Methylation Profiling of Biosynthetic Genes Reveals The Role of D-Galacturonic Acid Reductase In Ascorbic Acid Accumulation In Tomato Fruit

Yaru Wang
Huazhong Agricultural University
Ying Wang
Huazhong Agricultural University
Fangman Li
Huazhong Agricultural University
Lele Shang
Huazhong Agricultural University
Jinbao Tao
Huazhong Agricultural University
Xingyu Zhang
Huazhong Agricultural University
Haiqiang Dong
Huazhong Agricultural University
Wenxian Gai
Huazhong Agricultural University
Yuyang Zhang (yyzhang@mail.hzau.edu.cn)
Huazhong Agricultural University https://orcid.org/0000-0002-5341-0682
Zongjun Ren
Huazhong Agricultural University

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Abstract

Ascorbic acid (AsA) is an important nutrient component contributing to major flavor value of tomato fruit and human health. Although transcription regulation of AsA biosynthetic genes have been well demonstrated, epigenetic modification underlying AsA accumulation remains unclear. In this study, we exposed immature tomato fruits to a methyltransferase inhibitor (5-azacytidine) and detected the impacts on AsA accumulation. Inhibition of DNA methylation enhanced AsA accumulation in tomato leaves and fruits. We further isolated a AsA biosynthetic gene, *SlGalUR5*, which encodes a D-galacturonic acid reductase. *SlGalUR5* showed reduced DNA methylation levels and higher transcription levels in *Slmet1* mutant while have converse pattern in *Sldml2* mutant. 5-azacytidine treatment significantly decreased DNA methylation levels of *SlGalUR5* in fruits. Conversely, transcription profiles of *SlGalUR5* and enzyme activity of GalUR were enhanced in 5-azacytidine–treated fruits. Our finding revealed a new insight into epigenome modification of *SlGalUR5* involved in ascorbic acid accumulation and provide a potential means of increasing AsA levels for tomato breeding.

Introduction

In plants, L-ascorbic acid(AsA)is a multifunctional molecule that serves critical roles in development processes and stress response (Smirnoff, 2000; Arrigoni and Tullio, 2002; Barth et al., 2006; Gest et al., 2013). As a primary antioxidant, L-ascorbic acid keeps active oxygen under normal levels to protect plants in photosynthesis (Smirnoff, 2000). L-ascorbic acid is also involved in regulation of flowering, fruit development, senescence and response to biotic and abiotic stress (Conklin and Barth, 2004; Barth et al., 2006; Gallie, 2013). For human health, L-ascorbic acid provide beneficial effects, such as enhancing antioxidant and anticancer activities (Raiola et al., 2014; Macknight et al., 2017; Salehi et al., 2019). Unable to synthesize L-ascorbic acid, humans can only get L-ascorbic acid from plant-based foods, especially tomato. For this reason, L-ascorbic acid contributes to the major feature of fruit nutrition and antioxidant capacity (Law and Jacobsen, 2010).

In higher plants, major biosynthesis pathways of L-ascorbic acid have been clarified, including the D-mannose/L-galactose pathway, D-glucosone pathway, D-galacturonate and myo-inositol pathway (Wheeler et al., 1998; Lorence et al., 2004; Bulley and Laing, 2016). In tomato, multiple enzymes are involved in each biosynthesis pathway, including GDP-D-mannose pyrophosphorylase (GMP), GDP-D-mannose-3,5-epimerase (GME), GDP-L-galactose-phosphorylase (GGP), L-galactose-1-P phosphatase (GPP), L-galactose dehydrogenase (GalDH); L-galactono-1,4-lactone dehydrogenase (GLDH), Myo-inositol oxygenase (MIOX) and D-galacturonate reductase (GalUR) (Smirnoff and Wheeler, 2000; Davey et al., 2006; Zou et al., 2006; Mellidou and Kanellis, 2017; Munir et al., 2020). In plants, reduced L-ascorbic acid can be oxidized by ascorbate peroxidase (APX) and ascorbate oxidase (AO) (Bulley and Laing, 2016). Moreover, L-ascorbic acid accumulation also depends on conversion between oxidized and reduced L-ascorbic acid through the AsA-GSH cycle involving monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Chen et al., 2003; Gallie, 2013; Mellidou and Kanellis, 2017).

In recent years, several transcription factors and proteins have been reported in regulation of L-ascorbic acid biosynthesis (Wang et al., 2013; Mellidou and Kanellis, 2017). Some of these genes enhancing AsA accumulation through positively regulating the transcription of AsA biosynthesis genes. For instance, overexpression of AtERF98 increases AsA level by directly regulation expression of AsA synthesis genes in the D-Man/L-Gal pathway while the knockout mutant *erf98-1* displayed decreased AsA contents (Zhang et al., 2012). In Arabidopsis, mutation of KONJAC1 (KJC1) and KJC2 cause enhanced activity of VTC1 and GMP (Sawake et al., 2015). In tomato, two transcription factors, SlHZ24 and SlbHLH59, promote AsA accumulation via binding to promoters of AsA biosynthesis genes such as GDP-D-mannose pyrophosphorylase 3 (SlGMP3) (Hu et al., 2016; Ye et al., 2019). Nevertheless, others play negative roles on AsA biosynthesis. In Arabidopsis, AMR1 negatively affects expression of multiple genes encoding enzymes of the Man/l-Gal pathway, resulting in reduced AsA levels (Zhang et al., 2009). In tomato, SlNFYA10 modulates AsA biosynthesis through directly reducing expression of *SlGME1* and *SlGGP1* (Chen et al., 2020). Interaction of AtVTC1 and AtCSN5B causes ubiquitination-dependent AtVTC1 degradation with low AsA levels (Wang et al., 2013).

DNA methylation is a major form of epigenetic variations in plants and animals. This epigenetic modification of DNA occurs at the 5’ position of cytosine in symmetric sequence contexts (CG and CHG) and asymmetric sequence context (CHH) (Bender, 2004; He et al., 2011; Zhang and Zhu, 2012). In plants, DNA methylation contributes to many biological processes through altering gene expression and genome stability, such as fruit ripening, seed germination and stress response (Goll and Bestor, 2005; Dowen et al., 2012; Zhang et al., 2018; Liu and Lang, 2020). Establish of de novo DNA methylation is catalyzed by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) (Cao et al., 2003; Matzke and Mosher, 2014). CG and CHG methylation are maintained by METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3) respectively (Law and Jacobsen, 2010; Bewick et al., 2017). Over the years, DNA demethylation has been verified in developmental processes based on DNA demethylases, including Repressor of Silencing 1 (AtROS1) and DEMETER-LIKE 2 (DML2) (Lei et al., 2015; Tang et al., 2016; Liu and Lang, 2020).

In tomato, DNA methylation is tightly associated with development and fruit ripening (Seymour et al., 2013; Giovannoni et al., 2017; Zuo et al., 2020). Genome-wide mapping of tomato DNA methylome revealed that ripened fruit were governed with low DNA methylation levels (Zhong et al., 2013). Reduced DNA methylation in *Slmet1* knockout mutant, encoding a methyltransferase, resulted in defective inflorescence and small leaves (Yang et al., 2019). Moreover, loss-of-function of *SlDML2* inhibited fruit ripening by enhancing DNA methylation of ripening-induced genes, indicating the critical role of DNA methylation variation in fruit ripening (Lang et al., 2017). The ascorbic acid is synthesized along with fruit development and ripening. However, whether and how L-ascorbic acid accumulation is regulated by DNA methylation in tomato has not been elucidated. Here, we aimed to explore the link between DNA methylation and AsA accumulation.

Materials And Methods

Plant Materials And Treatment

‘Ailsa Craig’ (AC) tomato was selected for methyltransferase inhibitor treatment and ascorbic acid assay. The tomato materials were grown in greenhouse at 25°C with 65% relative humidity, under a 16 h/8 h light/dark photoperiod. For methyltransferase inhibitor treatment on fruits, 50 μl of 1 mM 5-azacytidine aqueous solution was injected into the flower pedicel at flowering stage. Water was selected as negative control. Fruits was sampled at four stages of immature green (IMG), mature geen (MG), breaker (BR), and red ripe (RR), respectively, after 23 days post anthesis (DPA) until ripening. For methyltransferase inhibitor treatment on leaves, AC seeds were cultured on 1/2 MS medium with 50 μl of 1 mM 5-azacytidine aqueous solution for treatment or 50 μl water for negative control. The third or fourth leaf from the top of 1-month-old tomato plants was taken every week.

Rna Extraction And Quantitative Real-time Pcr

RNA extraction and quantitative real-time PCR
Total RNA was extracted from fruits and leaves using TRIzol reagent. 1 μg of RNA was used to synthesize first-strand cDNA using a HiScript II 1st Strand cDNA Synthesis Kit (Vazyme). qRT-PCR reactions were performed with SYBR Green I Master Kit (Roche, http://www.roche.com/) by SYBR Light Cycler 480 instrument. The actin gene was used as an internal control. All reactions were performed with three biological replicates. The qPCR primers are listed in Table S4.

Ascorbic Acid Assay

The AsA levels were measured as previously described (Hu et al., 2016). The samples were collected and immediately ground to a fine powder in liquid nitrogen. Approximately 0.2–0.3 g of frozen tissue were added to one milliliter of ice-cold 6% trichloroacetic acid (TCA). After centrifuging at 16,000 × g for 10 min at 4°C, the supernatant was transferred to a new tube. For total AsA assay, 20 μl of 5 mM dithiothreitol (DTT) was added to equal volume of the supernatant. To convert the oxidized ascorbic acid into the reduced form, the plate was incubated for 20 min at 37°C. 10 mL of N-ethylmaleimide (NEM; 0.5% w/v in water) was added to remove the excess DTT. After incubating for 1 min at room temperature (approximately 25°C), 8 mL of the color reagent (see below) was then added to the mixture, followed by incubation for 1 h at 37°C. The absorbance was immediately detected at 550 nm by an Infinite M200 Pro instrument (Tecan; http://www.tecan.com/). The color reagents were prepared as follows: solution A, 31% orthopho-sphoric acid, 4.6% (w/v) TCA and 0.6% (w/v) iron chloride; solution B, 4% 2,2-dipyridyl (w/v in 70% ethanol). Solutions A and B were mixed at a ratio of 2.75:1 before use. For reduced AsA assay, the same volume of 0.4 M potassium phosphate buffer (pH 7.4) was used to replace DTT and NEM, while the rest of the procedure was the same with the total AsA assay.

Mcrbc-pcr

Genomic DNA was extracted from 5-azacytidine-treated and untreated fruits, quantified by Nanodrop (Thermo Fisher Scientific) and fractionated on a 1% agarose gel to check the integrity. The methylation state of the GalUR was determined using McrBC-PCR as previously described (Liu et al., 2020). The primer sequence was listed in Table S4.

D-galacturonic Acid Reductase (Galur) Activity Assay

Protein extraction from 5-azacytidine-treated and untreated tissues was carried out as described (Cai et al., 2015). 1 g of sample was ground to a fine powder in liquid nitrogen and extracted with 50 mM sodium phosphate buffer (pH7.2) consisted of 2 mM EDTA, 2 mM DTT, 20 % glycerol and PVPP. The supernatant obtained after centrifugation at 6,000 × g for 30 min at 4°C was used as the crude enzyme. GalUR activity was measured by the change in absorbance at 340 nm at 25°C after the addition of crude enzyme extract to the assay medium (1 ml). The medium contained 50 mM phosphate buffer (pH 7.2), 2 mM EDTA, 0.1 mM NADPH, 30 mM D-galacturonic acid and 2 mM DTT. One unit of GalUR activity in the crude enzyme extract was expressed as nmol of NADPH oxidized min^{-1} mg^{-1} protein.

Data Availability

NCBI Sequence Read Archive (raw sequence data): SRA046092, SRA046132, SRA046131, SRA053345 and SRA046480. Analyzed data can be accessed from the tomato epigenome database (http://ted.bti.cornell.edu/epigenome/).

Results

Methyltransferase inhibitor promotes AsA accumulation in tomato fruit

To test whether DNA methylation might affect AsA accumulation during tomato ripening, we introduced a general DNA (cytosine-5) methyltransferase inhibitor, 5-azacytidine (5-azaC), into tomato seeds and immature fruits. The total AsA content in seedling leaves at 4 weeks after 5-azaC treatment finally reached to 557.9 μg/g FW while that in untreated leaves was significantly lower (398.7 μg/g FW) (Fig. 1A). Changes of reduced AsA accumulation showed a similar pattern with total AsA content (Fig. 1C). To further investigate the role of DNA methylation in AsA accumulation during tomato ripening, we detected AsA contents in fruits at different stages under 5-azaC injection. As expected, treated fruits showed premature ripening (Fig. S1). In general, both the total AsA and reduced AsA contents in treated fruits at 38 DPA (day post anthesis) were significantly higher than control (Fig. 1B and 1D). All these results might indicate that inhibition of DNA methylation inducing AsA accumulation in tomato leaves and fruits.

Expression and DNA methylation patterns of AsA biosynthetic genes during tomato ripening

In tomato, major AsA biosynthesis pathways had been reported in recent years, including D-mannose/L-galactose pathway, myo-inositol pathway, the D-glucosone and D-galacturonate pathway. We identified 61 AsA biosynthetic genes in whole genome of tomato. To clarify the expression patterns of these AsA biosynthetic genes, we screened the transcript expression data (http://ted.bti.cornell.edu/) of tomato fruits during ripening (Fig. S2). According to the clustering analysis, expression abundance of AsA biosynthetic genes reached high levels at different stages, indicating that AsA biosynthesis was regulated with the coordination of AsA biosynthetic genes.

To further investigate the role of DNA methylation on AsA biosynthesis, we performed DNA methylation pattern analysis of AsA biosynthetic genes according to tomato methylomes during fruit development (http://ted.bti.cornell.edu/epigenome/). In total, AsA biosynthetic genes showed higher levels of CG methylation than non-CG methylation levels throughout fruit development (Fig. 2). The promoter and gene transcription regions of several primary AsA biosynthetic genes (*PMM*, *GME*, *GGP* and *GalUR*) had more CG methylation than down-stream region. These results showed that cytosine methylation significantly influences AsA biosynthesis.

The *Slmet1* and *Sldml2* mutations alter transcription levels of AsA biosynthetic genes

In tomato, variation of DNA methylation profiles is essential for fruit ripening. It has been demonstrated that methyltransferase 1 (MET1) is primarily responsible for maintenance of CG methylation. A previous study used CRISPR-Cas9 gene-editing system to generate stable *Slmet1* mutation which causes dramatical reduction in genome-wide CG methylation. To emphasize the influence of *Slmet1* mutation on AsA biosynthesis, we identified AsA biosynthetic genes from differentially expressed genes(DEGs)according to RNA-seq analysis of *Slmet1* fruits. In total, 9 AsA biosynthetic genes showed significant increased transcription profiles while 8 genes had decreased expression levels (Table S1).

SlDML2 has been demonstrated as a critical DNA demethylase for fruit ripening. To further clarify whether DNA demethylation affect AsA biosynthesis, we also analyzed the transcriptome of *Sldml2* mutant which cause global hypermethylation. Among the 16 AsA biosynthetic genes in DEGs, we found that *Sldml2* mutation cause reduced expression of 9 genes as well as several up-regulated genes (Table S2).

Considering that inhibition of DNA methylation induces AsA accumulation in tomato leaves and fruits, we aimed to find out the genes up-regulated in *Slmet* mutant (low methylation level) while down-regulated in *Sldml2* mutant (high methylation level). As expected, *GalUR5* and *AO*1 showed converse expression patterns in *Slmet1* and *Sldml2* mutants, and *GalUR5* exhibited the extreme difference, indicating that *GalUR5* is significantly regulated by DNA methylation in AsA biosynthesis (Table 1).

Table 1
The collectively regulated AsA biosynthesis genes by both DNA methylase SlMET1 and demethylase SlDML2

Gene ID	Gene Name	Fold change in *Slmet1* mutant	Fold change in *Sldml2* mutant
Solyc09g097960.2	*GalUR5*	7.02	-5.22
Solyc09g065900.2	*GR1*	-13.26	-1.20
Solyc01g097340.2	*GME1*	-55.54	-1.21
Solyc04g054690.2	*AO1*	9.170	-2.88
Solyc06g060260.2	*APX7*	-24.67	-1.19

Demethylation of *SlGalUR5* is required for AsA accumulation during tomato ripening

To confirm the epigenetic regulation of *GalUR5* in AsA biosynthesis, we analyzed methylation and transcription levels of GalUR5 in *Slmet1* and *Sldml2* mutants. As expected, *Slmet1* mutant displayed decreased methylation level of GalUR5 with increased transcription level (Fig. 3A and Table S3). In contrast, *Sldml2* mutant showed converse patterns (Fig. 3B and Table S3). Furthermore, we performed McrBC-PCR to detect methylation levels of GalUR5 in fruits with 5-azaC treatment at 25 and 38 DPA. A remarkable reducing of methylation level was observed in 5-azaC-treated fruits at 38 DPA (Fig. 4).

We further detected transcription and methylation levels of *GalUR5* in fruits under 5-azaC treatment. Consistent with elevated AsA contents, expression profiles of *GalUR5* in treated tissues noticeably increased (Fig. 5A). A previous study proved that the AsA content of tomato was coincident with the level of GalUR enzymatic activity variation. In this study, we further examined GalUR5 activity in 5-azaC-treated fruits. GalUR5 activity showed higher levels in 5-azaC-treated fruits at 38 and 44 DPA (Fig. 5B), indicating that GalUR5 is important for AsA accumulation. These results showed that demethylation of *SlGalUR5* induce AsA accumulation by enhancing its expression in tomato fruit.

Discussion

It has been well demonstrated that DNA methylation plays a critical role in fruit ripening control (Gallusci et al., 2016; Giovannoni et al., 2017). The dynamic variation of this epigenome modification is associated with transcription repression or activation in fruit development (Zhang et al., 2018; Liu and Lang, 2020). DNA methylation levels can be alternated by activation of key methyltransferase or demethylase. In tomato, loss-function of *SlDML2* cause genome DNA hypermethylation and activation of ripen-related genes (Lang et al., 2017). Application of a methyltransferase inhibitor 5-azacytidine can induce fruits prematuration because of demethylation in the 5' upstream region of *SlCNR* (Zhong et al., 2013). In this study, consistent with expected role of methyltransferase inhibitor, we found that 5-azacytidine promote tomato ripening. In addition, we also found that AsA contents in treated leaves and fruits have a remarkable increase (Fig. 1), suggesting a novel function of DNA demethylation in fruit ripening. Until

now, there are a few reports of DNA demethylation modulating AsA synthesis. Our results therefore provide a potential link between DNA demethylation and AsA accumulation.

AsA accumulation is fine regulated by transcription repression or activation of AsA biosynthetic genes (Wang et al., 2013). As a major approach of AsA biosynthesis, D-galacturonate pathway proceeds via D-galacturonic acid reductase (GalUR) converting D-galacturonic acid to L-galactonic acid. Although *GalUR* family genes have been identified in several plant species, such as strawberry, grape and sweet orange, the mechanism and function of *GalUR* members were mostly investigated on ectopic expression and transcriptional regulation. In strawberry, ectopic overexpression of *FaGalUR* in *Arabidopsis thaliana* enhances AsA content (Agius et al., 2003). Expression levels of *VvGalUR*, a homologous gene of *FaGalUR*, show consistent patterns with AsA contents during ripening in grape fruit, indicating the conserved function of *FaGalUR* (Cruz-Rus et al., 2010). In orange, *GalUR* genes show diverse expression patterns across different developmental stages, indicating variety of transcription regulation in AsA biosynthesis genes (Xu et al., 2013).

In our previous study, we found that ectopic expression of *FaGalUR* causes AsA accumulation and enhanced abiotic stress tolerance in tomato, indicating the presence of alternative D-galacturonate pathway for AsA biosynthesis (Cai et al., 2015). Here, we identified 11 *GalUR* paralogous genes in the tomato genome (Fig. S3 and S4), according to the conserved aldo_keto_reductase domain and evolutionary analysis within 5 plant species (*Arabidopsis thalian*, *Fragria vesca*, *Vitis vinifera*, *Solanum tuberosum* and *Solanum lycopersicum*) (Sanli et al., 2003). As expected, the transcription levels of these genes showed different patterns (Fig. S2), suggesting complicated regulation mechanisms in AsA accumulation. Surprisingly, almost all AsA biosynthetic genes had mCG methylation during ripening (Fig. 2), indicating important role of DNA methylation on AsA biosynthesis. We also found that *Solyc09g097960.2* (*SlGalUR5*) shared decreased DNA methylation levels in *Slmet1* mutant (demethylation status) and increased DNA methylation levels in *Sldml2* mutant (hypermethylation status), consistent with high transcription levels in *Slmet1* mutant and low transcription levels in in *Sldml2* mutant (Table S3). DNA methylation levels of *SlGalUR5* in 5-azaC–treated fruits were significantly reduced compared with untreated fruits. In contrast, transcription profiles of *SlGalUR5* and enzyme activity of GalUR enhanced in 5-azaC–treated fruits (Fig. 5), indicating that DNA demethylation promotes AsA accumulation by enhancing *SlGalUR5* expression.

In summary, our results demonstrated that DNA demethylation is critical for L-ascorbic acid accumulation in tomato fruit ripening. The epigenome modification of *SlGalUR5* is essential for L-ascorbic acid accumulation. Our results provide insight to epigenetic regulation of AsA biosynthesis in tomato fruit.

Declarations

Acknowledgment

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Supplementary Table

Supplementary table 4 is not available with this version.

Figures

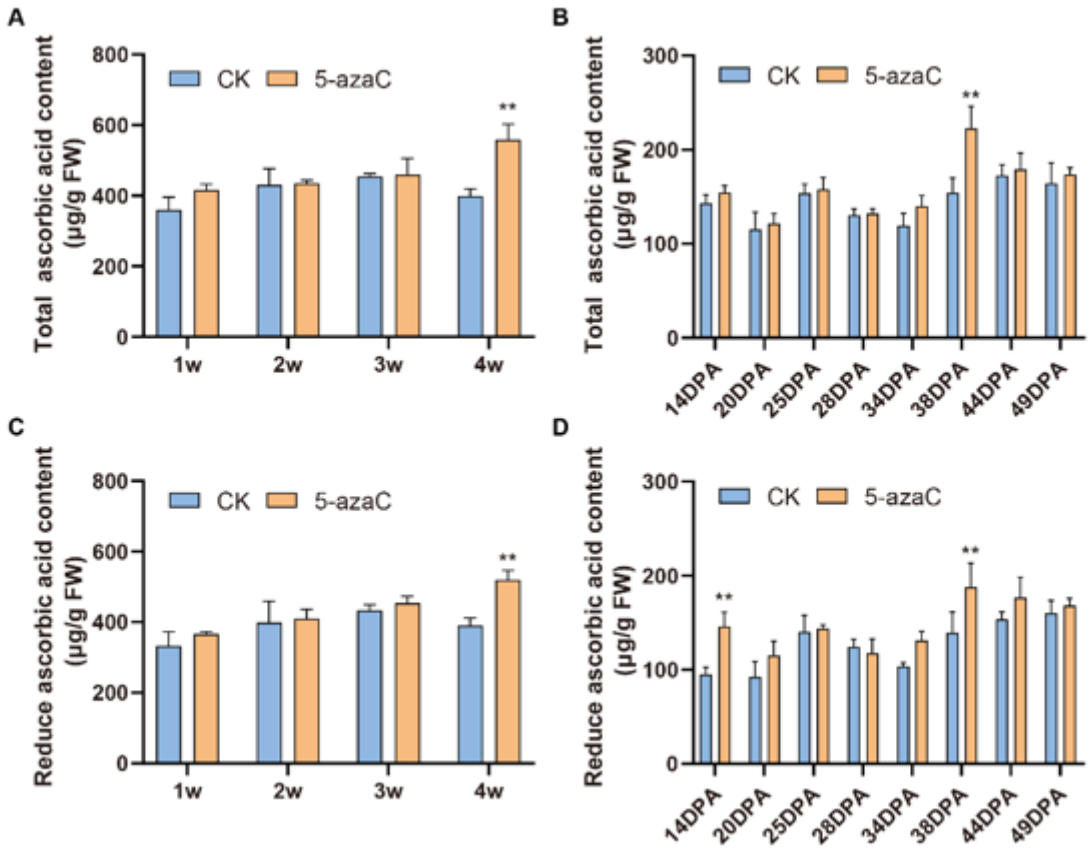

Figure 1

Application of DNA methylation inhibitor 5-azacytidine promotes ascorbic acid accumulation.

(A) Total ascorbic acid contents in leaves of 5-azacytidine–treated and untreated tomato plants. w, weeks. (B) Total ascorbic acid contents in 5-azacytidine–treated and untreated fruits. DPA, day post anthesis. (C, D) Reduced ascorbic acid contents in leaves (C) and fruits (D) of 5-azacytidine–treated and untreated tomato plants. Error bars, mean ± SD. The asterisks indicate a statistically significant difference (Student's *t*-test, $^{**}P < 0.01$).

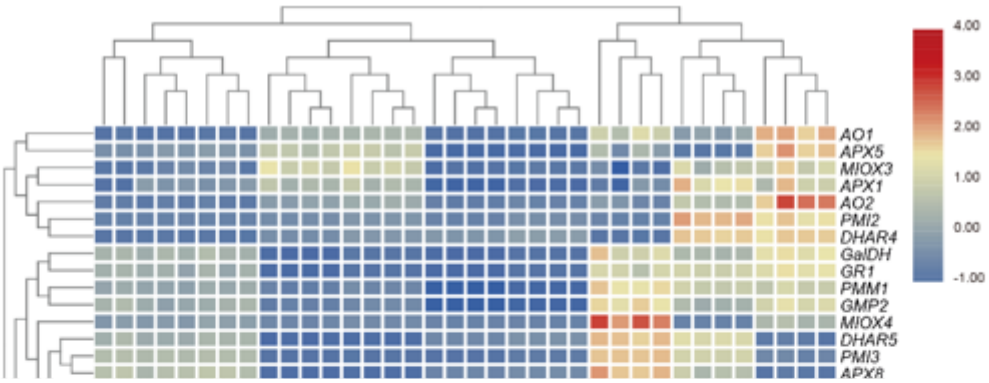

Figure 2

DNA methylation patterns of ascorbic acid biosynthetic genes during tomato ripening.

The cluster mapping is performed according to tomato methylomes during fruit development (http://ted.bti.cornell.edu/epigenome/). Methylation levels of three contexts (CG, CHG, CHH) are shown. The color of horizontal axis titles represents different regions of AsA biosynthetic genes. Purple indicates promoter region; orange indicates downstream region; green indicates gene coding region.

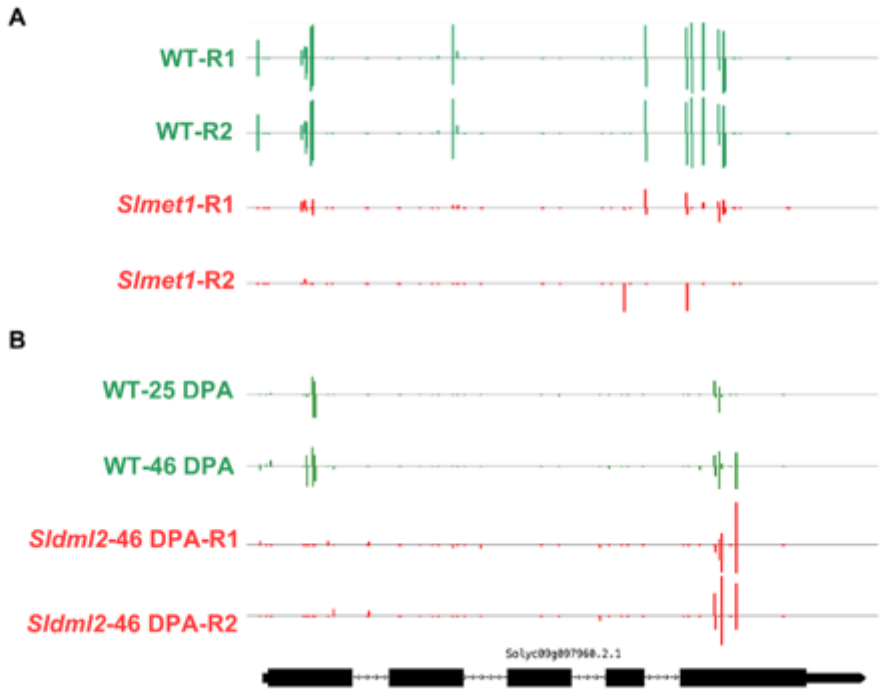

Figure 3

DNA methylation levels of *Solyc09g097960.2* in fruits of *Slmet1* and *Sldml2* mutants.

(A) Snapshots of *Solyc09g097960.2* loci showing DNA methylation in wildtype (WT) and *Slmet1* mutants. (B) DNA methylation levels of *Solyc09g097960.2* in WT and *Sldml2* mutants. DPA, day post anthesis. Vertical bars with the same scale indicate DNA methylation levels. Screenshots are shown with Integrative Genome Browser (IGB) display of whole-genome bisulfite sequencing data.

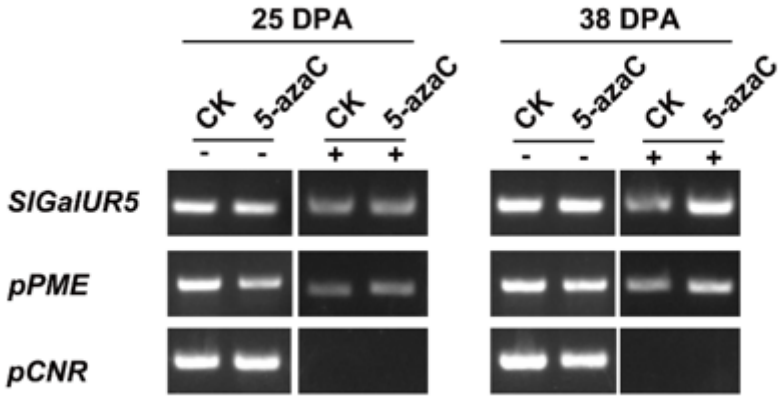

Figure 4

DNA methylation levels of *SlGalUR5* in 5-azaC–treated and untreated tomato fruits at two developmental stages.

DNA methylation levels were detected by McrBC-PCR in the presence (+) and absence (−) of GTP which allows McrBC to digest genomic DNA. The *PME* (*Solyc03g123630*) promoter is a known positive control with non-methylation status while CNR (*Solyc02g077920*) promoter is used for negative control with high-methylation levels.

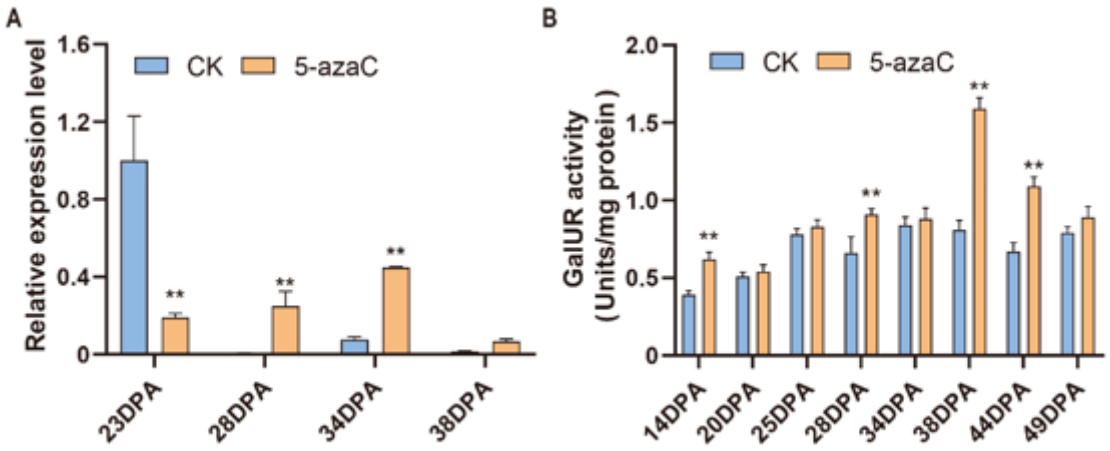

Figure 5

Expression levels of *SlGalUR5* and GalUR activity in 5-azacytidine–treated and control fruits.

(A) Relative transcription levels of *SlGalUR5* during tomato ripening under 5-azacytidine treatment. (B) The enzyme activity of GalUR in 5-azacytidine–treated and untreated control fruits. DPA, day post anthesis. Error bars, mean ± SD. The asterisks indicate a statistically significant difference (Student's *t*-test, **P < 0.01).

Supplementary Files

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