\geq 5$  in at least one sample. The input data had two columns: log<sub>2</sub>wfold-change WT-46dpa vs. WT-25dpa and log<sub>2</sub>wfold-change *slidml2*-46dpa vs. WT-46dpa. The Kmeans function of R was used for clustering.

The heatmap was generated using Matrix2png (45).

**Data Deposition.** The data discussed in this publication have been deposited in the NCBI's Gene Expression Omnibus and are accessible through the GEO Series accession number GSE94903. Previously published data used in this study were from Zhong et al. (27).

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