Methylation profiling of biosynthetic genes reveals the role of D-galacturonic acid reductase in ascorbic acid accumulation in tomato fruit

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Abstract

Ascorbic acid (AsA) is an important nutrient that contributes to the primary flavour of tomato fruits and human health. While the regulation of the transcription of AsA biosynthetic genes has been well demonstrated, the epigenetic alteration underlying AsA accumulation remains uncertain. In this study, a methyltransferase inhibitor (5-azacytidine) was applied to immature tomato fruit with a methyltransferase inhibitor (5-azacytidine) and effects on AsA accumulation were identified. Inhibiting DNA methylation increased the accumulation of AsA in tomato leaves and fruit. We also isolated a biosynthetic AsA gene, SlGalUR5, which encodes D-galacturonic acid reductase. SlGalUR5 showed a decrease in DNA methylation levels and higher transcription levels in the Slmet1 mutant while exhibiting inverted tendencies in the Sldml2 mutant. Treatment with 5-azacytidine dramatically reduced the DNA methylation levels of SlGalUR5 in fruit. In contrast, transcription profiles of SlGalUR5 and enzyme activity were improved in 5-azacytidine-treated fruit. Our finding reveals new insight into the epigenomic modification of SlGalUR5 involved in ascorbic acid accumulation and provides a potential means of increasing AsA levels for tomato breeding.

Keywords Ascorbic acid · Tomato · DNA methylation · 5-azacytidine · GalUR

Introduction

In plants, L-ascorbic acid (AsA) is a multifunctional molecule that plays a crucial role in development processes and stress response (Barth et al. 2006; Gest et al. 2013). As the principal antioxidant, L-ascorbic acid keeps active oxygen below normal levels to protect plants in photosynthesis (Smirnoff 2000; Dong et al. 2020). L-ascorbic acid also contributes to regulating flowering, fruit development, senescence and abiotic stress responses (Conklin and Barth 2004). For human health, L-ascorbic acid has positive effects, such as improving antioxidant and anticancer activities (Zhang and Hao 2020; Salehi et al. 2019). Incapable of synthesizing L-ascorbic acid, humans can only obtain L-ascorbic acid from foods of plant origin, particularly tomatoes. Consequently, L-ascorbic acid contributes to the primary characteristic of fruit nutrition and antioxidant capacity (Law and Jacobsen 2010).

Fruit maturation is accompanied by the accumulation of ascorbic acid, which contributes to the quality and flavour of the fruit (Bulley et al., 2016; Zhang et al., 2020). In tomato, the amount of ascorbic acid is associated with nutritional quality and stress tolerance (Law and Jacobsen 2010). The main biosynthetic pathways for L-ascorbic acid have been clarified, including the D-mannose/L-galactose pathway, D-glucosone pathway, D-galacturonate pathway, and myo-inositol pathway (Lorence et al. 2004; Bulley and Laing 2016). Several enzymes are involved in each biosynthesis pathway, including GDP-D-mannose pyrophosphorylase.
(GMP), L-galactose dehydrogenase (GalDH), Myo-inositol oxygenase (MIOX) and D-galacturonate reductase (GalUR) (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). Additionally, the accumulation of L-ascorbic acid also depends on conversion between oxidized and reduced L-ascorbic acid by the AsA-GSH cycle involving monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Mellidou and Kanellis 2017).

Over the past few years, a number of transcription factors and proteins have been reported in the regulation of L-ascorbic acid biosynthesis (Shinozaki et al. 2018; Mellidou and Kanellis 2017). In plants, reduced L-ascorbic acid can be oxidized through ascorbate peroxidase (APX) and ascorbate oxidase (AO) (Bulley and Laing 2016). For instance, overexpression of AtERF98 increases AsA level by directly enhancing expression of AsA synthesis genes in the D-Man/L-Gal pathway while the knockout mutant erf98-1 displays decreased AsA contents (Zhang et al. 2012). In tomato, two transcription factors, SIHZ24 and SlbHLH59, promote AsA accumulation via binding to the promoters of AsA biosynthesis genes such as GDP-D-mannose pyrophosphorylase 3 (SIGMP3) (Hu et al. 2016; Ye et al. 2019).

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 contexts (CHH) (Zhang and Zhu 2012; Grzybkowska et al. 2018). In plants, DNA methylation contributes to many biological processes by affecting gene expression and genome stability, such as fruit maturation, seed germination, and stress response (Zhang et al. 2018; Liu and Lang 2020). Establish de novo DNA methylation is catalyzed by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) (Matzke and Mosher 2014). CG and CHG methylation are maintained by METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3) respectively (Law and Jacobsen 2010). Over the years, DNA demethylation has been verified in developmental processes based on DNA demethylases, including Repressor of Silencing 1 (AiROS1) and DEMETER-LIKE 2 (DML2) (Tang et al. 2016; Liu and Lang 2020).

In tomato, DNA methylation is closely associated with fruit development and fruit ripening (Giovannoni et al. 2017). Genome-wide mapping of tomato DNA methylome reveals that ripened fruits were governed with low DNA methylation levels (Zhong et al. 2013). Reduced DNA methylation in the Slmet1 mutant leads to a defective inflorescence and small leaves (Yang et al. 2019). In addition, the loss of function of SIDML2 inhibits fruit ripening by increasing DNA methylation of ripening-induced genes, indicating the critical role of DNA methylation variation in fruit ripening (Lang et al. 2017). Ascorbic acid is synthesized as fruit develops and matures. However, it is unclear whether and how the accumulation of L-ascorbic acid is regulated by the methylation of DNA in tomato. 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 a 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 a negative control. Fruits were sampled at four stages of immature green (IMG), mature green (MG), breaker (BR), and red ripe (RR), respectively, after 14 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**

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 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.

**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 millilitre 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 the total AsA assay, 20 µl of 5 mM dithiothreitol (DTT) was added to an 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 h 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% orthophosphoric 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 as 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, Shinozaki et al., 2020).

**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 (pH 7.2) consisting 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 verify whether DNA methylation affects AsA accumulation during tomato ripening, we introduced a general DNA (cytosine-5) methyltransferase inhibitor, 5-azacytidine (5-azaC), into tomato seeds and immature fruits. Overall, the total amount of AsA in seedling leaves after 5-azaC treatment was higher than in untreated leaves (Fig. 1 A). Changes in reduced AsA content showed a similar tendency with total AsA content (Fig. 1 C). To further investigate the role of DNA methylation in AsA accumulation during tomato ripening, we detected AsA content in fruits at different stages under 5-azaC injection. As expected, the treated fruits exhibited premature maturation (Fig. S1). In general, both the total AsA and reduced AsA content in treated fruits at 38 DPA (day post anthesis) were higher than in control (Fig. 1 B and D). All these results indicated that inhibition of DNA methylation induces AsA accumulation in tomato leaves and fruits.

Expression and DNA methylation patterns of AsA biosynthesis and recycling-related genes during tomato ripening

In tomato, major AsA biosynthesis and recycling pathways had been reported in recent years, including D-mannose/L-galactose pathway, myo-inositol pathway, the D-glucosone and D-galacturonate pathway (Bulley et al., 2016). We identified 61 AsA biosynthesis and recycling-related genes in the whole genome of tomato (Table S1). To clarify the correlation between ascorbate content and expression patterns of these AsA biosynthesis and recycling-related genes, we screened the expression levels of these genes according to a previous transcriptome of tomato fruits during ripening (Ye et al. 2015) (Fig. S2). Based on the correlation analysis, the expression abundance of AsA biosynthesis and recycling-related genes showed complicated expression patterns at different stages. Among these genes, expression variation of several genes, including PMM3, GMP2, GGP2, GalUR5, GR2 and AO1, showed significant correlations with AsA content, indicating their important roles in AsA accumulation during fruit ripening. To further investigate the role of
DNA methylation on AsA biosynthesis, we performed DNA methylation analysis of AsA biosynthetic genes according to tomato methylomes during fruit development (http://ted.bti.cornell.edu/epigenome/). In total, AsA biosynthesis and recycling-related genes exhibited higher levels of CG methylation than non-CG methylation levels throughout fruit development (Fig. 2). The promoters and gene transcription regions of several primary AsA biosynthetic genes (PMM, GME, GGP and GalUR) had more CG methylation than the down-stream region. These results showed that cytosine methylation significantly affects AsA biosynthesis and recycling.

Slmet1 and Sldml2 mutations affect the transcript levels of the AsA biosynthetic genes.

In tomato, variation in DNA methylation profiles is critical for fruit ripening. Methyl transferase 1 (MET1) has been shown to be mainly responsible for maintaining GC methylation (Yang et al. 2019). A previous study used the CRISPR-Cas9 gene editing system to generate a stable mutation of Slmet1 that causes a dramatic reduction in CG methylation (Yang et al. 2019). To emphasize the influence of Slmet1 mutation on AsA biosynthesis, we identified AsA biosynthetic genes from differentially expressed genes (DEGs) based on RNA-seq analysis of Slmet1 fruits. A total of 9 AsA biosynthetic genes showed significantly increased transcription profiles while 8 genes had reduced expression levels (Table S2).

Sldml2 has been proven as a critical demethylase for fruit maturation (Lang et al. 2017). To determine whether demethylation of DNA affects AsA biosynthesis, the transcriptome of the Sldml2 mutant that causes global hypermethylation was also analyzed. Among the 16 genes associated with AsA biosynthesis and recycling in DEGs, we found that the Sldml2 mutation resulted in a reduction in the expression of 9 genes and several regulated genes (Table S3).

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 AO1 showed converse expression patterns in Slmet1 and Sldml2 mutants, and GalUR5 exhibited extreme differences, indicating that

| 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                        |

Table 1: The collectively regulated AsA biosynthesis genes by both DNA methylase SlMET1 and demethylase SLDML2.
GalUR5 is significantly regulated by DNA methylation in AsA biosynthesis (Table 1).

**Demethylation of SIGalUR5 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 displayed a decrease in GalUR5 methylation level with an increase in transcription levels (Fig. 3A and Table S4). In contrast, the SlDml2 mutant showed reverse patterns (Fig. 3B and Table S4). In addition, McrBC-PCR was performed to detect methylation levels of GalUR5 in fruits with 5-azaC treatment at 25 and 38 DPA. A notable reduction in methylation level was observed in 5-azaC-treated fruits at 38 DPA (Fig. 4).

Transcription and methylation levels of GalUR5 were also detected in fruits treated with 5-azaC. In accordance with the high level of AsA, the expression profiles of GalUR5 in the treated tissues increased significantly (Fig. 5A). A previous study demonstrated that the AsA content of tomato was coincident with the level of GalUR enzymatic activity variation (Cai et al. 2015). In this study, we further examined GalUR5 activity in fruits treated with 5-azaC. GalUR5 activity showed higher levels in fruits treated with 5-azaC at 38 and 44 DPA (Fig. 5B), indicating that GalUR5 is important for AsA accumulation. These results showed that demethylation of SIGalUR5 induces AsA accumulation by enhancing its expression in tomato fruit.

**Discussion**

It has been demonstrated that DNA methylation plays a crucial role in the control of fruit ripening (Gallusci et al. 2016; Giovannoni et al. 2017; Bai et al. 2021). The dynamic variation of this modification of the epigenome is associated with the repression of transcription or activation (Zhang et al. 2018; Liu and Lang 2020). DNA methylation levels can be alternated by activation of key methyltransferase or demethylase. In tomato, the loss function of SlDML2 results in hyper-methylation of genome DNA and activation of maturation-related genes (Lang et al. 2017). Application of a methyltransferase inhibitor 5-azacytidine may lead to fruit maturation due to demethylation in the 5′ upstream region of SlCNR (Zhong et al. 2013). In this study, we found that 5-azacytidine promotes maturation of tomatoes. In addition, we also found a noticeable increase in the AsA content of treated leaves and fruits (Fig. 1), suggesting a novel function of DNA demethylation in fruit ripening. Our findings thus provide a potential link between DNA demethylation and AsA accumulation.

As a major approach in AsA biosynthesis, D-galacturonic pathway proceeds via D-galacturonic acid reductase (GalUR) converting D-galacturonic acid to L-galactonic acid (Wang et al. 2013a, b; Kka et al. 2017). While GalUR family genes have been identified in several plant species, such as strawberry, grape and sweet orange, the mechanics and function of GalURs primarily focus on ectopic expression and transcriptional regulation. In strawberry, ectopic overexpression of FaGalUR in Arabidopsis thaliana enhances AsA content (Agius et al. 2003). The expression levels of VvGalUR, a homologous gene of FaGalUR, show consistent patterns with AsA contents during ripening in grape fruits, indicating the conserved function of FaGalUR (Cruz-Rus et al. 2010). In orange, GalUR genes show diverse expression patterns across different developmental stages, indicating the 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

![Fig. 5 Expression levels of SIGalUR5 and GalUR activity in 5-azacytidine-treated and control fruits](image-url)
abiotic stress tolerance in tomato, indicating the presence of D-galacturonate pathway (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 thaliana, Fragaria 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 regulatory mechanisms in AsA accumulation. Surprisingly, almost all genes associated with AsA biosynthesis and recycling exhibited mCG methylation (Fig. 2), indicating an important role of DNA methylation on AsA biosynthesis and recycling. We also found that Solyc09g097960.2 (SIGalUR5) shared decreased DNA methylation levels in Slmet1 mutant (demethylation status) and increased DNA methylation levels in Sldml2 mutant (hypermethylation status). These results are consistent with high transcription levels in Slmet1 mutant and low transcription levels in Sldml2 mutant (Table S4). Methylation levels of SIGalUR5 in 5-azaC-treated fruit were significantly decreased compared to untreated fruit.

In contrast, transcription profiles of SIGalUR5 and enzyme activity of SIGalUR5 were enhanced in 5-azaC–treated fruits (Fig. 5), indicating that DNA demethylation promotes AsA accumulation by enhancing SIGalUR5 expression.

In conclusion, our findings demonstrate that demethylation of DNA is essential for the accumulation of L-ascorbic acid in tomato ripening. The epigenetic modification of SIGalUR5 is essential for L-ascorbic acid accumulation. Our results provide insight into epigenetic regulation of AsA biosynthesis in tomato fruit.

Acknowledgements This work was supported by grants from the National Key Research & Development Plan (2021YFD1200210; 2018YFD1000800); National Natural Science Foundation of China (31972426; 31911882); Fundamental Research Funds for the Central Universities (2662022YLPY001); Key Project of Hubei Hongshan Laboratory (2021hszd007); International Cooperation Promotion Plan of Shihezi University (GJHZ202104).

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Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10725-022-00863-4.