Dynamic Changes of DNA Methylation Induced by Heat Treatment Were Involved in Ethylene Signal Transmission and Delayed the Postharvest Ripening of Tomato Fruit

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ABSTRACT: Deoxyribonucleic acid (DNA) methylation plays an important role in fruit ripening and senescence. Here, the role of DNA methylation of the CpG island of SIACS10, LeCTR1, LeEIN3, LeERT10, and SIERF-A1 genes induced by heat treatment (37 °C) in postharvest ripening of tomato fruit was studied. After heat treatment, the firmness and vitamin C content showed higher levels, the loss of aldehydes in volatile components was delayed, and the activities of methylase and demethylase decreased in tomato fruit. Moreover, in heat-treated fruit, significant changes in DNA methylation of SIACS10, LeCTR1, LeEIN3, LeERT10, and SIERF-A1 were induced, the expression of LeERT10 and LeEIN3 was inhibited, the expression of SIERF-A1 was increased, by which ethylene signal transmission might be suppressed and the postharvest ripening of tomato fruit was delayed. The present study provided valuable information for understanding the essential role of DNA methylation in the postharvest ripening of tomato fruit.

KEYWORDS: DNA methylation, ripening, ethylene, heat treatment, tomato

INTRODUCTION

Methylation, a covalent modification of genomic deoxyribonucleic acid (DNA), is the covalent addition of methyl groups to the fifth carbon of the cytosine pyrimidine ring or the sixth nitrogen of the adenine purine ring. Adding methyl to the DNA molecule can change the phenotype without altering the underlying nucleotide sequence to affect DNA activity. Meanwhile, the sequence context of cytosine is closely related to the mechanism of maintaining DNA methylation. In mammals, DNA methylation occurs mostly in the context of symmetric CG. It is mediated by the conserved DNA methyltransferase (DNMT) family and occurs primarily in the form of 5-methylcytosine (5mC) throughout the genome. DNA methylation plays an important role in a variety of cellular functions in mammals, such as tissue-specific gene regulation, aging, genomic imprinting, suppression of transposable elements, X chromosome inactivation, genome stability, and carcinogenesis. In plants, DNA methylation usually occurs in cytosine bases in all sequence contexts: CG, CHG (symmetric sites, H = A, C, or T), and the asymmetric CHH context, in which CpG was the most methylated region. After DNA replication and postreplication, maintenance methylation of CG sites is mediated by DNA methyltransferase MET1 (a homologue of the mammalian DNMT1). DNA methylation is the essential basis of DNA in all higher plants and plays an indispensable role in plant development, stress adaptation, and maintenance of genomic integrity. DNA methylation is also involved in fruit ripening and senescence. In tomato fruit, DNA methylation affected m6A modification by regulating the expression of N6-methyladenosine (m6A) demethylase gene, and m6A demethylase feedback regulated DNA methylation, thus regulated fruit ripening. In strawberry fruit, the expression level of DNA methylase genes was the highest during the turning stage (white to red), indicating that the DNA methylation level had a role in phenotypic changes of fruit ripening and senescence. Moreover, the DNA demethylation mediated by SfDML2 was crucial for tomato fruit ripening. When the function of SfDML2 was mutated, tomato fruit exhibited an obvious ripening-delayed phenotype.

Fruit ripening and senescence are important in fruit life cycles and are related to many physiological and biochemical changes. It always causes huge massive economic losses in postharvest fruit storage. As an effective and environmentally friendly technique, heat treatment has gained attention for maintaining the quality of postharvest fruit and vegetables and controlling postharvest decay and physiological diseases. Heat treatment has been effectively used to alleviate chilling injury in kiwi fruit, maintain sensory quality in cucumbers, and delay postharvest ripening of peach fruit. In addition, heat treatment at 42 and 48 °C decreased phenylalanine ammonia lyase (PAL) activity and delayed anthocyanin accumulation, thus delayed the ripening and senescence of postharvest strawberry fruit, respectively. Soaking in 42 °C hot water for 30 min had significant inhibitory effects on the...
decrease of hardness and red color development of tomato fruit at different maturity stages and also had a significant effect on delaying fruit ripening. To further extend the storage and shelf life of the fruit, it is important to understand the mechanism by which hot water treatment (HWT) delays fruit ripening and senescence. However, to date, scarce information is available about the epigenetic mechanism of DNA methylation involved in HWT-delayed fruit ripening.

Tomato is one of the most widely consumed fruits in the world and the main model for fleshy fruit biology research. In the present study, the effect of HWT on the postharvest ripening of tomato fruit was studied, and the dynamic changes of DNA methylation and CpG island methylation sites of \textit{SIACS10}, \textit{LeCTR1}, \textit{LeERN3}, \textit{LeERT10}, and \textit{SIERF-A1} genes involved in ethylene biosynthesis and signal transmission were detected. In addition, the epigenetic modification target sites involved in postharvest fruit ripening were analyzed.

## MATERIALS AND METHODS

### Plant Material

Tomato (\textit{Solanum lycopersicum} cv. Qirui F50) fruits at the mature green stage were randomly harvested from the experimental orchard of Tianjin Key Laboratory of Postharvest Physiology and Storage of Agricultural Products, put into a foam box, covered with a foam net cover to prevent rubbing injury, and immediately transported to the laboratory. All fruits were sorted based on size, maturity relatively consistent, without physical injuries or infections.

### Fruit Treatment

Tomatoes were randomly divided into two groups; each group was divided into six sampling time points (0, 2, 4, 6, 8, and 10 days) for analysis and treated as follows: (1) the selected tomato fruits were soaked in 37°C (±0.5°C) hot water for 15 min, dried, and stored at room temperature in dark storage; (2) control group, without any treatment and stored at room temperature in dark storage.

### Fruit Firmness

The fruit firmness was measured with a 2 mm diameter cylindrical probe at a horizontal axis using a TATXplus texture analyzer (Stable Micro System Ltd., Godalming, U.K.) according to the method of Hu et al. (10 mm depth). Six replications were performed.

### Total Soluble Solids (TSS), Total Acidity (TA), and Vitamin C (Vc)

Randomly select nine tomato fruits were homogenized in a centrifuge tube, then added 820 μL of PBS (pH 7.4). The frozen tissue was ground into powder under liquid nitrogen, and 0.4 g powder tissues were weighed and transferred to a 1.5 mL centrifuge tube, then added 820 μL phosphate-buffered saline (PBS) (pH 7.4). The samples were thoroughly mixed, the homogenate was centrifuged at 5000 g for 20 min, and the supernatant was collected for analysis.

### Respiration Rate (CO₂)

The respiration rate (CO₂) was measured according to the method of Oh et al. The fruits were weighed and placed in a sealed polypropylene container at room temperature for 2 h; then, the CO₂ content was measured using a portable residual oxygen meter (Check Piontl, Dansensor Co., Denmark). The respiration rate was expressed as mg kg⁻¹ h⁻¹.

### Ethylene Production

The ethylene production rate was measured according to the method of Zhang et al. Fruits were weighed prior to incubation in a 600 mL polypropylene container. After incubation at room temperature for 2 h, 10 mL of headspace gas was extracted from each container using a gas-tight syringe, and 1 mL of gas sample was injected into flame ionization detector (FID) gas chromatograph (GC-2010, Shimadzu Co., Kyoto, Japan) for analysis. The temperatures of the oven, injector, and detector were 60, 150, and 160°C, respectively. Ethylene production was calculated according to the ethylene volume fraction, tank volume, incubation time, and fruit weight, and the standard sample concentration was 50 μL L⁻¹.

### Electronic Nose (e-Nose) Analysis

The e-nose analysis was conducted using a PEN 3 e-nose (Airsense Analytics GmbH, Schwerin, Germany) according to the method of Qiu et al. It contains 10 different metal oxide sensors that can analyze different volatile components. The sensor arrays and their performance descriptions were detailed by Wang et al. The tomato fruit was placed in a 1 L beaker sealed with plastic wrap for 15 min. The setting of PEN 3 e-nose is as follows: sensor cleaning time, 70 s; automatic zeroing time, 10 s; sample preparation time, 5 s; sample test time, 50 s; sample measurement interval time, 1 s; internal flow rate, 100 mL min⁻¹; sampling flow rate, 100 mL min⁻¹. Six replications were performed.

### Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

The headspace solid-phase microextraction gas chromatography–mass spectrometry (HS-SPME–GC-MS) was combined with GC–MS (Finnigan Co.) to analyze the volatile components in tomato juice. The volatile components were measured according to the method of Wang et al. Briefly, the homogenized tomato fruits were centrifuged at 12,000 g for 10 min, filtered with four layers of gauze, and the supernatant (8 mL) was put in a 15 mL headspace bottle with a magnetic rotor (adding 2.5 g of NaCl). After incubation in a water bath at 55°C for 15 min, the headspace bottle was placed on a magnetic heating agitator at 55°C. The SPME fiber was inserted into the headspace part (about 1 cm from the liquid level), absorbed for 46 min, and immediately inserted into the GC–MS sample inlet desorbed at 250°C for 5 min.

### Methylation and Demethylation Activity Assays

Methylation and demethylation activities were determined using the Plant Methylation ELISA Kit and Plant Demethylation ELISA Kit (Jingkang Bioengineering Co. Ltd., Shanghai, China) following the manufacturer’s recommendations, respectively. Frozen tissue was ground to powder in liquid nitrogen, and 0.1 g of tissue powder was transferred to a 1.5 mL centrifuge tube with 960 μL of PBS (pH 7.4). The samples were thoroughly mixed, then centrifuged at 5000 g for 20 min, and the supernatant was collected. The optical density (OD) value was measured by a spectrophotometer at 450 nm wavelength.

### Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis

Total ribonucleic acid (RNA) was extracted from tomato fruit with Trizol reagent (Tiangen Biochemical Technology Co. Ltd., Beijing, China) following the manufacturer’s instructions, which includes RNase-free DNase I treatment to remove the genomic DNA. The gene primers used for qRT-PCR analysis are shown in Table S1, and the sequences of \textit{SIACS10}, \textit{LeCTR1}, \textit{LeERN3}, \textit{LeERT10}, and \textit{SIERF-A1} genes are shown in the Supporting Information. The relative expression levels of genes were calculated using GAPDH as an internal reference according to the method of Livak and Schmittgen. All reactions were performed in triplicate for each sample.

### DNA Preparation and Bisulfite Sequencing PCR (BSP)

Genomic DNA was isolated from tomato fruit by the phenol chloroform method. According to the manufacturer’s recommendations, the extracted high-quality DNA was modified using the EpiTect Bisulfite Kit (Qiagen, 59104). During the bisulfite conversion, all unmethylated uracil was transformed into thyme, while the methylated cytosine remained unchanged. To determine the DNA methylation changes of the \textit{SIACS10}, \textit{LeCTR1}, \textit{LeERN3}, \textit{LeERT10}, and \textit{SIERF-A1} genes in the supporting sequence, the BSP primers were shown in Table S2. The BSP reaction scheme is as follows: initial denaturation step (95°C, 5 min), 10 cycles (94°C, 30 s; 60–50°C, 30 s; 72°C, 30 s), 25 cycles (94°C, 30 s; 50°C, 30 s; 72°C, 30 s), and final extension.
step (60 °C, 30 min). PCR products were subjected to electrophoresis on 2% agarose gels and purified according to the Qiagen Adhesive Recovery Kit manual. The purified PCR products were cloned into the pTOPO-T cloning vector using the EZ-T Zero TOPO Cloning Kit (GenStar, T180-100) by T4 DNA ligase (New England Biolabs), and then, the recombinant clones were transferred to Escherichia coli cells. Finally, 10 positive clones were screened on the LB agar plate and sequenced. The results were analyzed by a BiQ analyzer. Clones with complete sequencing data and a minimum of 95% bisulfite conversion rate were included in subsequent analyses.

**Statistical Analysis.** The physiochemical data were analyzed by one-way analysis of variance (ANOVA). For e-nose analysis, sensor signals measured at 48–50 s were selected and subjected to linear discriminant analysis (LDA) using Win Muster software for the PEN 3 electronic nose. For the GC−MS analysis, by searching the NIST/Wiley standard spectrum library, qualitative analysis was carried out, and the relative contents of each compound were measured by peak area normalization method.

## RESULTS

**Effects of HWT on Fruit Ripening and Lycopene Content.** Fruits treated with heat showed obvious color breaking at 4 days and were full red at 10 days, which was 2 days later than that observed in control fruits (Figure 1A).

![Figure 1. Effect of HWT on fruit ripening and lycopene content. (A) Visual changes in pericarp and inner tissues during "Qirui F50" tomato fruit ripening. (B) Lycopene content. Treatment conditions: for heating, mature green tomato fruits were soaked in 37 °C (±0.5 °C) hot water for 15 min and stored at room temperature in dark storage. Symbols * and ** in the graph show the significant differences at P < 0.05 and P < 0.01, respectively.](https://example.com/image1)

Lycopene is the direct indicator of tomato ripening. In the present results, the accumulation of lycopene in heat-treated tomato fruit was significantly higher than that in control fruit. During storage from 2 to 10 days, lycopene in heat-treated fruit increased from 4.22 to 8.35 μg g−1 FW, while lycopene in control fruit increased from 4.64 to 8.35 μg g−1 FW (Figure 1B). The results showed that HWT treatment delayed the postharvest ripening of tomato fruit.

**Effects of HWT on Fruit Firmness, TSS Content, TA Content, Vc Content, Respiration Rate (CO2), and Ethylene Production.** Fruit firmness is an important sensory quality of tomato, which reflects its ripening process. As shown in Figure 2A, tomato fruit firmness exhibited a decreasing trend during storage. During storage from 2 to 10 days, the firmness of heat-treated fruit decreased from 10.05 to 5.61 N, while the firmness of control fruit decreased from 9.27 to 5.07 N (Figure 2A). These results indicated that HWT has a positive effect on maintaining fruit firmness.

During storage from 2 to 10 days, the TSS content gradually increased with fruit ripening. Compared with that in control fruit, exposure to HWT at 37 °C for 15 min increased the TSS content in fruit after being stored from 2 to 10 days. The TSS content increased from 4.10 to 5.60 °Brix in heat-treated fruit and from 4.57 to 5.73 °Brix in control fruit (Figure 2B). In addition, during storage from 2 to 10 days, the TA content in heat-treated fruit decreased from 0.91 to 0.51% and from 0.83 to 0.54% in control fruit (Figure 2C).

Vc is an effective antioxidant that protects the organism from free radicals. As shown in Figure 2D, the Vc content in heat-treated fruit was significantly (P < 0.05) higher than that in control fruit. During storage from 2 to 6 days, the Vc content in heat-treated fruit increased significantly from 4.65 to 8.34 mg 100 g−1 and decreased from 3.43 to 7.01 mg 100 g−1 in control fruit (Figure 2D).

Compared with that in control fruit, respiration was decreased in heat-treated fruit. The peak of respiratory rate was detected in both groups at 6 days. During storage from 2 to 6 days, the respiration rate in heat-treated fruit increased from 29.10 to 39.40 mg kg−1 h−1, while the respiration rate of control fruit increased from 30.79 to 43.54 mg kg−1 h−1 (Figure 2E). There was no significant difference in the respiration rate between control fruit and heat-treated fruit at 10 days (Figure 2E). Similarly, HWT effectively inhibited ethylene production and reduced the ethylene peak value. After storage for 6 days, the peaks of ethylene production were detected in both heat-treated fruit and control fruit. The ethylene production in heat-treated fruit and control fruit was 6.49 and 7.02 μL kg−1 h−1, respectively (Figure 2F).

**e-Nose Analysis.** Linear discriminant analysis (LDA) focuses on the spatial distribution of response values of volatile components and their distance from each other. As shown in Figure 3, the total variance of LDA was 92.62% under different storage times, but HWT did not significantly affect its ripening process. As shown in Figure 3, the distribution positions of ellipses in Figure 3 were similar among different storage times, but HWT did not significantly affect its ripening process. As shown in Figure 3, the distribution positions of ellipses in different storage times of the same treatment could be obviously separated, and it indicated that the volatile components of tomato vary significantly during storage. The results showed that LDA could distinguish the volatile components of tomato fruit under different storage times, but HWT did not significantly improve the volatile aroma components of tomato fruit during storage.

**Effect of HWT on Volatile Compounds during Postharvest Storage.** According to HS-SPME−GC−MS analysis, a total of 58 volatile organic compounds (VOCs) were detected during tomato ripening, including 15 alcohols, 11 aldehydes, 8 ketones, 5 terpenoids, 1 ester, 3 hydrocarbons, 4 phenols, 3 acids, 5 oxygen-containing heterocyclic compounds, and 3 other compounds (Table S3). Among
them, the relative contents of 3-hexen-1-ol, 2-hexenal, 2-methyl-4-pentenal, hexanal, methyl salicylate, 2-methyl-octadecane, and 2-methoxyphenol were higher than other VOCs (Table 1). The total relative content of detected flavor substances accounts for more than 95% of the total peak area, and VOCs with relative content not less than 0.10% were selected for analysis.

Compared with that in heat-treated fruit, alcohols were decreased by 1 kind and 3 kinds at 4 and 8 days, respectively (Table S3). At 4 days, the contents of 2-hexenal and 2-methyl-4-pentenal in control fruit were 18.3 and 8.16% (Table 1), while those in heat-treated fruit were 22.64 and 17.23%, respectively. During stored from 2 to 10 days, the contents of linalool, methyl salicylate, 2-methoxyphenol, and 2-methyl-octadecane in control fruit decreased from 4.02, 7.4, 32.32, and 11.39% to 0.47, 0.96, 0.31, and 0.62%, respectively (Table 1). Meanwhile, these components in heat-treated fruit were similar to those in control fruit, and the relative content decreased gradually with the ripening of the fruit. Moreover, terpenoids usually have a strong aroma, and linalool was the highest in terpenoids (Table 1). Linalool provides a strong smell of flower flavor. The content of linalool in control fruit was 4.02% at 2 days, and no linalool was detected in heat-treated fruit (Table 1). Ketones often have floral and fruit flavors. Eight important ketone volatiles were identified during fruit ripening. At 10 days, the contents of 6-methyl-5-hepten-2-one in control fruit and heat-treated fruit were 3.44 and 0.84%, respectively (Table 1).

**Effects of HWT on Methylase and Demethylase Activity.**
Methylase increased first, then decreased in both groups, and reached a peak at 6 days (Figure 4A). After treated for 6 and 10 days, the activities of methylase in heat-treated fruit were 0.87 and 0.35 U g\(^{-1}\) FW, respectively (\(P < 0.05\)) (Figure 4A). After treated for 10 days, the activity of methylase in control fruit was about three times higher compared with that in treated fruit (Figure 4A).

Compared with that in control fruit, demethylase activity in heat-treated fruit was lower, but the overall change was smaller. After treated for 2, 6, and 10 days, the activities of demethylase in heat-treated fruit were 8.35, 8.33, and 8.64 U g\(^{-1}\) FW, respectively (Figure 4B). However, the activities of demethylase in control fruit were 11.12, 18.30, and 9.20 U g\(^{-1}\) FW at 2, 6, and 10 days, respectively (Figure 4B).

**Effects of HWT on Gene Expression and DNA Methylation Levels.**
To understand the role of DNA methylation in fruit ripening, the expression and changes of DNA methylation of the CpG island of \(SlACS10\), \(LeCTR1\), \(LeEIN3\), \(LeERT10\), and \(SlERF-A1\) genes during fruit ripening were analyzed. As shown in Figure 5A, the expression levels of \(SlACS10\) gene in control fruit were 0.66 and 0.77 at 4 and 8 days, respectively. In heat-treated fruit, the expression level of \(SlACS10\) first increased, then decreased during storage, and reached 1.45 at 4 days (Figure 5A). The methylation sites −45 to +309 of the CpG island between the promoter and first exon regions in \(SlACS10\) were selected for BSP analysis.
Table 1. Aromatic Volatile Compounds Identified in This Study, with the Values of Different Letters in the Same Row Significantly Different (P < 0.05)

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<tr>
<th>volatile compound</th>
<th>CAS</th>
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<th>HWT group area (%)</th>
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<td>2 days</td>
<td>4 days</td>
</tr>
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<td>alcohols</td>
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<td>3.18b</td>
<td>3.77b</td>
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of LeEIN3 gene showed the same trend in heat-treated fruit and control fruit. The expression levels of LeEIN3 gene in heat-treated fruit and control fruit were 4.50 and 0.13 at 4 and 8 days, respectively (Figure 6D). Compared with control fruit, the expression level of LeEIN3 in heat-treated fruit showed lower level at 4 and 8 days (Figure 6D). The CpG island was located between +92 to +287 of the exon region of LeEIN3 (Supporting Information). The DNA methylation level of LeEIN3 gene in heat-treated fruit was significantly higher than that in control fruit (Figure 6E,F). After storage for 4 days, the methylation rates of LeEIN3 gene in heat-treated fruit and control fruit were 48.8 and 28.9%, respectively (Figure 6F). After storage for 8 days, the methylation rates of LeEIN3 gene in heat-treated fruit and control fruit were 26.7 and 13.3%, respectively (Figure 6F).

Compared with the control fruit, the expression of LeERT10 gene in heat-treated fruit was downregulated (Figure 7A). The expression level of LeERT10 in heat-treated fruit was 0.81 and 0.06 at 4 and 8 days, respectively (Figure 7A). The 122 bp CpG island was located +141 to +263 of the exon region of LeERT10 gene (Supporting Information). The methylation rate of CpG island of LeERT10 in heat-treated fruit was 0.4 and 1.3% at 4 and 8 days, respectively (Figure 7B,C). However, DNA methylation of CpG island of LeERT10 in control fruit was not detected at 4 days (Figure 7B,C). Compared with that in the control fruit, the expression of SIERF-A1 gene in heat-treated fruit was also downregulated at 4 days (Figure 7D). The expression level of SIERF-A1 was 0.54 and 0.66 in heat-treated fruit, and was 0.65 and 0.52 in control fruit at 4 and 8 days, respectively (Figure 7D). The 344 bp CpG island was located +334 to +678 of the exon region of downstream of SIERF-A1 gene (Supporting Information). After treated for 4 days, the DNA methylation rates of CpG island of SIERF-A1 gene in control fruit was 2.6 and 48.9%, respectively. No DNA methylation was found in the heat-treated fruit at 8 days (Figure 7E,F).
DISCUSSION

Tomato is a typical respiration climacteric fruit. The ripening of most climacteric fruit is characterized by many obvious physiological and morphological changes including tissue softening, increased TSS content, enhanced color development, and increased ethylene production. After the fruit entered the respiratory climacteric period, the storage period shortened, and the fruit was perishable in the process of postharvest storage and transportation. A number of studies have indicated that proper heat treatment could delay the postharvest ripening of fruit. For example, heat treatment inhibited the incidence of anthracnose and stem-end rot of papaya fruit, effectively delayed the fruit softening, and reduced the decay rate, thus maintaining the quality of papaya. In peach fruit, heat treatment reduced ROS synthesis and interfered with Ca transport by activating the ascorbate–glutathione cycle, thereby strengthening tissue cell walls and improving peach fruit quality. In tomato fruit, heat treatment at 38 °C inhibited ethylene production, lycopene synthesis, and chlorophyll and cell wall degradation. Heat treatment improved the transcription level of LeARG1 and LeARG2 genes and effectively alleviated chilling injury of tomato fruit. In the present study, HWT maintained the Vc content and firmness of tomato fruit (Figure 2A), which was in line with the report on heat-treated apples. In addition, HWT reduced the peak respiratory rate and ethylene production of tomato fruit, which was similar to that in the heat-treated apple fruit. The decreased ethylene production and lycopene content caused by HWT may be one of the reasons for delaying fruit ripening.

Fruit ripening and senescence are accompanied by the production of aroma substances. Volatiles are usually produced by a series of lipid, amino acid, and carotenoid precursors and have a positive effect on flavor. C6 short-chain volatiles in tomato fruit, such as 2-hexenal, are produced by specific fatty acids including linoleic and linolenic acids. Among them, 2-hexenal is the most abundant volatile substance, which provides the green, grassy notes associated with plant tissues and plays an important role in the flavor of tomato fruit.
many fruits and vegetables. In the present study, HWT decreased the loss of the relative content of 2-hexanal in the early stage of storage, which was the main flavor substance in aldehydes. Among all VOCs, the variety of ester was much lower than other components, and methyl salicylate (MeSA) was mainly detected (Table 1). MeSA is synthesized from salicylic acid (SA), which contributes to the unique flavor of tomato fruit. MeSA could enhance the resistance of fruit decay caused by Botrytis cinerea and inhibit the ripening of tomato fruit. During storage, the content of MeSA was higher in heat-treated fruit than that in control fruit and significantly decreased in control fruit (Table 1), which indicated that maintaining the content of MeSA might be one of the reasons for HWT delaying fruit ripening. Moreover, acid were detected only at 0 to 6 days in heat-treated fruit and control fruit (Table 1). The effect of HWT maintained the relative content of acid might be related to the higher TA content and delayed fruit ripening. Lycopene is the immediate precursor of 6-methyl-5-hepten-2-one. In heat-treated fruit, HWT effectively delayed the accumulation of lycopene in tomato fruit. Compared with that in control fruit, HWT inhibited the relative content of 6-methyl-5-hepten-2-one in tomato fruit, which indicated that the synthesis of 6-methyl-5-hepten-2-one might be related to the accumulation of lycopene and involved in fruit ripening. Interestingly, compared with that in control fruit, HWT inhibited the activities of methylase and demethylase in tomato fruit. We speculated that the downregulation of methylase and demethylase induced by HWT might lead to more stable life characteristics of tomato fruit and might be one of the reasons for delaying fruit ripening.

Ethylene plays an important role in fruit ripening. In ethylene biosynthesis, the main regulatory point occurs at the level of ACS transcription. In the present results, there were 24 sites in the CpG island of SlACS10 (Supporting Information), and about 1.1% of CpG island in both groups were methylated at 8 days (Figure 5C). In climacteric fruit, ACS plays an important role in regulating the ethylene production during ripening and senescence. At least eight ACS genes have been identified in tomato fruit, among which SlACS2 and SlACS4 are mainly responsible for the autocatalytic ethylene production during fruit ripening and senescence. The increase of ethylene production in tomato fruit treated with brassinolide was related to the higher transcription levels of SlACS2 and SlACS4. In the present results, HWT effectively inhibited ethylene production and reduced the ethylene peak value (Figure 2F), which might be related to DNA methylation of SlACS10 gene. Therefore, the delayed postharvest ripening in heat-treated tomato fruit might be related to DNA methylation of SlACS10 gene.
CTRL, a key negative regulator in the ethylene pathway, can effectively inhibit the ethylene signaling cascade and is involved in fruit ripening and senescence.55 In the present results, the expression levels of LeCTRL gene was very high, and the DNA methylation level was very low in both heat-treated fruit and control fruit at 0 and 4 days (Figure 6A–C). The expression level of LeCTRL gene decreased and the level of DNA methylation increased in tomato fruit after heat treatment for 8 days (Figure 6A–C). The changes of three DNA methylation sites of the CpG island of LeCTRL gene in heat-treated fruit at 8 days might have been involved in inhibiting the expression of LeCTRL gene, by which ethylene signaling was activated and fruit ripening was delayed.

EIN3 is a key positive transcription factor in ethylene signal transduction, which consists of 10 putative Ser/Thr phosphorylation sites, and is involved in fruit ripening and senescence.46 In Arabidopsis, the loss of EIN3 and EIN3-like1 (its near homologues) led to delayed leaf senescence.47 In peach fruit, PpEIN3 was involved in the development and ripening, and the expression of PpEIN3 increased with fruit ripening.48 LeEIL is a homologous gene of EIN3 in Arabidopsis and plays a positive regulatory role in the ethylene signaling pathway.49 In the present results, the LeEIN3 gene was highly expressed during fruit ripening from 0 to 4 days (Figure 6D). The increased 23 DNA methylation sites (26.7%) of the CpG island of LeCTRL gene in heat-treated fruit might have been involved in inhibiting its expression, by which ethylene signaling might be suppressed and fruit ripening at 8 days was delayed.

In Arabidopsis, a full-length cDNA clone was isolated using the cDNA fragment ERT1, and the clone was named ERN1 (for ethylene-regulated nuclear protein) because of the nuclear localization of the ERN1 encoding protein.50 ERN1 is a downstream component of ethylene signaling. To date, the function and mechanism of ERTs in ethylene-regulated fruit ripening have not been thoroughly studied. LeERT10 may be located downstream of the ethylene signaling pathway and may regulate the ethylene reaction by an unknown mechanism. In the present results, compared with that in control fruit, the expression level of LeERT10 gene was significantly decreased in heat-treated fruit at 4 and 8 days, and the DNA methylation level of LeERT10 was also increased in heat-treated fruit from 4 to 8 days, which might be one of the reasons for HWT-delayed fruit ripening.

Ethylene response factors (ERFs) act downstream of the ethylene signaling pathway to regulate the expression of ethylene-responsive genes.43,51 Unlike EIN3/EILs, ERFs is one of the largest transcription factor gene families in plants and has positive or negative regulatory functions.52,53 ERFs from different plants are involved in a variety of processes including ethylene response and fruit ripening.51,52 In apple fruit, MdERF2 was associated with fruit ripening as a negative regulator.54 The blue light treatment increased the expression of PpERF2 in peach fruit and promoted fruit softening.55 In tomato fruit, SIERF.B3 was responsive to both ethylene and auxin, and the overexpression of its dominant repressor version (ERF.B3-SRDX) led to the change of auxin sensitivity and the decrease of chlorophyll accumulation.56 In the present results, the expression level of SIERF-A1 gene decreased during fruit ripening from 0 to 8 days (Figure 7D). It might play a negative regulatory role in the ethylene signaling pathway. Compared with that in control fruit, the expression level of SIERF-A1 was significantly increased in heat-treated fruit at 8 days, and the DNA methylation level of SIERF-A1 was also decreased in heat-treated fruit from 4 to 8 days, which might be one of the reasons for HWT-suppressed ethylene signaling and delayed fruit ripening.

In conclusion, the changes of DNA methylation of the CpG island of SIACS10, LeCTRL, LeEIN3, LeERT10, and SIERF-A1 genes in tomato fruit induced by HWT might play important roles in postharvest ripening of tomato fruit. The present results offered a foundation for genetically modifying the epigenetic target sites and controlling fruit ripening.

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c02971.

Forward and reverse primers used in qRT-PCR analyses, DNA methylation PCR primers, and forward and reverse primers used in qRT-PCR analyses (PDF)

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**Notes**
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**ABBREVIATIONS USED**
ANOVA, analysis of variance; BSP, bisulfite sequencing PCR; DNMTs, DNA methyltransferases; ERFs, ethylene response factors; e-nose, electronic nose; GC—MS, chromatography—
mass spectrometry; HS-SPME, headspace solid-phase micro-extraction; HWT, hot water treatment; LDA, linear discriminant analysis; SmC, 5-methylcytosine; MeSa, methyl salicylate; m6A, N6-methyladenosine; PAL, phenylalanine ammonia lyase; qRT-PCR, quantitative real-time polymerase chain reaction; SA, salicylic acid; TA, total acidity; TSS, total soluble solids; Vc, vitamin C; VOCs, volatile organic compounds

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