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A DEMETER-like DNA demethylase protein governs tomato fruit ripening

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Author contributions:

REL: T2 plant phenotype analysis, metabolomics, isoprenoid analysis, molecular biology analysis**, LS:** Plant transformation, gene expression analysis, phenotype of T1 and T2 plants, **ET:** McrBC PCR Analysis, plant phenotype, writing paper**, AHK, JT**: BS pyrosequencing of NOR, CNR and PSY1 promoter fragments; **NC:** targeted BS sequencing; **CDG**: DML gene expression analysis; **SH:** T1 transgenic plants; **AB:** carotenoid analysis; **DR, MM:** primary metabolite analysis; **ML, MB:** ethylene analysis; **YH:** VIGS experimental design and writing paper; **JK, CW**: VIGS

experiments; **C H**: rin, nor, cnr and WT microarrays **GBS:** targeted BS seq, writing paper; **JG**: data analysis, writing paper **PG:** experimental design, work coordination, McrBC PCR analysis and writing paper.

Keywords: DNA methylation, fruit ripening, tomato, Demeter like

Abstract

In plants, genomic DNA methylation which contributes to development and stress responses can be actively removed by DEMETER-like DNA demethylases (DML). Indeed, in Arabidopsis DMLs are important for maternal imprinting and endosperm demethylation, but only few studies demonstrate the developmental roles of active DNA demethylation conclusively in this plant. Here we show a direct cause and effect relationship between active DNA demethylation mainly mediated by the tomato DML, SlDML2, and fruit ripening; an important developmental process unique to plants. RNAi SlDML2 knock-down results in ripening inhibition via hypermethylation and repression of the expression of genes encoding ripening transcription factors and rate-limiting enzymes of key biochemical processes such as carotenoid synthesis. Our data demonstrate that active DNA demethylation is central to the control of ripening in tomato.

Significance Statement

This work shows that active DNA demethylation governs ripening, an important plant developmental process. Our work defines a molecular mechanism, which has until now been missing, to explain the correlation between genomic DNA demethylation and fruit ripening. It demonstrates a direct cause and effect relationship between active DNA demethylation and induction of gene expression in fruits. The importance of these findings goes far beyond understanding the developmental biology of ripening and provides a completely new strategy for its fine control through fine modulation of epimarks in the promoters of ripening related genes. Our results have significant application for plant breeding especially in species with limited available genetic variation.

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Introduction

Genomic DNA methylation is a major epigenetic mark that is instrumental to many aspects of chromatin function, including gene expression, transposon silencing or DNA recombination (1-4). In plants, DNA methylation can occur at cytosine both in symmetrical (CG or CHG) and non-symmetrical (CHH) contexts, and is controlled by three classes of DNA methyltransferases, namely, the DNA Methyltransferase 1, Chromomethylases and the Domain Rearranged Methyltransferases (5-7). Indeed, in all organisms cytosine methylation can be passively lost after DNA replication in the absence of methyltransferases activity (1). However, plants can also actively demethylate DNA via the action of DNA Glycosylase-Lyases, the so-called DEMETER-Like proteins that remove methylated cytosine which is then replaced by a non-methylated cytosine (8-11). Initially identified as enzymes necessary for maternal

imprinting in Arabidopsis thaliana (12), DML implication has since been established in various processes like limiting extensive DNA methylation at gene promoters (13), determining the global demethylation of seed endosperm (8, 14) and promoting plant responses to pathogens (15). Noteworthy, Arabidopsis ros1, dml2 and dml3 single, double or triple mutants showed little or no developmental alterations (9, 16, 17), suggesting that active DNA demethylation is not critical for development in this species. However, as mentioned above, genomic DNA methylation is an important mechanism that influences gene expression, and methylation at promoters is known to inhibit gene transcription (5, 18). Hence, it is likely that the active removal of methylation marks is an important mechanism during plant development and plant cell fate reprogramming, leading to the hypomethylation of sites important for DNA-protein interaction and gene expression as already observed in human cells (19).

Indeed, accumulating evidence suggests that active DNA demethylation might play a greater role in controlling gene expression in tomato. In support of this idea, recent work describing the methylome dynamics in tomato fruit pericarp revealed substantial changes in the distribution of DNA methylation over the tomato genome during fruit development, and demethylation during ripening at specific promoters such as the NOR and CNR promoters (20, 21). This is consistent with previous studies indicating that genome cytosine methylation levels decrease by 30% in pericarp of fruits during ripening, although DNA replication is very limited at this stage (22).

Here we investigated active DNA demethylation as a possible mechanism governing the reprogramming of gene expression in fruit pericarp cells at the onset of fruit ripening.

Manuscript text

The tomato genome contains four DNA glycosylase genes with specific expression patterns.

The tomato genome contains four putative DML genes encoding proteins with characteristic domains of functional DNA glycosylase-lyases (23) (**SI Appendix, Fig.S1A, C; Table S1**). SlDML1 and 2 are orthologous to Arabidopsis AtROS1 gene, SlDML3 to AtDME whereas SlDML4 has no closely related Arabidopsis ortholog (**SI Appendix, Fig.S1B**). All four SlDML genes are ubiquitously expressed in tomato plants although SlDML4 is expressed at a very low level in all organs analyzed**.** In leaves, flowers and young developing fruits, they present coordinated expression patterns characterized by high expression levels in young organs that decrease when organs develop. However, unlike SlDML1, SlDML3 and SlDML4 that are barely expressed during fruit ripening, SlDML2 mRNA abundance increases dramatically in ripening fruits, suggesting an important function at this developmental phase (**Fig. 1**).

Transgenic plants with reduced DML gene expression present various fruit and plant phenotypes.

The physiological significance of tomato DMLs was addressed through RNAi-mediated gene repression using the highly conserved HhH-GPD domain specific to DML proteins as a target sequence (**SI Appendix, Fig.S2A**). Our goal was to repress simultaneously all tomato SlDML genes, anticipating potential functional redundancy among these four genes. 23 independent T0 transgenic lines were generated and 22 showed alterations of fruit development including delayed ripening, modified fruit

shape, altered color, shiny appearance, parthenocarpy or combinations of these phenotypes (**Fig. 2A**).

Lines 2 and 8 that showed delayed and inhibited ripening phenotypes were chosen to investigate the possible link between ripening and DNA demethylation. In both cases 10 to 25 T1 and T2 plants were grown that showed maintenance and strengthening of the non-ripening phenotypes in subsequent generations coincident with the presence of the transgene. The loss of the RNAi transgene in segregating lines led to reversion to a wild type (WT) phenotype indicating a lack of memory effect across generations when fruit ripening is considered (**Fig. 2A-B; SI Appendix, Fig.S3A)**. In plants of both RNAi lines, analysis of SlDML gene residual expression in 20 days post anthesis (dpa) fruits indicates that only SlDML1 and SlDML2 are repressed to 40 to 60% of the WT level, whereas SlDML3 and SlDML4 are either unaffected or induced as compared to WT (**Fig. 3A**). This is most likely due to the lower homology level of these two genes with SlDML1 in the part of the gene used for the RNAi construct (**SI Appendix, Fig.S2A**). During ripening, SlDML2 expression is reduced to 10 % of WT at the Br stage and remains low at 55 dpa (Br+16), but increases slightly at 70 dpa (Br+31) (**Fig. 3B, SI Appendix, Fig.S2B)** coincident with the partial ripening observed in transgenic RNAi fruits (**Fig. 2C; SI Appendix, Fig.S3B**). Whether the increase in SlDML2 expression at late ripening stages is due to a weaker effect of the RNAi remains unclear. None of the three remaining genes, SlDML1, SlDML3 and SlDML4, which are weakly expressed during ripening, displayed significantly reduced expression as compared to WT fruit of the same age indicating that observed ripening phenotypes are likely due to SlDML2 gene repression. This hypothesis was further confirmed using VIGS to specifically repress the SlDML2 gene. 17.5 % of the fruits injected with a PVX/SlDML2 vector presented non ripening sectors contrary to those injected with a control PVX virus that all ripened normally (**Fig. 2E**; **SI Appendix, Fig.S4A**). Indeed, SlDML2 was down regulated in non-ripening sectors of fruits injected with the PVX/SlDML2 vector, whereas none of the three other SlDML genes was repressed (SI Appendix, Fig.S4B), demonstrating that the specific knock down of SlDML2 is sufficient to inhibit ripening.

It was noteworthy that some plants from line 2 developed additional phenotypes affecting plant growth, leaf shape, flower development and fruit carpel number that were not observed in T0 and T1 generations (**Fig. 2D**, **SI Appendix, Fig.S3B-C)**. The screening of additional lines revealed other independent transgenic lines that presented flower, fruit and plant phenotypes similar to line 2 **(SI Appendix, Fig.S3D)**. These observations indicate that the severity of the phenotypes increases over generations, and suggest that DMLs may also be involved in other aspects of tomato plant development beyond fruit ripening.

All aspects of fruit ripening are delayed and limited in RNAi transgenic lines

Fruits of transgenic lines 2 and 8 were further analyzed to investigate the consequences of DNA demethylation on the ripening process. Indeed, in fruits of both transgenic lines, the onset of fruit ripening was delayed from 10 to 20 days as compared to WT or Azygous revertant fruits, and ripening of transgenic fruits was never completed even after 45 days or longer maturation times (**Fig. 2B-C**; **SI Appendix, Fig.S3B**). The ripening defect is further demonstrated by the late and extremely reduced total carotenoids and lycopene accumulation, and the delayed chlorophyll degradation

(Fig. 4A). Primary metabolite composition was also modified as visualized by Principal Component Analysis (PCA) using the absolute concentration of 31 primary metabolites issued from ^{1}H-NMR analysis **(Fig 4B, SI Appendix, Fig.S5A).** The first two Principal Components (PC), explain more than 54 % of total variability. During early development (20, 35 and 40 dpa), WT and transgenic samples follow parallel trajectories as highlighted by the PCA in which the second principal component (PC2) explains 21% of the total variability. However at 55 dpa and later ripening stages, PC1 which accounts for 33.67% of the global variability, separates WT fruits from all other samples. Hence, WT fruit samples harvested at 55 dpa and older stages are clearly distinct from transgenic fruit samples of the same age. Metabolic differences between ripening WT and transgenic fruits are mainly due to over accumulation of malate and reduction or delayed accumulation of compounds typical of ripening fruits including glucose, fructose, glutamate, rhamnose and galactose (**SI Appendix, Fig.S 5B-D**). Climacteric rise of ethylene production was also dramatically reduced in fruits of both DML RNAi lines, though low ethylene accumulation occurred to a degree and timing consistent with the late and limited ripening process of RNAi fruits (**SI Appendix, Fig.S 6**).

Fruit ripening defects are correlated with the repression and hypermethylation of genes necessary for this developmental process.

To demonstrate a causal relationship between fruit ripening defects of transgenic lines and the impairment of active DNA demethylation, the expression of COLOURLESS NON RIPENING (CNR) (21), RIPENING INHIBITOR (RIN) (24), NON RIPENING (NOR) (25) and PHYTOENE SYNTHASE 1 (PSY1) (26,27) genes was assessed in RNAi transgenic plants. These genes were selected among others because they are necessary for the overall ripening process (CNR, RIN, NOR), or specifically govern carotenoid accumulation (PSY1), an important quality trait of mature tomato fruit. Moreover, their promoter regions showed reduced methylation levels during fruit ripening in WT tomato (20,21). It is noteworthy that CNR gene induction was delayed 15 days in transgenic fruits and all three other genes showed a dramatic reduction in expression level consistent with the ripening defect of the transgenic lines (**Fig 5A, SI Appendix, Fig.S7**). To assess whether repression of CNR, RIN, NOR and PSY1 gene expression in ripening fruit results from the maintenance of a high cytosine methylation status of their promoter upon down-regulation of SlDML2, McrBC-PCR analysis of the corresponding promoters was performed. This approach revealed a ripening-associated demethylation of the RIN, NOR and PSY1 promoters in WT and Azygous revertant fruits but not in SlDML RNAi fruits (**Fig. 5B**). No detectable variations of methylation in the CNR promoter during ripening of WT fruits were revealed with this method. The putative Differentially Methylated Regions (DMRs) in the NOR and PSY1 promoter regions were subsequently analyzed by gene specific Bisulfite Pyrosequencing (28). Methylation analysis of the CNR promoter was targeted to a region known to be methylated at all stages (CNR1, SI Appendix, Fig.S9C) used here as a control for methylation and to a previously identified DMR (CNR2, SI Appendix, Fig.S9C) (20, 21). For all 3 promoters, cytosines that became demethylated in ripening WT fruits but not in transgenic fruits of the same age were identified (**Fig. 6A; SI Appendix, Fig.S9**). Two distinct situations were observed: (i) sequences corresponding to putative RIN Binding Sites (RIN BS) in the CNR and NOR promoters (20) where methylation is high at 20 and 35 dpa in all plants analyzed and drops to very low levels during ripening of

WT fruits but is maintained to high levels in RNAi fruits of the same age; (ii) sequences that are hypermethylated in transgenic fruits at all stages analyzed compared to WT fruits. These latter sequences include a newly identified DMR in the PSY1 promoter and cytosines upstream and downstream to the RIN BS in the NOR and CNR promoters**.** These data demonstrate the absolute requirement of promoter demethylation in critical genes for ripening to occur. They also suggest multiple patterns of cytosine demethylation occurring either specifically during ripening or at earlier stages.

Discussion

Previously reported analysis of DNA cytosine methylation and RIN binding during fruit development in WT and in the rin and Cnr tomato ripening mutants suggested a significant role for DNA methylation during ripening and a feedback loop between methylation and ripening transcription factors (20, 21, 29). Here we demonstrate for the first time that active DNA demethylation is an absolute requirement for fruit ripening to occur and show a direct cause and effect relationship between hypermethylation at specific promoters and repression of gene expression. In this context SlDML2 appears to be the main regulator of the ripening associated DNA demethylation process. (1) It is the only SlDML gene induced concomitantly to the demethylation and induction of genes that control fruit ripening, (2) its specific knock down in VIGS treated fruits leads to inhibition of fruit ripening similar to DML-RNAi fruits and (3) the hypermethylayed phenotype described in the Cnr and rin mutants (20) is associated with the specific repression of SlDML2; none of the other SlDML genes being down regulated (Fig 6B).

Indeed, we cannot formally rule out that SlDML1, which is repressed in the transgenic RNAi lines, also participates in the genomic DNA demethylation in fruits. However, SlDML1 is mainly expressed at early stages of fruit development and only at very low levels during fruit ripening. Hence, this protein may also be involved in demethylation events, but mainly those occurring at the early stages of fruit development.

In addition to genes encoding major fruit ripening regulators, those encoding enzymes involved in various aspects of fruit ripening are also likely to be demethylated as suggested by the observation that PSY1 gene expression also requires demethylation. Combined transcriptomic,methylome and metabolome analysis of the transgenic lines described here will now be required to determine the network of genes and metabolic processes primarily targeted by demethylation in tomato fruit.

SlDML2 is the likely focal point of a feedback regulation on ripening-associated DNA demethylation, as this gene is clearly down regulated in fruits of the rin, nor and Cnr mutants, contrary to the other SlDML genes that are normally expressed (**Fig. 6B, C; Dataset S1).** It is plausible that timing and extent of demethylation may represent an important source of variation in the diversity of kinetics and intensity of ripening found among tomato varieties, thus presenting a frontier for further investigation. Controlling the timing and kinetics of active DNA demethylation in fruits may therefore provide new strategies to enhance fruit shelf life. In addition, engineering DNA demethylation in tomato fruits would be an innovative and novel strategy for the improvement of traits of agronomical relevance in a species with little genetic diversity (30). Finally, the recent demonstration that hypermethylation of a Myb promoter blocks anthocyanin accumulation during pear and apple ripening (31, 32) supports the notion of a more

general role for demethylation in fruits. However, whether this mechanism occurs similarly during the ripening of all fleshy fruit species requires now further investigation.

Materials Methods

Plant material and experimental plan

All experiments were performed using a cherry tomato variety (Solanum lycopersicum, cv WVA106), that was grown in greenhouse conditions, except for VIGS experiments that were performed on Solanum lycopersicum , cv Ailsa Craig grown in growth chambers as described (21). For the array experiments fruit pericarp of Ailsa Craig and near isogenic mutants rin, nor and Cnr were collected at 13 stages of fruit development and ripening with three independent biological replicates per line and immediately frozen in liquid nitrogen for RNA extraction and array analysis. Details of tomato transformation, selection of line 2 and 8 used in this study and of VIGS experiments are provided in SI Appendix, SI materials and methods.

For all analysis, two independent transgenic T2 plants (DML2A, B and DML8A, B for line 2 and 8 respectively) and an azygous plant obtained from line 8 were used. Additional T2 plants were eventually used as control for the phenotypes of these 4 plants. T2 plants from line 2 presented dramatic alterations of flower development, not visible in previous generations, and were backcrossed to allow fruit development. This resulted in a limited number of fruits (see below). For this reason not all developmental stages could be analyzed for this line.

The experimental plan was designed to span tomato fruit development and ripening in cv West Virginia 106 (WVA106) and transgenic DML RNAi plants over a period of 85 days from fruit set to account for the strongly delayed ripening phenotype of the transgenic fruits. At stages following mature green, the DML RNAi fruits diverge from the wild type, as they are significantly delayed in ripening induction and almost completely ripening inhibited. As it was not possible to select stages equivalent to the Breaker (39 dpa) or red ripe stages in the transgenic lines we have chosen to analyze fruits identically staged which allows comparing changes in the context of a developmental parameter (days post anthesis) that can be precisely measured. Two independent cultures were performed. (1) Plants from line 2 and the relevant WT control (WT1): fruits were harvested at 20, 35, 55 (Br+16), 70 (Br+31) and 85 (Br +46) dpa. As fruit yield was reduced in line 2, a sufficient number of fruits at the Br stage could not be harvested and older fruits were preferentially selected to allow the analysis of late effects of demethylation inhibition. (2) Line 8 was grown together with its own WT control (WT2) and an azygous plant. As there were more fruits available for this line the Br stage (39 dpa) was harvested in addition of the stages used for line 2.

For all fruit samples, 2 individual T2 plants were used, and for each sample a minimum of six fruits separated in 3 biological replicates were processed and stored at -80°C until used.

Molecular and metabolites Analysis

Details of molecular (gene expression, microarrays, McrBC-PCR analysis of gene DNA methylation and gene targeted Bisulfite sequencing) and metabolites (Carotenoid, ethylene, ^{1}H-NMR) analysis are provided in SI Appendix, SI materials and methods.

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Authors have no competing financial interest.

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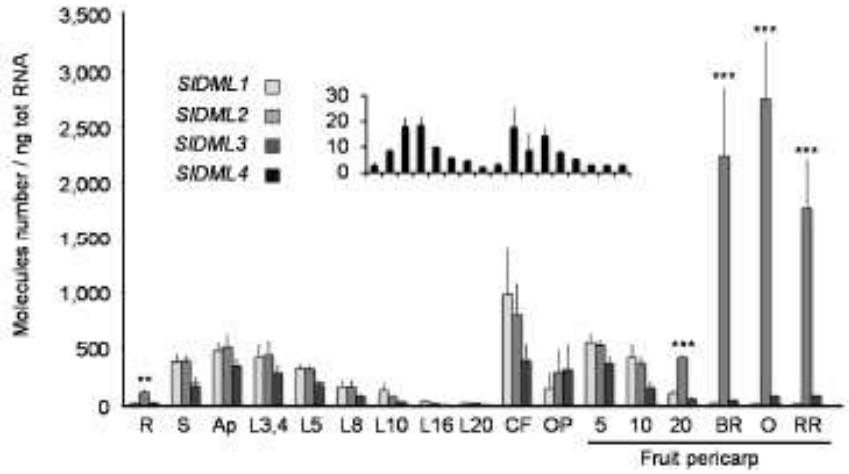

Figure 1: Differential expression of SlDML genes in tomato organs. Absolute quantification of SlDML1, SlDML2, SlDML3 and SlDML4 mRNA (33); SlDML4 gene expression is presented in a separate diagram because of its very low expression level. R: Roots, S: Stem from whole seedlings, Ap: stem apex; L: leaves at position 3-4, 5, 8, 10, 16, 20 from apex; CF: closed flowers; OF: open flowers, 5, 10, 20; Fruit pericarp at 5, 10, 20 dpa, and at Breaker (Br-39 dpa), Orange (O) and Red Ripe (RR). Stars indicate significant difference (student's t test (n=3)) between SlDML2 and all other SlDML genes (*: $p<0.05$; **: $p<0.01$; ***$p<0.001$). Error bars; mean+/- sd.

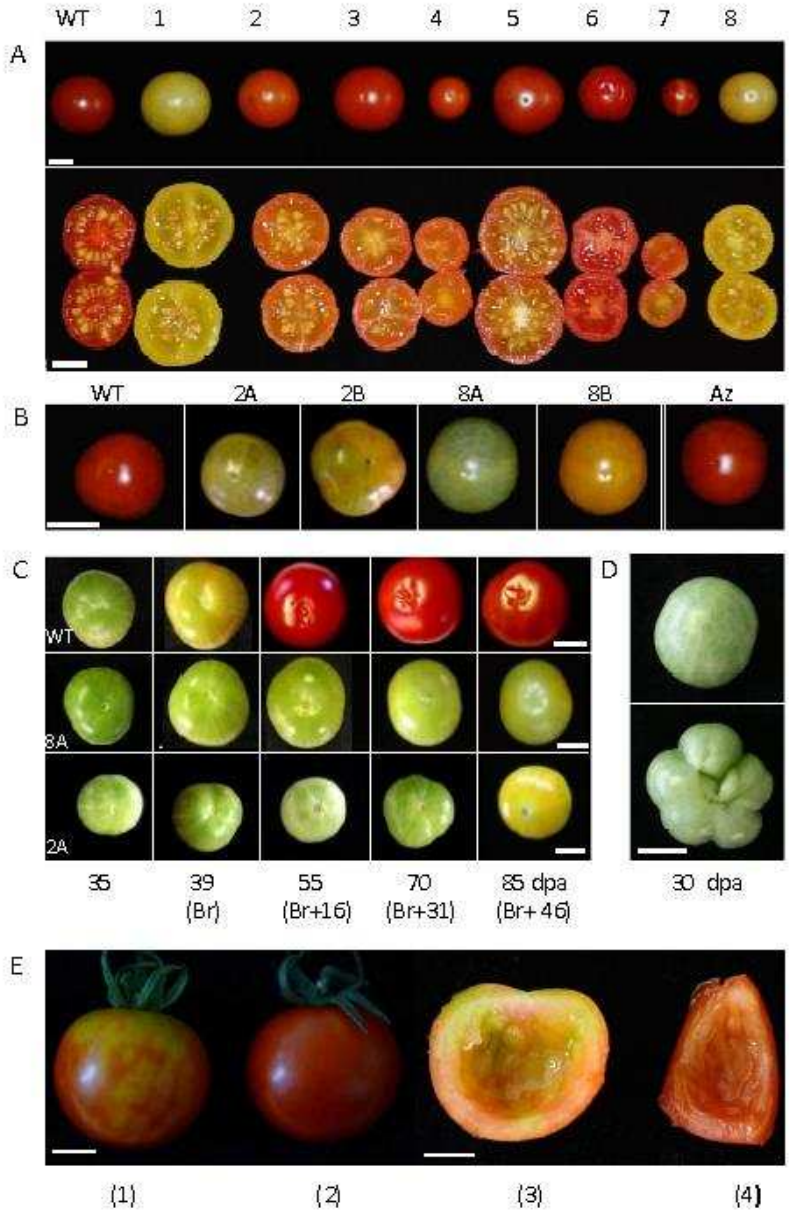

Figure 2: Phenotypes of tomato DML RNAi fruits. (A) 70 days post anthesis (dpa) old fruits (upper lane) or fruit sections (lower lane) from 8 independent representative T0 RNAi plants. (B) Fruits (85 dpa) from T2 plants (left to right); WT, line 2 plants DML2A, DML2B and line 8 plants DML8A, DML8B and an azygous plant (AZ). (C) Ripening kinetics of WT (upper) DML8A (middle) and DML2A (bottom). (D) WT bicarpel (top) DML2B multi-carpel fruits (bottom). (E) VIGS experiment on 47dpa (Br + 5) old fruits injected with PVX/SlDML2 (1, 3) or PVX (2, 4) at 12dpa, (3-4) inside of fruits (1) and (2) respectively. Bars: 1cm.

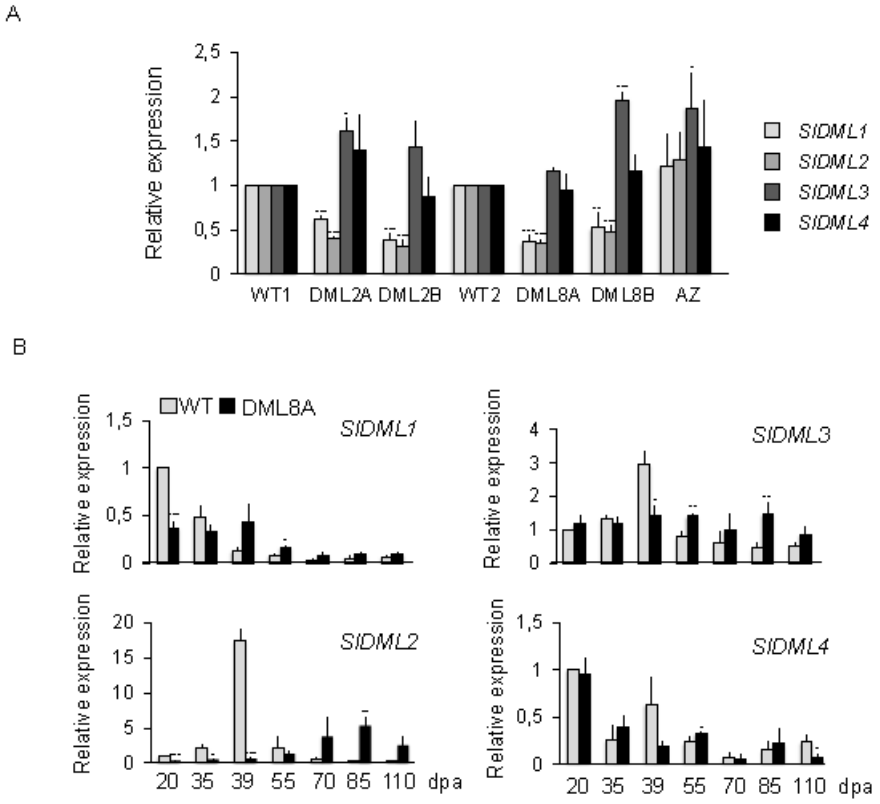

Figure 3: Residual expression of SlDML genes in fruits of transgenic DML RNAi plants. Normalized expression of the SlDML genes (A) in 20 dpa transgenic fruits of plants from line 2 (DML2A, 2B), line 8 (DML8A, 8B), an azygous plant (AZ) and the respective WT1 and WT2 controls (B) in WT2 and DMLA8A fruits at 7 developmental stages. Expression of the SlDML genes was normalized to EF1 and to the corresponding WT fruits at 20dpa. For each SlDML gene, stars indicate significant difference (student's t test (n=3)) between transgenic plants and WT controls respectively at 20 dpa (a) or at the same age during fruit development (b). (*: $p<0.05$; **: $p<0.01$; ***$p<0.001$). Error bars; mean+/- sd.

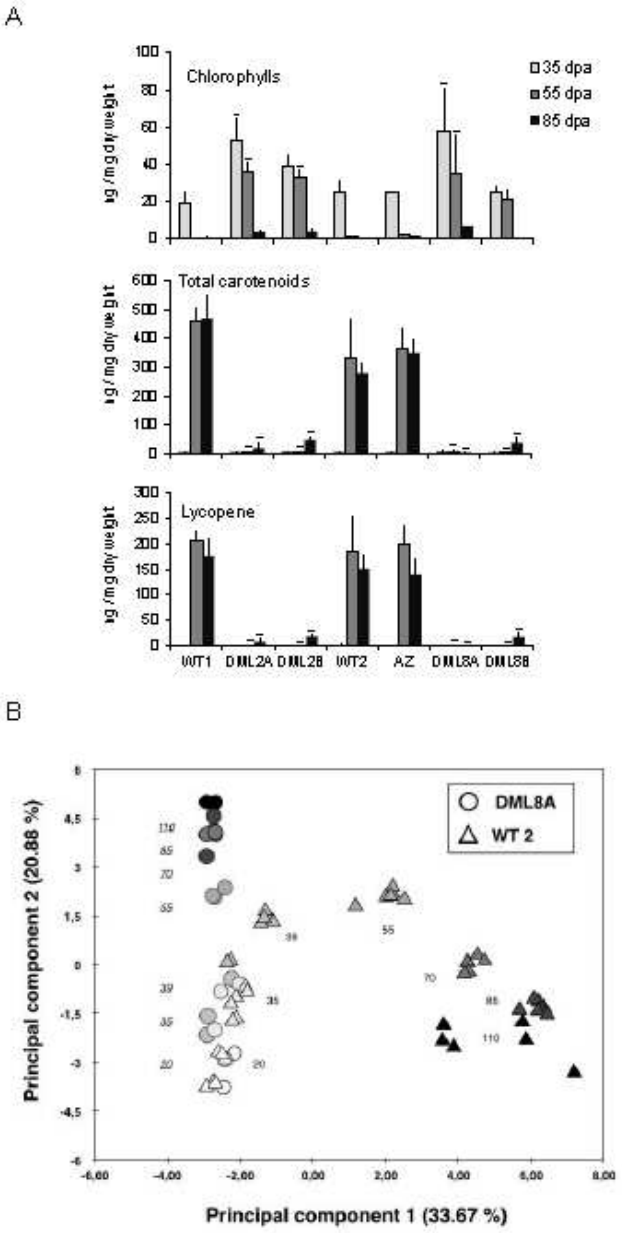

Figure 4: Metabolic profiling of carotenoids and primary metabolites in transgenic DML RNAi fruits. (A) Chlorophylls (upper panel), total carotenoids (middle panel) and lycopene (lower panel) content. Stars indicate significant difference (student's t test (n=3)) between DML2A, B, DML8A, B and WT1 and WT2 respectively at the same age (*: $p<0.05$; **: $p<0.01$; ***$p<0.001$). Error bars; mean+/- sd. (B) Principal Component Analysis using primary metabolites in WT2 (∆) and DML8A (o) fruits at 7 developmental stages.

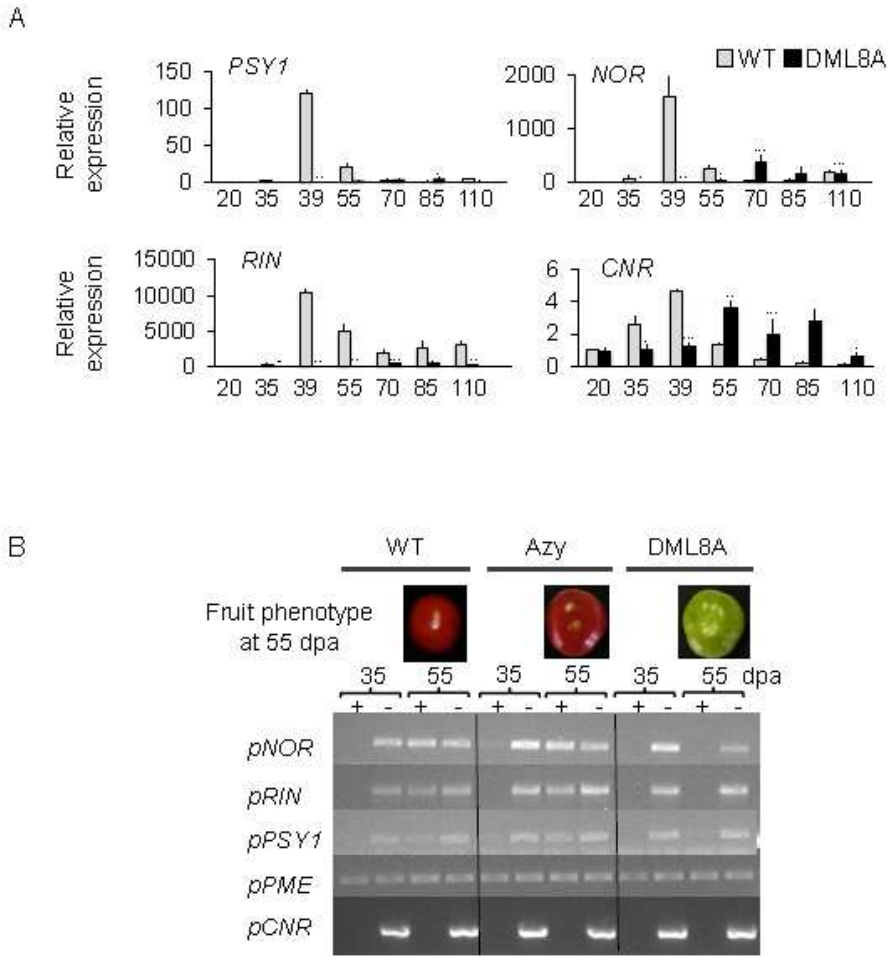

Figure 5: Expression and demethylation at key genes controlling ripening are inhibited in DML RNAi plants. (A) Expression of the RIN, NOR, CNR, PSY1 genes in transgenic DML8A and WT fruits normalized to EF1 and to WT fruits at 20dpa. Stars indicate significant difference (student's t test (n=3)) between WT and DML8A samples at a given stage (*: $p<0.05$; **: $p<0.01$; ***$p<0.001$); Error bars; mean+/- sd. (B) McrBC - PCR analysis of selected promoter fragments in fruits of WT, azygous (Azy), and DML8A plants. 1µg genomic DNA was digested with McrBC (NEB) during 5h (+). (–) indicate negative control for the digestion reaction that was performed without GTP. In the WT and azygous plants the part of NOR, RIN and PSY1 promoter regions analyzed are methylated at 35 dpa (no amplification) but are demethylated at 55 dpa (amplification). In DML8A plants, the three promoter regions behave similarly to WT at 35 dpa, but remained methylated at 55 dpa (no amplification in both cases). The pectin-methyl esterase (PME) promoter is used as an un-methylated control and the CNR promoter fragment used here was found to be sufficiently methylated at all stages for complete digestion by McrBC.

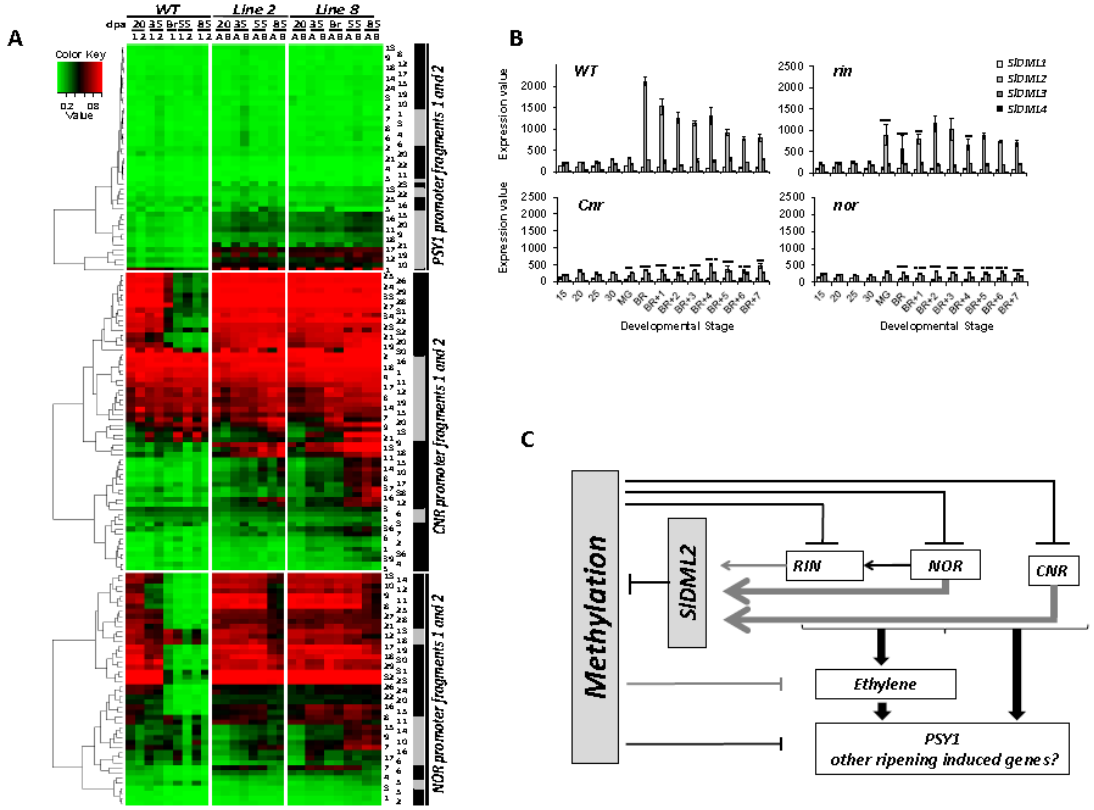

Figure 6: Bisulfite sequencing analysis at the NOR, CNR and PSY1 promoter fragments in WT and transgenic DML RNAi plants. (A) Heat map representation of DNA methylation at selected NOR, CNR and PSY1 promoter regions (SI Appendix, Fig.S8) in fruits of control (WT1, WT2) and transgenic (DML2A, 2B, 8A, 8B) plants at 5 developmental stages. For each promoter, two fragments have been analyzed (Fragment 1: grey box; Fragment 2: black box), the position of which are shown in SI Appendix, Fig. S8 and Fig. S9. The position of the Cs within each promoter fragment is also shown (number in the columns on the right side) as defined in SI Appendix, Fig.S8. For each promoter, Cs have been clustered considering the two PCR fragments analyzed together (B) Changes in expression of SlDML genes in fruits of Ailsa Craig (WT) and near isogenic mutant lines rin, Cnr and nor as determined by microarrays analysis. For fruit development days post anthesis (dpa) are shown. Mature green is 40 dpa in Ailsa Craig and then Breaker is 49 dpa. For non-ripening mutants Br onward are 49 dpa + 1 to 7 days. Stars indicate significant difference (Variance ratio F- tests) between WT and mutant lines for the SlDML2 gene only to avoid overloading the figure (*: $p<0.05$; **: $p<0.01$; ***$p<0.001$). Details of expression results and statistical analyses for all 4 genes are provided in Dataset S1. Error bars; mean+/- sd (C) Proposed function of DNA demethylation in the control of fruit ripening, SlDML2 is necessary for the active demethylation of the NOR, CNR RIN and PSY1 promoter region thereby allowing these gene expressions. SlDML2 gene expression is reduced in the rin, nor and Cnr background suggesting a regulatory loop. There is at this time no evidence of direct regulation of the SlDML2 gene by the RIN, NOR or CNR protein. SlDML2 may

control the expression of additional ripening induced gene as shown in this study for the PSY1 gene and suggested by the demethylation of several promoters during fruit ripening (20). Arrows: activation, line: repression, Black: direct effects, grey: direct or indirect effects.