Materials and methods

1 Plant handling

“Micro-Tom” is an experimental model cultivar used extensively for postharvest research

[[1]](#endnote-1) [[2]](#endnote-2) [[3]](#endnote-3). Fruit developmental stages include Mature Green (MG), Breaker (B), Turning (T) and Red Ripe (R)[[4]](#endnote-4) (**Figure 1**). Wild-type Micro-Tom tomato seeds was from UCD Tomato Genomic Resources Center (TGRC). Seeds were soaked in 2.7 % (v/v) sodium hypochlorite for 45 minutes, rinsed thoroughly in running water to get rid of chemical left, placed into petri dishes with damp paper towels and located in a 20°C (± 2°C) room under 16/8 hour-photoperiod for one week. Routine watering was applied every other day. Seedlings were transferred into greenhouse under standard growth condition. After nearly three months, fruit were randomly harvested from a group of 80 wild type Micro-Tom plants. Harvested plants were soaked in 0.25% (v/v) sodium hypochlorite for three minutes and rinsed with nanopore water, placed into paper towels to wait until dry.

A picture containing food

Description automatically generated

**Fig 1. Fruit developmental stages.** Mature Green (M); Breaker (B); Turning (T); Ripen(R) and Over Ripe (OR). Fruit quality improves with ripening. In addition, demethylation increases, and methylation decreases with ripening.

2 Fruit sampling

Treatment groups are described in **Figure 2**. After specific treatment, fruit pericarp was sampled with autoclaved sterile knives, tweezers and tubes, put into liquid nitrogen directly and then stored at -80 freezer. Six individual fruits were randomly chosen from a group of 20 middle- shape fruits, and they were pooled together as one biological replicates. Three biological replicates were used.17

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**Fig 2. Fruit treatment.** FHM, FHT: Tomato fruits are fresh harvested at Mature green and Turning (T) stage; 20T and 12.5T: Fruits were harvested at MG, stored at 20 or 12.5℃, and sampled until T; 5M: Fruits were harvested at MG, stored at 5℃ for two weeks, and directly sampled; 5T: Fruits were harvested at MG, stored at 5℃ for two weeks and then rewarmed at 20℃ until T.

3 Quality assessment

3.1 Firmness

For each treatment groups, six fruits were picked up randomly. Fruit firmness was measured using Texture Analyzer (TA. XT Plus, Texture Technologies, Scarsdale, NY) by compressing the middle of the whole fruit 3mm. The maximum force for each measurement was recorded.

3.2 Objective Color

The three parameters of L, a and b was recorded for each individual fruit using a Konica Minolta colorimeter (Chroma Meter CR-400, Konica Minolta Sensing Americas, Ramsey, NJ, USA) (2° observer and standard illuminant C) in a three-dimensional color space. Standard calculation for hue [Arc tan(b/a)] was done[[5]](#endnote-5).

3.3 Total Soluble Solids (TSS) and Reducing Sugar

Total soluble solids include carbohydrates, organic acids, proteins, fats and minerals of the fruit. Degree brix of each fruit was evaluated to test TSS content, using a portable digital brix refractometer (Hanna Instruments, Inc.). To further test sugar content in tomato fruits, reducing sugar (fructose and glucose) was tested. Six randomly-chose fruits were pooled together as one biological replicate. The protocol is based on one published paper with some modification in our lab[[6]](#endnote-6).

3.4 Starch content

Quantification of starch content was applied on pooled fruit pericarp. Three-day protocol was used based on published work[[7]](#endnote-7). Six fruit pericarp strips were finely grinded together in 75% (v/v) ethanol and then boiled three times in 10 mL each time. The starch pellet without any supernatant was totally dried overnight to get rid of ethanol left. Then the pellet was grinded again and resuspended in 5ml sterile water. Equally disturbing 500 ul homogenate into three screw-up 2mL tubes, all tubes were autoclaved for 45 min. Same amount of 200 mM sodium acetate (PH 5.5) was added. α-amyloglucosidase and α-amylase were added into two of three tubes, and all samples were incubated at 37 °C overnight to fully digest starch into glucose. One left non-enzyme tube was as control. 3,5-dinitrosalicylic acid (DNS) reagent was used for assaying glucose content. The concentration of each sample was determined by glucose standard curve. The reading of control was subtracted from corresponding enzyme-treated ones.

4 Gene expression

4.1 RNA isolation

Prechilled mortar and pestle was used for grinding frozen pericarp tissue. Liquid nitrogen was applied to make sure no melting during grinding. RNA was isolated from 100mg grinded power using homemade Trizol. DNase treatment was applied during extraction using TURBO DNA-free™ Kit (Life Technologies, Carlsbad, CA, USA). The absorbance of 260/280 was between 1.9-2.1 and 260/230 were above 2.0, which means good RNA quality. By running 1% Agarose gel at 84 V for 30min, there was clearly two separated 23s and 15s bands, which represents rarely RNA degradation.

4.2 cDNA synthesis

cDNA was synthesized from 1ug of RNA using random primers in 20ul reaction with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

4.3 Semiquantitative RT-PCR

Semiquantitative RT-PCR is an efficient method for quantification of specific RNA transcripts and the detection of any variation in their expression level under specific experimental conditions[[8]](#endnote-8). This method also costs less than quantitative real-time PCR (RT-qPCR) with relatively high accuracy, which has been verified by other works[[9]](#endnote-9) [[10]](#endnote-10). Based on this, preliminarily analyzing relative gene expression level with semiquantitative RT-PCR is reasonable and efficient. 1 ul cDNA (100mg) product was amplified with 1 unit of Ampli Taq polymerase in the corresponding buffer. ripening related RIN and demethylation related DML2 were tested. The tomato actin gene (SlACT7) was used as an internal control. The amount of tested gene primers and primers of controlled should be extractly same. Designed primers and product length are listed in Table 2. Reactions were carried out in the Gene Amp PCR system 9600. The annealing temperature was optimized to fit well in both two primer pairs. Since the amplicon length is short, cycling numbers as 30 were considered best for quantification analysis. PCR products were directly loaded in Ethidium Bromide- stained, 2% Agarose gel with, running for 45min at 84 V. Two amplified bands were separated clear for each sample. Image J was applied for data analysis.

Table 2 Primers in Semiquantitative RT-PCR

|  |  |  |  |
| --- | --- | --- | --- |
| Gene ID | Name | Nucleotide sequence (5’-3’) | Product length (bp) |
| Solyc03g078400.2 | SlACT7-F | GCTATCCAGGCTGTGCTTTC | 157 |
| SlACT7-R | CAGTAAGGTCACGACCAGCA |
| Solyc05g012020 | RIN-F | ATTGGGCACAAAAGACTTGG | 212 |
| RIN-R | CACTTTGCTCACCACAATGC |
| Solyc10g083630 | DML2-F |  |  |
| DML2-R |  |  |

4.4 Quantitative real-time PCR (RT-qPCR)

To further accurately test DML-2 expression level, RT-qPCR was applied. cDNA was diluted 80-fold and RT-qPCR was performed in a 10 uL reaction using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). Applied Biosystems 7300 Real Time PCR system was applied. Primers were designed based on the cDNA sequences[[11]](#endnote-11) (Table 3). Validation experiment was designed for testing the efficiency of two primer pairs. The efficiency for all pairs of primes was close to 100%, so the comparative Ct Method (ΔΔCT Method) was able to apply for analyzing data. Three biological and three technical replicates were used for each experimental condition.

Table 3 Primers in quantitative real-time PCR

|  |  |  |  |
| --- | --- | --- | --- |
| Gene ID | Name | Nucleotide sequence (5’-3’) | Product length (bp) |
| Solyc03g078400.2 | SlACT7-F | GCTATCCAGGCTGTGCTTTC | 157 |
| SlACT7-R | CAGTAAGGTCACGACCAGCA |
| Solyc10g083630 | DML2-F | GCAGCAGTTCATGCTTACCA | 95 |
| DML2-R | CCCTTTGGCATTTATGCTGT |

5 Methyl-sensitive amplification polymorphism (MASP)

5.1 DNA extraction

Genomic DNA from tomato fruit pericarp was extracted by optimizing published CTAB protocols

[[12]](#endnote-12). Fruit tissue was first used prechilled mortar and pestle in liquid nitrogen. 300mg frozen powder was added 1ml chilled prewash buffer to reduce carbohydrates inside samples, put on the ice for 10 min, and centrifuged 2 min to get rid of all supernatant. Then 65 degree Celsius prewarmed 1ml CTAB buffer was added into each sample. The following step is according to the well-established CTAB extraction protocol, in which chloroform and 95% ethanol were applied[[13]](#endnote-13). DNA pellet was dissolved using 50ul nuclear-free water. The absorbance ratio 260/280 nm and 260/230 nm were between 1.8 and 2.0. Further, DNA appeared on the agarose gel as a single high molecular weight band. Together, these data signify that the DNA was of high quality.

**5.2** **Methyl-sensitive amplification polymorphism (MSAP)**

MSAP is a low-cost method used to provide a global overview of changes in genome methylation status including percent methylation level and changes in methylation sites [[14]](#endnote-14) [[15]](#endnote-15). In MSAP, *Hpa*II and *Msp*I recognize the same CCGG sequence, but have differential sensitivity to methylation at the inner or outer cytosine[[16]](#endnote-16). Changes in DNA methylation can be determined by analyzing a subset of these fragments.

5.2.1 Genomic DNA digestion

Two copy of 500 ng genomic DNA from each sample was treated by 1U each of HF-*EcoR*Iand either methylation-sensitive *Hpa*II or methylation-insensitive *Msp*I with cusmart buffer in a total volume of 10ul. One was labeled as H, and the other was labeled as M. The reaction was ongoing at 37 degree Celsius for 4 hours and followed by heating at 80 degree Celsius 10 min to deactivate the digestion enzyme.

5.2.2 Adaptor ligation

Two pairs of adaptors were designed for *EcoR*I and *Hpa*II*/Msp*I. (See table 4) First, annealing adaptor pairs in two separate tubes. One was for *EcoR*I*,* and one was for H/P. 60pmol of each complementary primer in a total volume of 40ul was heat up to 72 degree centigrade for 10min and then cooled down in a well-closed box overnight. Then the ligation buffer containing 10ul digested DNA, annealed adaptor pairs, T4 ligase and ligase buffer, was incubated overnight at 18 degree Celsius.

5.2.3 Preselective PCR amplification

Ligation reaction was diluted four times, and then 3ul from each one was added to PCR reaction, which includes *EcoR*Ipre-selected primer, *Hpa*II*/Msp*I pre-selected primer, dNTPs, *Taq* DNA polymerase and PCR buffer I. The reaction conditions: 94, 56. 72, for 20 cycles.

9ul PCR reactions was checked in agarose gel electrophoresis 1% at 84V for 45 min. The long smear bands were tested, which verified successful digestion and ligation.

5.2.4 Selective PCR amplification

Collected reaction above was diluted 10 times as a template in the following PCR reaction. Two pairs of selective primers were applied separately and a touchdown program was optimized to get decent band amount, which was as following: 94, 66. 72. The annealing temperature falls 1 degree Celsius in each cycle.

5.2.5 Analyzing selective PCR band number and size.

The 2100 Agilent Bioanalyzer, which uses a micro-capillary based electrophoretic cell, will be used to analyze PCR bands. The Bioanalyzer gives more precise information on the number and sizes of DNA fragment than agarose gel electrophoresis. Selective PCR products were purified using QIAQGEN MinElute@ PCR purification kit, collecting 70bp to 4Kb PCR bands. Then PCR fragments were diluted between 1-10ng/ul as required by Agilent High Sensitivity DNA kit. 1ul of each sample was analyzed in Agilent 2100 bioanalyzer.

Table 4 Adaptors and primers used in MSAP

|  |  |
| --- | --- |
| Name | Nucleotide sequence (5’-3’) |
| *EcoR*I adaptors | CTCGTAGACTGCGTACC  AATTGGTACGCAGTCTAC |
| HpaII/MspI adaptors | GATCATGAGTCCTGCT  CGAGCAGGACTCA TGA |
| *EcoR*IPreselective primer | GACTGCGTACCAATTC |
| *Hpa*II*/Msp*I Preselective primer | ATCATGAGTCCTGC TCGG |
| Selective primer pair 1 | GACTGCGTACCAATTCACC  ATCATGAGTCCTGCTCGGTCAA |
| Selective primer pair 2 | GACTGCGTACCAATTCACC  ATCATGAGTCCTGCTCGGTCCA |

5.2.6 Data analysis

Positive tested PCR bands shown in at least once among three biological replicates, were considered as effective. The restriction digestion sites of each primer pair were recognized first. The presence and absence of bands cut by H and M separately are considered as “1” and “0”. Based on the mechanism of two enzymes, there are different type of situations[[17]](#endnote-17). (Table 5)

Table 5 MSAP site types and methylation status

|  |  |  |  |
| --- | --- | --- | --- |
| Site type | MspI | HpaII | Methylation status |
| I | 1 | 1 | Demethylated |
| II | 1 | 0 | Fully methylated internal C |
| III | 0 | 1 | Hemi-methylated external C |
| IV | 0 | 0 | Fully methylated both C or hemi-methylated internal or external C |

6 Whole Genome Bisulfite sequencing (WGBS)

Since MSAP does not identify specific sequences and only provides data on a relatively small subset of the genome. Differential Methylated Regions (DMRs) in the tomato genome will be assessed at a higher resolution with WGBS [[18]](#endnote-18), [[19]](#endnote-19). The data derived from MSAP and WGBS will be compared in overall methylation level.

6.1 DNA extraction

6.2 Library preparation and sequencing

6.3 Data analysis

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