Understanding postharvest induced fruit quality changes associated with DNA methylation level in tomato (*Solanum lycopersicum* L.)

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ABSTRACT

Tomato (*Solanum lycopersicum* L.) is one of the most popular vegetable crops, and rich in vitamins, antioxidants and fiber. Tomato fruit are highly perishable, and require proper postharvest handling techniques to extend shelf-life. However, postharvest practices may negatively affect fruit quality. Tomato is also a well-established model for studying fleshy fruit ripening. DNA methylation, a kind of epigenetic modification, is involved in initiating tomato fruit ripening, with demethylation occurring in many ripening-related genes as fruit ripen. DNA methylation is also responsive to chilling stress and may regulate gene expression due to changes in external environments. However, the relationship between changes in DNA methylation, and fruit quality due to postharvest handling is still unclear. A better understanding of fruit ripening mechanism with a focus on DNA methylation may help find novel solutions to reduce fruit quality loss due to postharvest.

Therefore, this work aimed to uncover the connection between tomato fruit DNA methylation status and fruit quality changes relating with two types of common postharvest practice, i.e., early harvest and low-temperature storage, using approaches such as Methyl-sensitive amplification polymorphism (MSAP) for DNA methylation and assessing fruit quality biomarkers. The results illustrated that early harvest and low-temperature storage could induce global DNA methylation changes. Furthermore, the alteration in DNA methylation is associated with quality parameters shown by multivariate analysis. This finding is not a comprehensive

assessment, but it advances the understanding for tomato fruit ripening under postharvest handlings. In the future, I propose to connect the DNA methylation dynamics, transcripts abundance of the genes with fruit physiological changes altered by postharvest via more elaborate methods, i.e., fruit transcriptomic analysis and whole genome bisulfite sequencing for fruit DNA methylation.

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CHAPTER ONE: Literature Review

1. Importance of tomato fruit

Tomato (*Solanum lycopersicum* L.) is one of the most produced and consumed vegetables globally (USDA, 2019; FAOSTAT, 2019). There has been a continual increase in global production from 109.3 to 180.9 million tons over the last 20 years (FAOSTAT, 2019). Tomatoes are low in calories and rich in vitamins, micronutrients, minerals and antioxidants such as carotenoids and polyphenols (Vats et al., 2020; Martí et al., 2016; Young & Lowe, 2018). Two types of carotenoids, i.e., ß-carotene, a precursor of vitamin A, and lycopene, a non-provitamin A compound, are the major contributors to the healthful properties of tomato, which include lowering the incidence of certain cancers (Jacob et al., 2008). Tomato fiber is also a good source of dietary fiber that is associated with reducing mortality risk (Padayachee et al., 2017; Navarro-González et al., 2011).

Tomato is also an important functional genomic model for the study of climacteric fleshy fruit development and ripening (Klee & Giovannoni, 2011). The short lifecycle, relatively easy transformation, the availability of a high-quality sequenced genome with the relatively small size (~950Mb) and well-studied natural and induced mutants, enable advances in the exploration of fruit ripening systems at the molecular and physiological levels (Giovannoni, 2004; Mueller et al., 2005; Seymour et al., 2013).

There are generally two types of commercial tomatoes - fresh market and processing. Processing tomatoes are mainly used for tomato pastes, sauces, canned products and ketchup (AGMRC, 2018). They are usually harvested at red ripe and are processed mechanically and immediately after harvest. In contrast, tomatoes for fresh consumption are mainly handpicked before they are fully ripened, and they are sold on the open market (Costa & Heuvelink, 2005). The proportion of processing and fresh market tomato grown differs by country (USDA, 2019). Fresh market tomatoes normally fetch higher prices and require more elaborate postharvest operations compared to processing tomatoes (Zhengfei Guan, 2018).

2. Postharvest technology

Tomato fruit are highly perishable, and sensitive to handling techniques and storage conditions after harvest (Beckles, 2012). Postharvest losses in quantity and quality commonly occur in fresh market tomatoes (Suthar et al., 2019). The irreversible and continuous changes after fruit harvest leads to cell death and senescence, which makes some postharvest losses unavoidable (Kader, 2002). However, some losses are caused by improper postharvest practices, such as mechanical injury or pathological breakdown (Passam et al., 2007). Appropriately applying postharvest techniques could slow fruit senescence and therefore, maintain fruit quality from vine to consumers. While the specific recommendations for postharvest handling are dependent on the desired fruit shelf-life and vary among the produced and consumed regions of tomatoes, some examples of commonly applied tomato postharvest practices are discussed below.

2.1 Harvesting maturity

Fruit maturity at harvest plays an important role in its postharvest quality. Tomato fruit could be harvested between the stages of mature green and fully ripe, depending on the demand for fruit shelf-life. For the tomato produced for local markets, producers harvest fruit close to the fully ripe stage. Tomato destined for sale in distant markets, may be harvested earlier (after 'Mature green' stage), followed by off-the-vine ripening to effectively satisfy the market need for an extended shelf-life. Tomato fruit that is harvested early, i.e., 'Mature green' fruit is able to fully ripen, but it loses its carbon and energy source from the mother plant, resulting in quality loss (Davis & Gardner, 1994). Reduced fruit quality including loss of sugar, a lower ratio of TSS/TA, and changes in volatiles have been reported in off-the-vine ripening fruit compared with optimal harvest (Klein et al., 2010; Zhang et al., 2020).

2.2 Storage and temperature management

Storage of the fresh market tomato after harvest provides stability to the supply chain. Precise temperature management is powerful in fruit storage, and the prescribed temperature may differ by fruit cultivar, harvesting stage, expected storage period, or, transportation (Arah et al., 2016). Before storage, precooling of freshly harvest fruit is used to remove 'field heat', ameliorating the rise in metabolic activities in fruit (Cherono et al., 2018). For short-periods of storage, tomato fruit can be placed at room temperature under good ventilation, while, for longer storage time, early harvesting followed by low-temperature storage is a possible strategy, but this will negatively reduce the fruit flavor (Resurreccion & Shewfelt, 1985). When sensitive produce are exposed to low, non-freezing temperature (≤10°C) for long periods, postharvest chilling injury (PCI) may occur (Biswas et al., 2016). PCI symptoms in tomato fruit include a failure to ripen,

uneven ripening, surface pitting, decay, and high rates of ethylene and respiration upon rewarming (Hobson, 1987).

There are other approaches to calibrate the rate of fruit ripening during storage. Applying controlled and modified atmospheres could delay fruit ripening during postharvest storage. Controlling relative humidity (RH), and modifying the relative proportion of oxygen (O_2) and carbon dioxide (CO_2) in the atmosphere can extend the shelf-life of tomato fruit without a significant loss of flavor (Yang & Chinnan, 1988).

For greater flexibility, ripening can be further controlled by regulating postharvest ethylene. When fruit are exposed to ethylene, ripening would speed up (Chomchalow et al., 2002). Conversely, reducing ethylene production will maintain fruit quality and prolong shelf-life, and several commonly examples are as following: (1) precooling or heat treatment is efficient in slowing down ethylene production during storage (Martínez-Romero et al., 2007); (2) high rates of CO_2 is able to inhibit autocatalytic ethylene biosynthesis (Martínez-Romero et al., 2007); (3) applying 1-Methylcyclopropene (1-MCP) would delay fruit ripening by binding to the ethylene receptors (Watkins, 2006), however use of these chemicals may affect fruit quality (Cliff et al., 2009); (4) calcium chloride ($CaCl_2$) is successfully used in inhibiting ethylene production after harvest (Senevirathna & Daundasekera, 2010); (5) ethylene biosynthesis and sensitivity are affected by low-temperature postharvest storage (Rugkong et al., 2011).

3. Tomato fruit quality

Consumers have been complaining about the quality of store-bought, conventional varieties of tomatoes over the last 30 years (Causse et al., 2002; Klee, 2010). Understanding the physiological basis of fruit quality has therefore become the main focus of tomato researchers, as this information would support the breeding of new tomato cultivar with better quality (Tieman et al., 2017; Klee & Giovannoni, 2011). Fruit quality characteristics depend on the fruit developmental and physiological pathways which are dynamically regulated in the ripening process (Klee & Giovannoni, 2011).

The quality of the tomato fruit is mainly determined by its color, texture and flavor. Tomato fruit color is not only an appealing eating quality for consumers, but also a commonly used indicator of the fruit ripening stages. During ripening in most tomato cultivars, chloroplasts are converted to chromoplasts accompanied by chlorophyll breakdown and carotenoids accumulation, resulting in fruit color transition from green to red (Figure 1). The color of the ripe fruit is red primarily due to lycopene and the accumulation of a smaller amount of the orange or yellowish β-carotene (Salunkhe et al., 1974). The dynamic interplay of carotenoid and chlorophyll metabolism dictates tomato fruit color (Barry & Pandey, 2009; Manoharan et al., 2017).

Figure 1. United States Department of Agriculture tomato color chart (photographed by Kader, Adel. (*ANR Repository*, 2011)). There are six ripening stages represented, classified by the percentage of the fruit

surface manifesting the colors described i.e., Mature Green (100% green), Breaker (Less than 10% yellow, pink or red); Turning (10% to 30% tannish-yellow, pink or red), Pink (30% to 60% pink or red), Light Red (60% to 90% pinkish-red or red), Red (more than 90% red).

Fruit texture is a sensory, multi-parameter characteristic that can be perceived through the consumer's senses, i.e., vision, touch, and taste (Szczesniak, 2002). Tomato fruit texture is commonly described by the attributes of firmness, juiciness, and mealiness (Chaïb et al., 2007). There are various methods of measuring fruit texture, in which puncture and compression are commonly used destructive methods, and non-destructive approaches may include ultrasonic and optical techniques (Chen & Opara, 2013). Firmness of tomato fruit is considered as the main determinant of texture, and the decline of firmness leads to the fruit softness (Payasi et al., 2009). Fruit softening is a natural phenomenon in ripening, and critical to seed dispersal and attracting animals (Oltman et al., 2014; Shipman et al., 2021). A certain degree of fruit softening is desirable in fresh market tomatoes, but too much softening is unwanted (Wang et al., 2018), as it limits fruit shelf-life and increases susceptibility to decay. Tomato fruit softening is associated with disassembly of the cell wall matrix, involving depolymerization and solubilization of the cell wall polysaccharides components, i.e., cellulose, hemicellulose and pectin (Saladié et al., 2007). This event is mainly controlled by the activity of many cell wall degrading enzymes, such as polygalacturonase, pectin methylesterase , galactanase, or remodeling proteins, like expansin (Yang et al., 2017; Wang et al., 2018).

Tomato fruit flavor is determined by a complex interaction of many biochemical compounds, including sugars, acids, amino acids, with aroma volatiles (Yilmaz, 2001). Tomato aroma volatiles are often complex secondary metabolites, and they are derived from carotenoids and

simpler primary metabolites precursors such as amino and fatty acids (Klee, 2010). There are more than 400 volatiles discovered in tomato, but only a limited proportion influences tomato flavor (Baldwin et al., 2000). The non-volatile compounds determine fruit taste. Sugar, mainly the reducing sugars glucose and fructose, makes the largest contribution to tomato taste. There are also organic acids in tomato fruit, predominantly citric and malic acids. The ratio of sugar to acid in fruit is an important indicator of sweetness perception (Kader, 2008; Beckles, 2012). It is well established that tomato flavor is highly interdependent on the relative levels of sugars, acids, and key volatiles, and no single chemical controls quality (Klee & Tieman, 2013).

4. Carbohydrate metabolism in tomato fruit.

Carbohydrate metabolism is important in determining tomato fruit postharvest quality (Luengwilai et al., 2010). Fruit are "sink" tissues (Koch, 2004), that require carbohydrates supplied by photosynthetic "source" tissues (Osorio et al., 2014). The chloroplasts in green tomato fruit have active photosynthetic ability (Egea et al., 2010), thus, green tomato fruit is able to fix carbon (Tanaka et al., 1974; Obiadalla‐Ali et al., 2004). The carbon dioxide from respiration is the major source of green fruit photosynthesis (Y. Zhang & Fernie, 2018). Fruit fixed carbon accounts for 10-15% of that used by the fruit, and may be critical for seed development (Lytovchenko et al., 2011), and maximal carbohydrate and carotenoid accumulation (Powell et al., 2012).

The carbohydrate metabolic pathway in developing tomato fruit is mediated by the activities of numerous enzymes and sugar transporters (Schaffer et al., 2000; Hou et al., 2019; Beckles et al., 2012) (See Fig. 2 for details). Sucrose, the main translocated assimilate, is taken up by tomato fruit (Yelle et al., 1988). In early fruit development, starch synthesis is the main flux; fruit starch level reaches its peak at the mature green stage, and it is then degraded during ripening (Schaffer

& Petreikov, 1997). Starch synthesis and breakdown coexists throughout fruit development, with net synthesis occurring prior to the mature green stage, and net degradation to sugars occurring during ripening (Luengwilai & Beckles, 2009). Starch degradation therefore contributes to sugar accumulation in ripening fruit (Petreikov et al., 2009; Centeno et al., 2011).

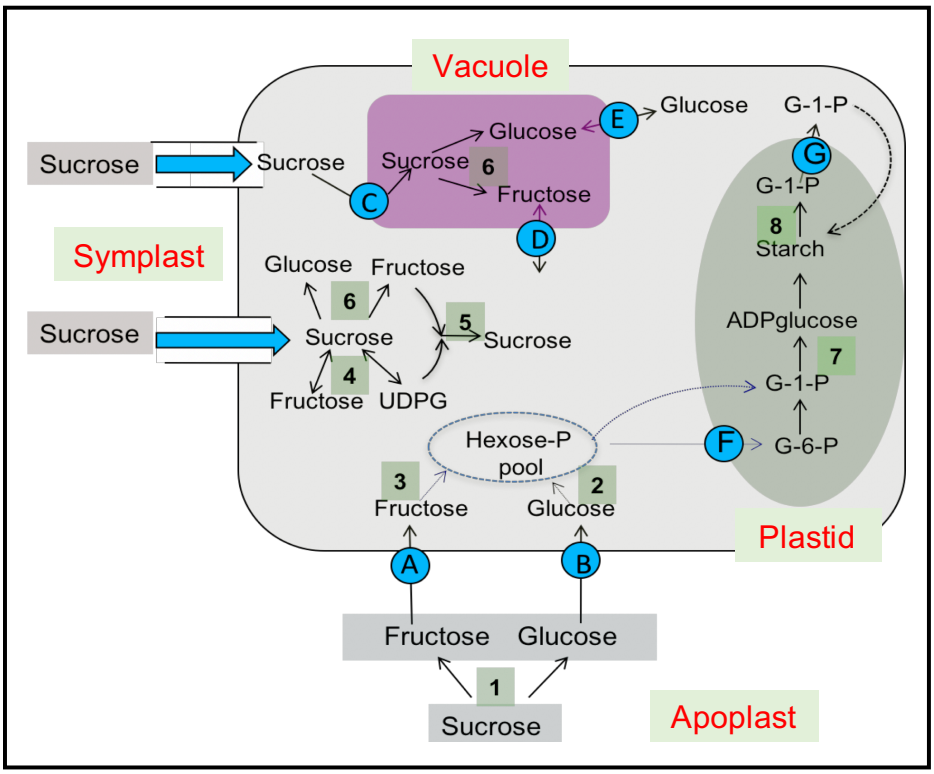

Figure 2. The pathway of developing tomato fruit carbohydrate metabolism adapted from (Beckles et al., 2012). Sucrose is imported to fruit cell by apoplast or symplast. Sucrose may be stored in the vacuole and starch is stored in plastid. Enzymes that involved in this pathway are described as numbers: (1) apoplastic invertase, (2) glucokinase, (3) fructokinase, (4) sucrose synthase, (5) sucrose phosphate, (6) invertase, (7) ADPglucose pyrophosphorylase, (8) starch phosphorylase. The transporters are indicated as letters: A and B are apoplastic hexose transporters; C is the sucrose vacuolar transporter; D and E are hexose vacuolar transporter; F is the plastidic hexose phosphate transporter; G is glucose 1-phosphate transporter. Although mitochondria and chloroplast are not shown in this figure, during the early fruit development and ripening, respiration mainly happened in cytosol and mitochondria, which provides

ATP and carbon dioxide and consumes carbohydrate. In the late ripening stages, chromoplasts that are differentiated from chloroplast are also able to produce ATP in a process called chromorespiration (Renato et al., 2014).

5. The current tomato fruit ripening model

Fruit ripening is initiated after seed maturation, which is the last stage of fruit development (Gillaspy et al., 1993). During tomato ripening, fruit size does not change but there are a myriad of biochemical and physiological processes occurring, which collectively determine fruit quality (Quinet et al., 2019). To improve fruit quality and ameliorate postharvest losses, a deeper understanding of fruit ripening from a physiological, and molecular perspective is necessary. The current tomato fruit ripening model is focused on ethylene, master ripening regulators (transcription factors, TFs) as well as epigenetic changes (Chen et al., 2020; Hiwasa-Tanase, 2016) (Fig. 3).

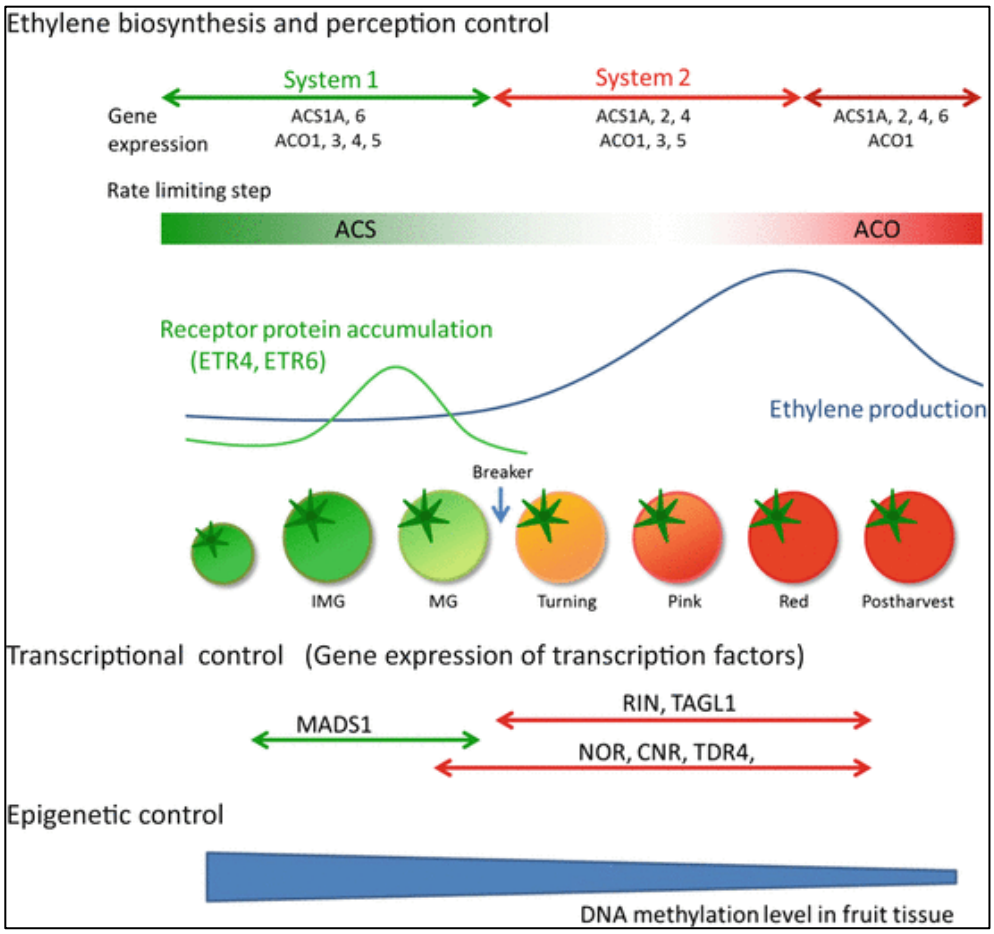

Figure 3. Tomato ripening regulatory model adapted from (Hiwasa-Tanase, 2016). The three panels, i.e., ethylene biosynthesis and perception control, transcriptional control, epigenetic control especially DNA methylation are the three components of tomato fruit ripening. The details are discussed below.

5.1 The role of ethylene in climacteric type of fruit

Ethylene is chemically, the simplest plant hormone. It regulates flowering, shedding of leaves and the ripening and senescence of fruit (Iqbal et al., 2017). Increased rates of ethylene and respiration production during fruit ripening is a defining feature of the climacteric fruit (Paul et al., 2012).

Due to the importance of ethylene, its biosynthesis, signaling pathway in model plants, e.g., Arabidopsis and tomato, and other valuable vegetable and ornamental crops have been well documented over recent decades (Liu et al., 2015; Lelièvre et al., 1997; Karagiannis et al., 2018). The current model of ethylene biosynthesis involves two steps. S-adenosyl-L-methionine (SAM) is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS), and then ethylene will be produced by ACC oxidase (ACO) from ACC (Houben & Van de Poel, 2019). There are two systems of ethylene production characterized in plants. In system 1, ethylene is autoinhibited; while, in system 2, ethylene biosynthesis is controlled in an auto-inductive manner (Fig. 3) (Lelièvre et al., 1997). However, the factors that regulate the transition from system 1 to system 2 ethylene production are still relatively unknown (Liu et al., 2015).

5.2 Master transcription factors (TFs) regulating tomato fruit ripening

Some ripening-related transcription factors (TFs) are involved in ethylene production, but others are functionally independent of ethylene regulation (Klee & Giovannoni, 2011). In tomato fruit, the three master ripening regulators i.e., RIN, CNR and NOR have been extensively studied (Fujisawa et al., 2011; Wang et al., 2020; Lai et al., 2020; Gao et al., 2020). The naturally occurred mutants, i.e., *rin* (*ripening inhibitor*), *nor* (*non-ripening*) and *Cnr* (*Colorless non-ripening*) are all unable to initiate normal ripening, indicating their critical role in tomato fruit ripening (DellaPenna et al., 1989; Thompson et al., 1999). In addition, the three mutants are all considered as 'ethylene-independent' due to the lack of an 'ethylene burst' during fruit ripening (Klee & Giovannoni, 2011).

With the advancement of gene editing technologies, the function of these TFs have been studied in greater detail using CRISPR/Cas9 (Ito et al., 2015; Ito et al., 2017; Ito et al., 2020; Gao et al.,

2019). The CRISPR/Cas9 induced mutation in the coding regions of *RIN*, *CNR*, *NOR* only show partially delayed ripening, indicating that *rin*, *nor* and *Cnr* are all "gain-of-function" mutants and that compensatory functions may exist (Ito et al., 2015; Ito et al., 2017; Ito et al., 2020; Gao et al., 2020).

There are also many other TFs involved tomato ripening, such as TAG1, TAGL1, AP2a, and etc. Some TFs would interact together as a protein complex, and regulate fruit ripening cooperatively (Bemer et al., 2012). Considering the complexity of the transcriptional regulation of tomato fruit ripening, targeting multiple TF genes at the same time by generating spontaneous allele mutation seems to be necessary to better understand the fruit ripening model, and improve fruit quality (Wang, Angenent, et al., 2020; Wang, Lammers, et al., 2020).

5.3 Dynamics of DNA methylation in tomato fruit ripening

Epigenomic modifications, especially DNA methylation of gene promoter regions, are considered to play a critical role in triggering tomato fruit ripening (Zhong et al., 2013). DNA methylation is characterized by the addition of a methyl group to the 5' position of cytosine. The cytosines that are methylated occur in either a symmetrical CHG and CG, or an asymmetrical CHH context (where H is A, C or T) (Finnegan et al., 1998). Symmetrical and asymmetrical DNA methylation are regulated by different genes (Law & Jacobsen, 2010; Matzke et al., 2015). DNA demethylases are able to remove methyl groups from cytosines, a process termed DNA demethylation. In plants, DEMETER-Like demethylases (DMLs) are a group of enzymes that can activate gene promoter demethylation, by the activity of DNA Glycosylase-Lyases (Zhu,

2009). There are four DML isoforms in tomato, of which SlDML2 is most important in fruit (Liu et al., 2015). Silencing this gene inhibits tomato fruit ripening (Lang et al., 2017).

DNA methylation in plants regulates molecular processes such as gene expression and transposon silencing, which in turn modulates biological and developmental processes, and response to biotic and abiotic stress (Zhang et al., 2018; Gallego-Bartolomé, 2020). During tomato fruit ripening, DNA demethylation is active, such that the global DNA methylation level is reduced (Giovannoni et al., 2017). Demethylation is especially active in the promoter of ripening genes that contain the RIN binding sites (Zhong et al., 2013).

Changes in DNA methylation occur when tissues are exposed to environmental stress. Postharvest handling strategies may be viewed as types of stress on the tomato fruit (Zhang et al., 2018), but there are few reports related to postharvest induced changes in the tomato fruit methylome, especially early harvest and low-temperature storage. Based on current knowledge, postharvest chilling leads to hypermethylation of the promoter of ripening genes in red tomato fruit (Zhang et al., 2016), suppressing their expression, and partially explaining the poor quality of refrigerated tomato. This chilling-induced methylation is reversible when fruit is rewarmed (Zhang et al., 2016). Furthermore, Zhang et al. (2020) followed up and demonstrated that postharvest ethylene is able to stimulate increases in *SlDML2* transcripts and DNA demethylation in tomato. Still in tomato, besides DNA methylation, there are also additional epigenetic regulatory mechanisms influencing fruit ripening. Methylation-directed mRNA changes, i.e., epitranscriptomic regulation that is mainly governed by mRNA demethylase, is also widespread in fruit ripening (Zhou et al.,

2019). Another epigenetic mark- histone modifications after translation, is commonly conserved in fleshy fruit species regulating ethylene biosynthesis (Lü et al., 2018; Liang et al. 2020). Histone lysine demethylase encoded by *SlJMJ6* could regulate this histone methylation, therefore, promote tomato fruit ripening (Li et al., 2020).

In conclusion, postharvest practice is needed to control tomato fruit ripening speed, however, it may negatively reduce fruit quality. To maintain fruit quality as well as extend its shelf-life, a deep understanding on fruit ripening mechanism is necessary. The tomato ripening network is complex. There are raising questions regarding DNA methylation on tomato fruit ripening: (1) though, the recent studies shown that postharvest chilling and ethylene have effect on global DNA methylation levels and fruit volatiles content, they didn't focus on the postharvest practices that are commonly used in fresh tomato industry, i.e., early harvest and low-temperature storage; (2) the correlation between postharvest induced DNA methylation and important fruit quality biomarkers including sugar, acid, firmness, and etc. is still unclear; (3) the global DNA demethylation appears to take place during tomato fruit ripening, and is triggered by demethylase. However, the key players initiating demethylase gene expression, i.e., *SlDML2* and *SlJMJ6* are unknown. This could be studied by focusing on the transcriptional regulation of demethylase gene; (4) the current knowledge only considers that DNA demethylation in the gene promoter activates ripening genes expression. However, the ripening transcription factors may also regulate DNA methylation by binding *SlDML2*. The potential evidence is that the ripening mutants, including *rin*, *nor* and *Cnr*, all have decreased *SlDML2* expression level in fruit ripening. A further study is needed for testing their regulatory relationship; (5) hormone, a key player in fruit ripening, may play an important role in regulating DNA demethylation during ripening. Their relationship is still undetermined.

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CHAPTER TWO:

Postharvest handling induces changes in fruit DNA methylation status and is associated with alterations in fruit quality in tomato (*Solanum lycopersicum* L.)

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Contributions:

Bixuan Chen, a MS graduate in the Graduate Group of Horticulture & Agronomy, initiated the experimental design, sampled and treated some of the fruit. Dr. Karin Albornoz, also a Ph.D. graduate from the Graduate Group of Horticulture & Agronomy, advised on postharvest methodology. I developed the experimental plan, optimized the MSAP and executed all other experiments, performed data analysis, wrote the original draft and edited the manuscript. Dr. Diane Beckles directed all phases of the work. All authors edited the manuscript.

ABSTRACT

Postharvest handling of tomato (*Solanum lycopersicum* L.), specifically low-temperature storage and early harvest, are used to extend shelf life but often reduce fruit quality. Recent work suggests that DNA methylation dynamics influences fruit ripening through the demethylase SlDML2 gene. However, the influence of postharvest handling on DNA methylation in relation to fruit quality is unclear. This work aimed to clarify these issues by analyzing DNA methylation using methyl-sensitive amplification polymorphism (MSAP), semi-quantitative transcriptional analysis of marker genes for fruit quality (*RIN*; *RIPENING INHIBITOR*) and DNA methylation (*SlDML2*; *Solanum lycopersicum* L. *DNA demethylase 2*), and, fruit biochemical quality biomarkers. Multivariate analysis of these data suggested that fruit DNA methylation state was associated with different postharvest handling techniques. Chilled postharvest fruit were distinct in their DNA methylation state and quality characteristics, which implied that these three phenomena i.e., chilling, methylation, and quality are highly connected. In addition, different postharvest handling methods modulated *SlDML2* transcript levels but had little effect on the level of *RIN* transcripts in fruit that reached the Turning stage after early harvest, and cold storage. Although not a comprehensive global assessment, these data collectively helped to advance our interpretation of tomato fruit ripening. In conclusion, our findings revealed that postharvest-induced variation in fruit quality is in relation to DNA methylation. Long-term this work will help better connect physiological changes in tomato fruit to events happening at the molecular level.

Keywords: tomato fruit ripening; postharvest handling; fruit quality; DNA methylation; methyl-sensitive amplification polymorphism.

Abbreviations: MSAP, methyl-sensitive amplification polymorphism; ‘FH’, fresh-harvested fruit; ‘M’, Mature green; ‘T’, Turning; ‘5M’, fruit harvested at Mature green and stored at 5°C for 14 days; ‘5T’, ‘5M’ fruit stored at 20°C until Turning; ‘20T’ and ‘12.5T’, fruit harvested early at Mature green, and stored at 20°C and 12.5°C, respectively; SRT-PCR, Semiquantitative Reverse Transcription-Polymerase Chain Reaction; RT-qPCR, Quantitative real-time PCR; TA, titratable acidity; *SlDML2*, *Solanum lycopersicum* L. *DNA demethylase 2*; *RIN, RIPENING INHIBITOR*.

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the world's most popular fresh-market vegetables (Strange et al., 2000; Gould, 2013), and is also an important research model for fleshy-fruit development (Giovannoni et al., 2017). Tomato is highly perishable after harvest (Sloof et al., 1996), and determining adequate postharvest handling and storage conditions is important for extending fruit shelf-life and reducing postharvest losses (Nasrin et al., 2008), which is necessary given the complicated modern supply chain for fresh produce (Shipman et al., 2021).

Early harvest and low-temperature storage can delay fruit ripening and extend shelf-life, but there can often be unintended consequences, such as lower fruit sensory quality, reduced consumer satisfaction, and as a result, fewer repeat purchases (Hobson, 1987; Heuvelink, 2005; Klein et al., 2010). For example, Mature green fruit ripened off-the-vine at temperatures lower than ambient will have maximal shelf-life, but they may not have a fully realized sensory profile (Majidi et al., 2014). These poor sensory attributes compared to 'on-the-vine' ripened tomato occur because fruit nutrient supply from the mother plant is prematurely disrupted, and there may be additional losses of flavor- and taste-associated compounds occurring during storage which collectively leads to postharvest waste (Beckles, 2012). Further, exposing tomatoes to temperatures below 10°C can severely disrupt normal ripening leading to postharvest chilling injury (Biswas et al., 2016; Albornoz et al., 2020). This physiological disorder is cumulative in its effect, as the consequences are normally presented during rewarming, and include unusual

softening, poor flavor and taste, and, visual defects such as uneven ripening, pitting and decay (Albornoz et al., 2019).

Tomato quality attributes are determined by many genetic, physiological and biochemical factors that occur as the fruit ripens. This leads to the characteristic and desirable changes in color, texture, flavor and taste. Measurements of fruit firmness can act as a proxy for texture and juiciness (Saladié et al., 2007), color is an indicator of ripening stage and visual quality (Stommel et al., 2005), and the ratio of sugar-to-acid contributes to an appealing tomato taste and is used as a marker of this attribute (Anthon et al., 2011; Beckles, 2012). The biochemical changes that lead to these and other events are controlled by programmed developmental pathways (Klee & Giovannoni, 2011) that are disrupted by off-the-vine ripening and low-temperature storage (Biswas et al., 2016).

Tomato fruit ripening, and therefore quality is mediated in part, by upstream changes in DNA methylation (Stower, 2012). The promoter region of many ripening genes remains methylated during early fruit development until the onset of ripening (Zhong et al., 2013). In tomato, DNA demethylation is critical for fruit ripening and quality, while in contrast, postharvest chilling reverses this process and promotes methylation of many ripening genes, and it is associated with reduced quality (Zhang et al., 2016). Two genes have important roles in these observations: *SlDML2* and *RIN* which both increase in expression during tomato fruit development. *SlDML2* encodes a DNA demethylase, that activates hundreds of ripening-related genes in tomato fruit by removing the methyl-group from their promoter region (Lang et al., 2017). One important target of *SlDML2* is *RIN (*Liu et

al., 2015; Lang et al., 2017). RIN is a central fruit ripening transcription factor (Ito et al., 2008; Li et al., 2011; Qin et al., 2012; Martel et al., 2011; Karlova et al., 2014; Ito et al., 2020; Li et al., 2020) that is also, powerfully regulated by the DNA methylation levels of its promoter regions (Liu et al., 2015; Lang et al., 2017). RIN and RIN-induced genes and transcription factors (TFs) are suppressed through hypermethylation, in pre-ripened fruit or fruit exposed to chilling (Zhang et al., 2016).

The dilemma raised here is that consumers prefer full-flavored tomato fruits (Bruhn et al., 1991), but postharvest practices designed to extend shelf-life often reduce fruit quality. The former observations provide a cornerstone for understanding how postharvest techniques result in the loss of flavor in tomato fruit. Here, we researched if changes in fruit DNA methylation status are induced by different postharvest practices i.e. early harvest at Mature green, and low-temperature storage, and, if there is a relationship between changes in DNA methylation and fruit quality parameters. An unbiased overview of changes in ripening-associated genome methylation was determined using Methyl-sensitive amplification polymorphism (MSAP) (Xu et al., 2000). This is a simple method that indicates changes in a limited set of methylation sites across genomes (Yaish et al., 2014). In MSAP, two restriction enzymes *Hpa*II and *Msp*I that recognize the same CCGG sequence, but with differential sensitivity to methylation at the inner or outer cytosine are used. DNA methylation status is then determined first, by analyzing the number and sizes of the generated fragments after enzyme digestion, and second, by comparing fragments from tissues at different developmental stages, environmental

treatment etc. to indicate how these conditions influence global methylation status (Chen et al., 2019).

To sum up, our aim was to use MSAP to clarify how industry practices influence fruit global DNA methylation levels. This is a first step to extend shelf-life while helping improve fruit quality. Long-term, reducing postharvest losses and increasing market consumption could be possible if the relationships of fruit quality and DNA methylation are understood.

2. Materials and methods

2.1 Plant handling

'Micro-Tom' tomato seeds were obtained from the UCD Tomato Genetics Resource Center (TGRC). Seeds were soaked in 2.7% (v/v) sodium hypochlorite for 45 min, rinsed thoroughly in running water, 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 the greenhouse at UC Davis in 2018 and 2019 under the growth temperature between 25°C to 30°C. Fruit were randomly harvested from over one hundred 'Micro-Tom' plants.

2.2 Fruit sampling

All harvested fruits were soaked in 0.25% (v/v) sodium hypochlorite and rinsed with nanopore water, wrapped with paper towels until dry before storage or further analysis. To explore potential changes at the molecular level, and in tomato fruit quality due to

postharvest practices, specifically, early harvest and low-temperature storage, we harvested fruit at Mature green (the earliest harvest stage), and stored them at different temperatures (20°C, 12.5°C) until they reached the Turning stage (Fig. S1 and Fig. 4) (Takizawa et al., 2014). Turning is the ripening stage just before Red ripe for 'Micro-Tom', which is similar to the Pink stage for conventional tomatoes (United States Department of Agriculture, 1975). To investigate postharvest chilling injury, we stored Mature green fruit at 5°C for 14 days in order to induce this disorder (Albornoz et al., 2019). Tomato fruit are unable to ripen at this temperature, so after chilling they were allowed to recover at 20°C until they reached Turning.

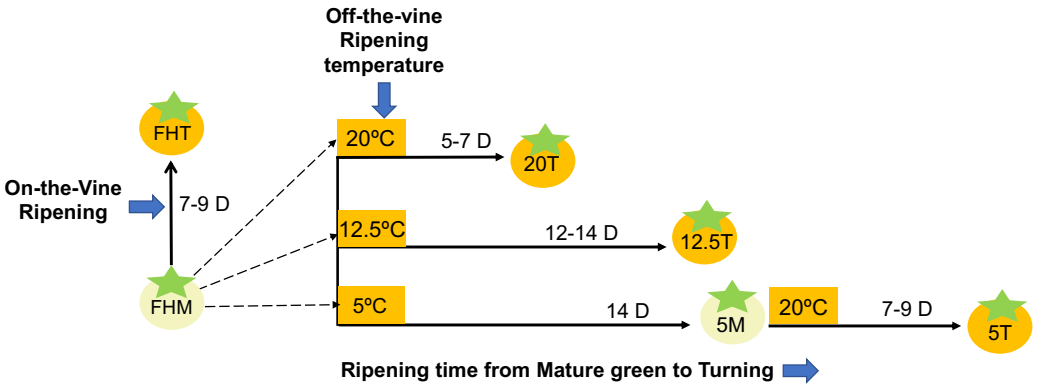

Figure 4. A diagram to illustrate the different fruit treatments used in this study. Different comparisons were made based on the following: 1) Fruit development: Tomato fruit were freshly harvested at Mature green (M) and Turning (T) stages and are described as 'FHM' and 'FHT', respectively. 2) Temperature treatment: Fruit were harvested at M, stored at 20°C or 12.5°C, and sampled until Turning. These fruit are described as '20T' and '12.5T', correspondingly. 3) Chilling injury: '5M' represents fruit that were harvested at M, stored at 5°C for 14 days, and directly sampled. These '5M' fruit were rewarmed at 20°C until Turning and described as '5T'. 4) Ripening time: The numbers beside each line indicate the timeframe between samples.

Fresh fruit were used for firmness, color and Total Soluble Solids (TSS) analysis, while the pericarp from the remaining sampled fruit were fresh frozen in liquid nitrogen and stored at -80°C for further assessment. Six randomly chosen fresh fruit were measured for firmness, color and TSS analyses as six biological replicates, and three biological replicates were used for titratable acidity. For reducing sugar, starch, gene expression and DNA methylation assessment, six individual fruits were randomly chosen from a group of twenty fruits of uniform size, and those six fruits were pooled together as one biological replicate. A minimum of three biological replicates were prepared in each assessment.

2.3 Quality assessment

Firmness. Fruit firmness was measured using a Texture Analyzer (TA; XT Plus, Texture Technologies, Scarsdale, NY) by compressing the middle of the whole fruit 3 mm, using a 5 mm flat probe. This method was adapted from the paper published by El-Mogy et al., (2018). The maximum force for each measurement was recorded.

Fruit Color. Objective parameters of color i.e. L, a* and b* were recorded for each individual fruit using a Konica Minolta colorimeter (Chroma Meter CR-400, Konica Minolta Sensing Americas, Ramsey, NJ, USA). Readings were obtained using a 2° observer and standard illuminant C setting in a three-dimensional color space. Standard calculations for Hue [Arc tan(b/a)] were made as described by Mclellan et al., (1995).

Total Soluble Solids (TSS) and Reducing Sugars. Tomato TSS include carbohydrates, organic acids, proteins, fats and minerals contents. Degrees Brix (used to assess TSS content) of each fruit was evaluated, using a portable digital Brix refractometer (Hanna Instruments, Inc.). To further test sugar content in tomato fruit pericarp, reducing sugars (fructose and glucose) were measured (Miller, 1959).

Titratable acidity (TA). An aliquot of the pericarp (1-2 g) from a single fruit pericarp was added to 10 mL water and incubated at 80°C for 20 min, centrifuged at 4,000 x g for 15 min. The supernatant was transferred to a beaker, and water added to 75 mL. The solution was equally divided into three tubes (technical replicates) and one drop of 0.5% (w/v) Phenolphthalein Indicator (La-Mar-Ka, Inc) was added to each. TA was obtained by titrating 0.05 M NaOH manually into the solution until a faint color was visible for a few seconds.

Starch content. Fruit tissues were treated, homogenized and gelatinized as described by Luengwilai et al., (2010). A 500 µL digestion mixture of 200 mM sodium acetate (pH 5.5), 1-unit α-amyloglucosidase and 12 units α-amylase were added into two tubes containing the homogenized starch solution, with a third serving as a non-enzyme control. All samples were incubated at 37°C overnight to fully digest starch into glucose. The 3,5-dinitrosalicylic acid (DNS) reagent was used for assaying glucose content as described by Dong et al., (2018).

2.4 Gene expression

RNA isolation. RNA was isolated from 100 mg ground power using a Trizol-based protocol (Leterrier et al., 2008). DNase treatment was applied during extraction using TURBO DNA-free™ Kit (Life Technologies, Carlsbad, CA, USA). RNA quality and integrity were assessed by microvolume spectrophotometer and 0.8% (w/v) agarose gel electrophoresis.

cDNA synthesis. cDNA was synthesized from 2 µg of RNA using random primers in 20 µL reaction with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

Semiquantitative RT-PCR (SRT-PCR). The PCR program and cDNA input amount were optimized to ensure that the targeted gene (*SlDML2* and *RIN*) and *SlACT7* (used as an internal control) PCR bands intensity on the gel were within a linear range for quantitative analysis. One microliter of cDNA product was amplified with AmpliTaq polymerase in the corresponding buffer. Designed primers and product sizes are listed in Table S1. Reactions were carried out in the Gene Amp PCR system 9600 (Applied Biosystems, USA) with the following program: one cycle of 10 min at 95°C, 45 s at 57°C, 45 s at 72°C, and then 25 cycles of 45 s at 95°C, 45 s at the annealing temperature (optimized for each pair of primer: 57°C for *RIN* and *SlACT7*, and 52°C for *SlDML2* and *SlACT7*), 1 min at 72°C, followed by 72°C extension for 2 min. PCR products were directly loaded into a 2% (w/v) agarose gel, electrophoresed for 45 min at 84 V, and stained with ethidium bromide. Two amplified bands were separated for each sample. The relative expression level was calculated by the ratio of intensity areas in agarose gel

between the targeted gene and *SlACT7* by Image J (Schneider et al., 2012). All enzymes and buffers were from Applied Biosystems (USA).

Quantitative real-time PCR (RT-qPCR). cDNA created as described was diluted 80-fold. RT-qPCR was performed in a 10 μL reaction using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). Applied Biosystems 7300 Real Time PCR system (Applied Biosystems, USA) was used. Primers were designed based on the cDNA sequences (Table S2). The efficiency for all pairs of primers was close to 100%, so the comparative Ct Method (ΔΔCT Method) was applied for analyzing data. Three biological replicates and three technical replicates per bio-replicate were used for each experimental condition.

2.5 Methyl-sensitive amplification polymorphism (MSAP)

A schematic of the MSAP procedure is shown in Fig. 5, while detailed steps in the protocol are described below.

DNA extraction. Three biological replicates were used for MSAP analysis, each derived from a pool of six individual fruits. Genomic DNA from tomato fruit pericarp was extracted by optimizing published CTAB protocols (Yan et al., 2018). Three hundred milligram of frozen powder was added to 1 mL of chilled washing buffer (100 mM Tris-HCl (pH 8.0), 5 mM ethylene-diaminetetraacetic acid (EDTA, pH 8.0), 0.35 M glucose, 1% (w/v) polyvinylpyrrolidone (PVP), 2% (v/v) β-mercaptoethanol), put on ice for 10 min, and centrifuged 2 min to remove all supernatant. This step helps remove high levels of polysaccharides and polyphenols. Then 1 mL 65°C prewarmed CTAB buffer (100 mM Tris-HCl (pH 7.5), 25 mM EDTA, 1.5 M NaCl, 2% (w/v) CTAB, and 0.3% (v/v) β-

mercaptoethanol) was added into each sample. The remaining steps were done as described by Healey et al., (2014). The DNA pellet was dissolved using 50 µL nuclease-free water. The absorbance ratio 260/280 nm and 260/230 nm were between 1.8 and 2.0. DNA appeared on an 0.8% (w/v) agarose gel as a single high molecular weight band.

Genomic DNA digestion. Two aliquots of 1 µg genomic DNA from each sample were treated by 20 U each of HF-*EcoR*I and either methylation-sensitive *Hpa*II or methylation-insensitive *Msp*I with Cutsmart buffer in a total volume of 30 µL. The reaction was held at 37°C for 4 hours, followed by heating at 80°C for 10 min to deactivate the digestion enzyme. All restriction enzymes and buffers were from New England Biolabs (USA).

Adaptor ligation. Two pairs of adaptors were designed for HF-*EcoR*I and *Hpa*II/*Msp*I (See Table S3). Fifty pmol of each adaptor pair was placed in a total volume of 40 µL, heated at 72°C for 10 min and all tubes were then cooled in a tightly closed box overnight. Then, 15 µL of digested DNA, annealed adaptor pairs, T4 ligase and ligase buffer, in a total volume of 30 µL, was incubated overnight at 18°C.

Preselective PCR amplification. Twenty-five µL of the ligated product was added to the PCR reaction, which included the HF-*EcoR*I pre-selected primer, *Hpa*II/*Msp*I pre-selected primer, dNTPs, AmpliTaq DNA polymerase (Applied Biosystems, USA) and PCR buffer I, in a total volume of 30 µL. The program was comprised of one cycle of 5 min at 72°C, 3 min at 94°C, and then 36 cycles of 30 s at 94°C, 1 min at 56°C, 1 min at 72°C, and 10 min at 60°C. Nine microliters of the PCR reaction product was checked on

a 1.5% (w/v) agarose gel after electrophoresis at 84 V for 45 min. A DNA 'smear' of even intensity was observed among all DNA samples, verifying successful digestion and ligation.

Selective PCR amplification. The pre-selective PCR product was diluted 10-fold and used as a template in the following PCR reaction. Each template was loaded twice into two different tubes applying two pairs of selective primers separately for generating diverse data. Each PCR reaction also included a pair of selective primer, dNTPs, AmpliTaq DNA polymerase (Applied Biosystems, USA) and PCR buffer I, in a total volume of 20 μL. All primer sequences were listed in Table S1. The touchdown program was set as following: 1 cycle of 45 s at 94°C, 30 s at 65°C, and 1 min at 72°C, 12 cycles decreasing the annealing temperature by 0.7°C per cycle , and then 20 cycles of 30 s at 94°C, 30 s at 55.9°C, and 1 min at 72°C.

Analyzing selective PCR bands number and size. PCR products were purified using the MinElute@ PCR purification kit (QIAGEN, USA) which collects fragments from 70 bp to 4 kb. The fragments were diluted to reach a concentration range of 1-10 ng/μL as required by the Agilent High Sensitivity DNA kit. A 2100 Bioanalyzer (Agilent Scientific Instruments, USA), with a micro-capillary based electrophoretic cell, was used to analyze the PCR bands.

Data analysis. Three biological replicates were performed for each tomato treatment. A "1" or "0" indicates the presence and absence of a DNA band respectively. If at least two

replicates of the three showed a PCR band, this was classified as a "1", while, if only one or no replicate showed a band, this was considered as "0". Alteration in DNA methylation of tomato fruit was determined by comparing the site type (see Fig. 5B and Table S4) from two different treatments, and the definition of "de novo methylation", "demethylation" and "no change" was according to Chen et al., (2019).

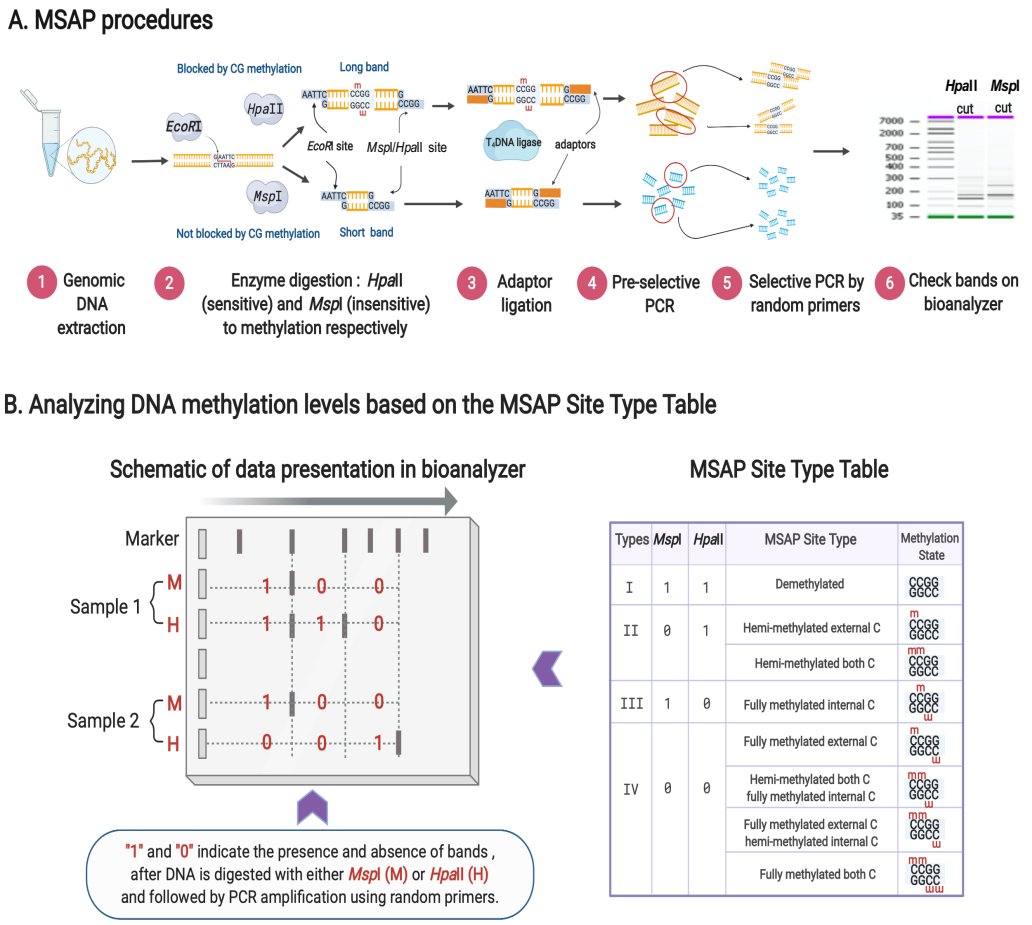

Figure 5. Methyl-sensitive amplification polymorphism (MSAP) procedures. (A) Individual steps used in the MSAP protocol. For details refer to Section 2.5 in the materials and methods. (B) Schematic representation of the DNA fragments and their classification as one of four 'site-types' determined by the *Msp*I (M) and *Hpa*II (H) digestion pattern. Each pattern represents a different methylation state shown in the MSAP Site Type Table (adapted) (Fulneček & Kovařík, 2014; Guarino et al., 2019).

2.6 Statistical analysis

All statistical analyses were done using the R platform (R Core Team, 2020). Box plots for fruit quality and gene expression, and the heatmap of DNA methylation patterns (Fig. 6) were generated by ggplot2 (Wickham, 2009). Compact Letter Display of Pairwise Comparisons (CLD) (Piepho, 2004) were used for testing all pairwise comparisons of least-squares mean, with the significance level set at 0.05. Hierarchical Clustering analysis (HCA) was performed using "Euclidean distance" by the function of "dist" and "hclust" (Murtagh & Legendre, 2014). Principal Component analysis (PCA) for MSAP data were created by "mixOmics" (Rohart et al., 2017) and "tidyverse" (Wickham et al., 2019), and PCA for quality parameters was completed by MetaboAnalystR (Pang et al., 2020).

3. Results

3.1 DNA methylation variation due to ripening and postharvest handling

The DNA methylation data generated by MSAP were grouped into one of three classes based on treatment-induced changes i.e., de novo methylation, demethylation, or no change in methylation status. These data were depicted in two ways; first, showing details of the relative abundance of individual sites (DNA bands) that led to the above classification (Fig. 6), and second, providing an overview of the data i.e. the percentage of each methylation class induced by the treatment (Fig. 7).

We found distinctive global DNA methylation levels in fruit at the same ripening stage i.e. Turning, due to postharvest handling. This conclusion was drawn based on the MSAP data (Fig. 6D and Fig. 7D), which indicated that there were different de novo methylation and demethylation events occurring across assorted Turning fruit, and only a limited number of the examined sites did not change. 'FHT' fruit were ripened under optimal condition. Early-harvested fruit that were ripened at 20°C ('20T') was the most similar in methylation patterns to 'FHT', while the most contrastable DNA methylation fragmentation state was observed between 'FHT' and '5T'.

Comparing the methylation levels in 'FHM' vs. 'FHT', we found that 26.13% of the genomic sites tested represented DNA demethylation events, which is greater than the percentage representing de novo methylation events (23.42%) (Fig. 6A and Fig. 7A). This indicates that the overall cytosine methylation was reduced during 'on-the-vine' ripening.

To focus on the chilling effect on Mature green fruit, chilled '5M' were compared to those that were freshly harvested 'FHM' (Fig. 6B and Fig. 7B). The data showed that 39.64% of the bands underwent de novo methylation, 35.14% of the bands were unchanged, and 25.23% of bands were demethylated in '5M' relative to 'FHM'. Thus, the data from 111 generated DNA fragments show that DNA methylation status was influenced by chilling.

After transferring previously chilled fruit to room temperature, fruit were able to resume ripening. Under this recovery treatment, more genomic sites (39.64%) were demethylated, compared to those that underwent de novo methylation (Fig. 6C and Fig. 7C). The high percentage of demethylation events during rewarming (from '5M' to '5T') is similar to the trend seen during normal ripening (from 'FHM' to 'FHT'). This suggested that the normal demethylation events that occured during ripening recovered when the fruit were rewarmed, which allowed the fruit to reach Turning after two weeks chilling ('5T').

To summarize all observations generated by the MSAP analysis, (a) postharvest handling induced changes in fruit global DNA methylation status, and the rank of their methylation status based on similarity to "on-the-vine" ripened fruit at Turning was '20T' > '12.5T' > '5T'; (b) demethylation occurred as the fruit ripened; (c) chilling at the onset of fruit ripening inhibited the demethylation trend in normal fruit ripening; and (d) DNA demethylation occurred in the following rewarming process, but some of these demethylated sites were different to the fruit under normal ripening.

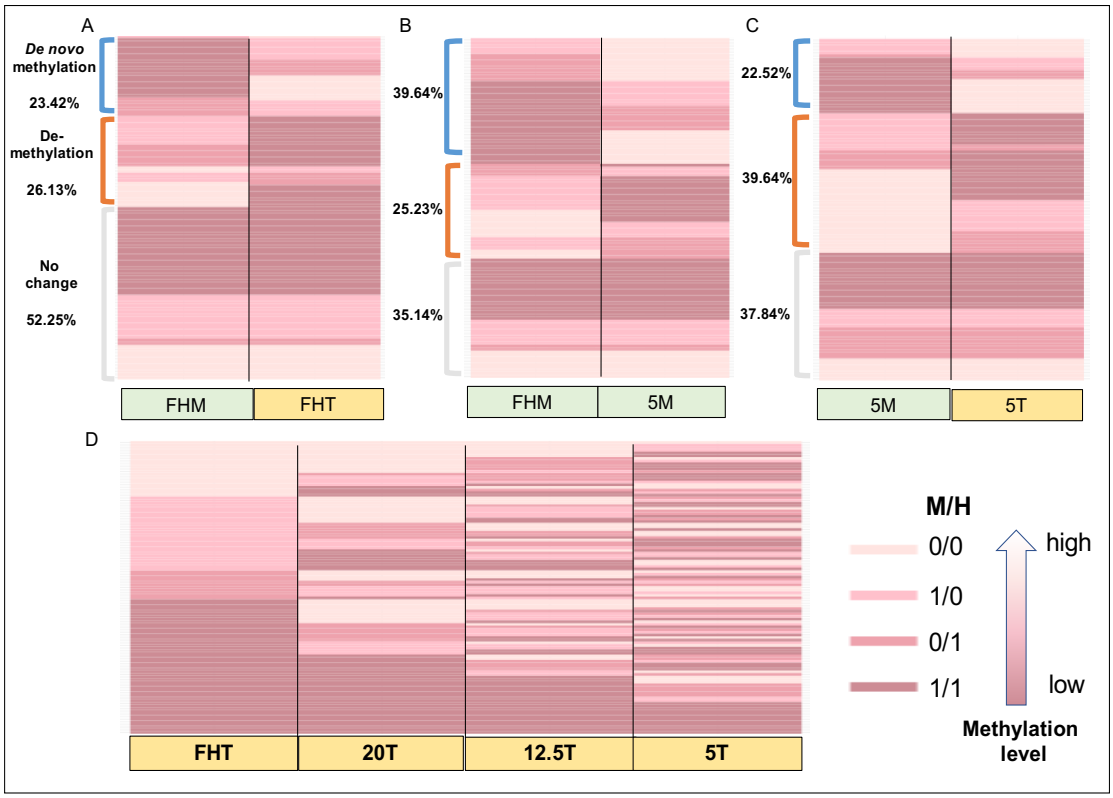

Figure 6. Global DNA methylation status as determined by the banding pattern generated by MSAP. Bands are shown as one of four colors from dark to light pink indicating low to high DNA methylation levels respectively. Each color indicates a distinct DNA methylation state, illustrated in the MSAP site type table (Fig. 5B and Table S4). Each row represents one band of a specific size generated by restriction with *Msp*I (M) or *Hpa*II (H). A total of 111 bands (rows) were generated by the MSAP. All figures show the same 111 bands but arranged differently for ease of comparison. In Figs. 6A, 6B and 6C, bands were ordered and organized based on the three DNA methylation patterns observed when the two samples were compared i.e. 'No change' in methylation status (grey bracket), 'Demethylation' (orange bracket) and 'de novo methylation' (blue bracket). For Fig. 6D, bands were organized by their similarity to 'FHT'. Comparisons examined were: **(A) Normal ripening**: comparison between fresh harvested Mature green fruit 'FHM' and fresh harvested fruit at Turning i.e. 'FHT'. **(B) Chilling effect**: fresh harvested Mature green fruit 'FHM' compared to Mature green fruit stored at 5°C i.e. '5M'. **(C)**

Rewarming effect: when '5M' fruit were stored at 20°C until Turning i.e. '5T'. **(D) Ripening and Treatment**: all samples at Turning stage i.e. 'FHT', '20T', '12.5T' and '5T', were compared.

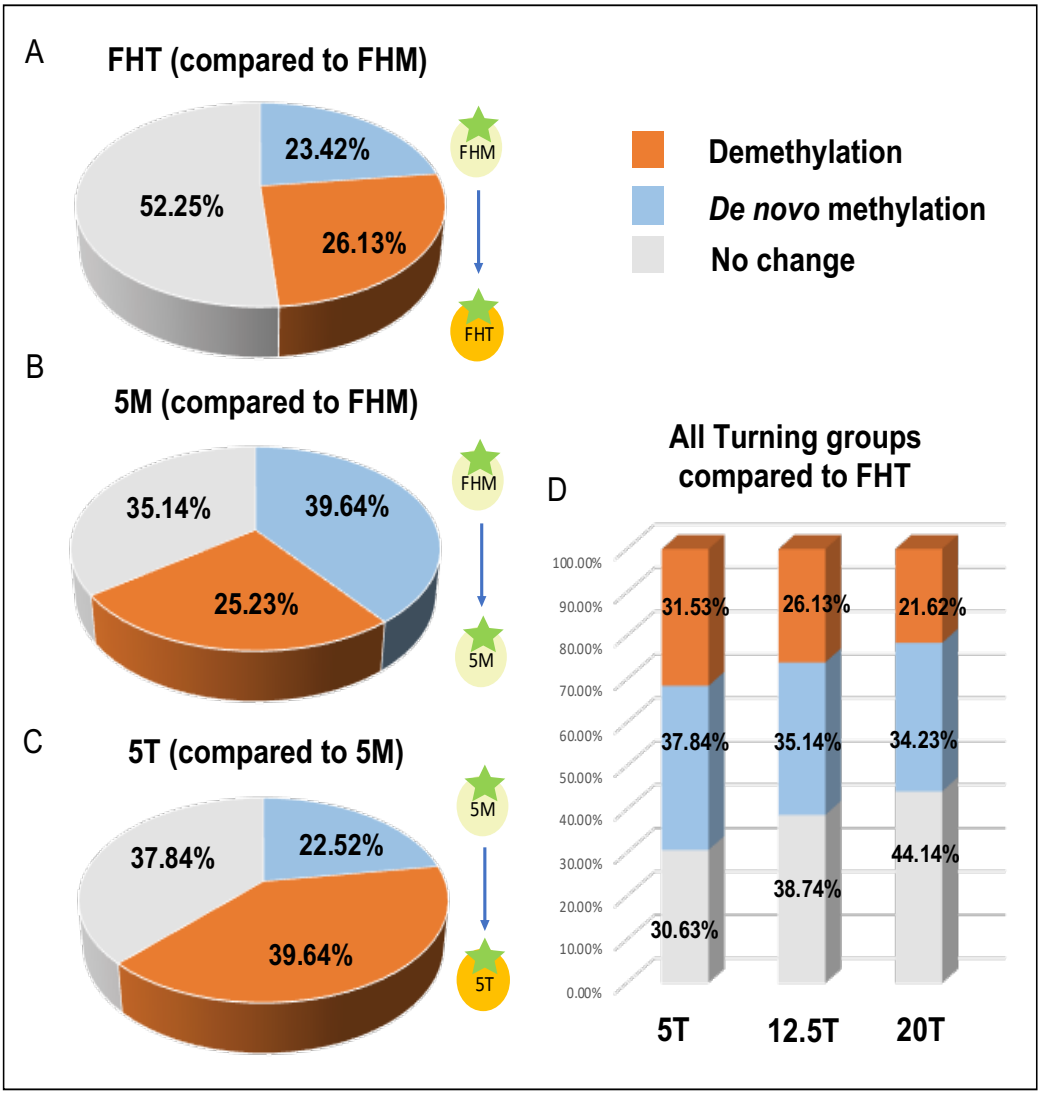

Figure 7. Comparisons of fruit DNA methylation status due to ripening and chilling. The following factors were examined: **(A) Normal ripening**: fruits harvested at T ('FHT') were compared to fruits at M ('FHM'). **(B) Chilling**: fruits at Mature green stored at 5°C for 14 days ('5M') were compared to fruit before they were chilled ('FHM'). **(C) Rewarming**: chilled fruit rewarmed at 20°C until T ('5T') were compared to chilled fruits ('5M'). **(D) Postharvest**

ripening. Comparisons were made between fruit harvested at Mature green and allowed to ripen under different conditions until Turning ('5T', '12.5T', '20T') compared to 'FHT'.

3.2 Tomato fruit quality is largely influenced by early harvest and low-temperature storage

We examined the quality of the fruit harvested and stored, using common postharvest markers i.e. firmness, total soluble solids (TSS), reducing sugars, objective color, titratable acidity, and starch content. We observed that 'FHT' and '20T' showed optimal quality, while '5T' was comparatively the worst compared with other Turning fruit (Fig. 8). The group of fruit stored at 12.5°C ('12.5T'), was better than '5T' but lower than '20T' based on the measured quality parameters.

Time to ripen. Low-temperature storage slows down fruit ripening, while chilling temperatures i.e. < 10°C arrest the process (Gonzalez et al., 2015). In this work (Fig. 8), fruit harvested at Mature green and stored at optimum ripening temperature 20°C ('20T') took on average 5 to 7 days to reach Turning, which is faster than 'on-the-vine' ripening (7-9 days). Tomato chilled at 5°C for 2 weeks reached Turning when stored at 20°C, taking 7 to 9 days on average. Remarkably, non-chilling, but low-temperature storage resulted in delayed ripening in '12.5T'. It took around 12 to 14 days to reach Turning, which is the longest timespan of all groups.

Color and firmness. Fruit ripening stages are often defined by external color (Gonzalez et al., 2015), e.g., in our study, all fruit were categorized as reaching 'Turning' when they attained the established predetermined color that defines that stage (Fig. S1). Objective

color (hue angle) was used to quantify the green to red hues characteristic of ripening; hue angle is low in red, and high in green fruit respectively. In Fig. 8A, hue angle was identical in the green 'FHM' and '5M' fruit, and higher than all fruit at Turning. Notably, the hue angle of '20T' fruit was lower (redder), compared to all other Turning fruit including 'FHT'. Ripening and carotenoid accumulation may have been accelerated in harvested fruit stored at 20°C in a controlled environment (see Fig. 8; Suslow & Cantwell, 2002). 'FHT' ripened in the greenhouse would have experienced variability in ambient light and temperature, which may have retarded carotenoid accumulation (Gautier et al., 2008), relative to '20T'. It should be noted that although fruit color was used here as a marker for fruit ripening stage and quality, other parameters that are also important for quality may not change in concert with color, especially after interference of the ripening program by postharvest treatments (Deltsidis et al., 2018; Shewfelt et al., 1988; Lana et al., 2005).

All Mature green fruit were firmer than those at Turning (Fig. 8B). 'FHM' fruit were firmer than '5M' fruit, while '5T' fruit was softest of all the fruit examined. The abnormal changes in fruit firmness were caused by chilling in '5M' (Biswas et al., 2016), and the excessive softening in '5T' fruit is a classic symptom of postharvest chilling injury which appears after the transfer of produce from low to warm temperatures (Cheng & Shewfelt, 1988).

Total Soluble Solids (TSS), Sugar and titratable acidity (TA). Tomato fruit at Turning have increased TSS, sugar and decreased starch content compared to the onset of ripening

(Luengwilai & Beckles 2009; Luengwilai et al., 2010). Our data were in accordance: 'on-the-vine' ripening was associated with higher TSS and higher reducing sugar when 'FHT' fruit was compared to 'FHM' (Figs. 5C and 5D). However, TSS content increased marginally from 4.56% in 'FHM' to 5.93% in 'FHT', which may be due to the high acid content in Micro-Tom which contributes to TSS (Luengwilai et al., 2010).

The sugar-to-acid ratio influences fruit taste, and is also a fruit maturity indicator, i.e. lower ratio indicates retarded maturity (Tigist et al., 2013; Beckles, 2012). '5M' and '5T' had significantly lower sugar compared to 'FHM', indicating that chilling and early harvest results in radical sugar consumption (Fig. 8D). Interestingly, after rewarming, the sugar content of '5T' was similar to that in '5M' fruit, which means that chilling resulted in a depletion of sugars that could not be restored when the fruit were allowed to ripen at room temperature. This observation is consistent with the lower glucose and fructose content in 'Micro-Tom' fruit induced by chilling (Gómez et al., 2009).

TA content from 'FHM' to 'FHT' decreased significantly ($p < 0.05$), i.e. from 12.0 and 6.7 meq. 100g-1 FW (see Fig. 8E), which is in agreement with other work (Teka, 2013). However, decreased acidity, a normal feature of tomato fruit ripening did not occur in any other fruit samples i.e. '5T', '12.5T' and '20T', indicating poorer quality induced by the postharvest handling. The TSS-to-TA ratio and Sugar-to-TA ratio (Figs. 8G and 8H) illustrate the rank of fruit taste as 'FHT' > '20T' > '12.5' > '5T'.

Starch. Green fruit store high levels of starch, which is degraded to sugars during ripening. When fruit are harvested at Mature green, starch becomes the primary carbon and energy source for 'off-the-vine' fruit development (Beckles, 2012). The data shown in Fig. 8F followed what was expected, but remarkably, starch content at '5T' and '5M' were identical. Thus, rewarming previously chilled fruit didn't influence starch and reducing sugar content significantly, which is unlike the trends in '12.5T', '20T' and 'FHT' fruit. This phenomenon was also seen in Albornoz et al., (2019), where starch degradation occurred during cold-storage fruit, but there was no resumption during the rewarming period. In many plant tissues, chilling accelerates starch degradation presumably as a response to stress (Dong & Beckles, 2019).

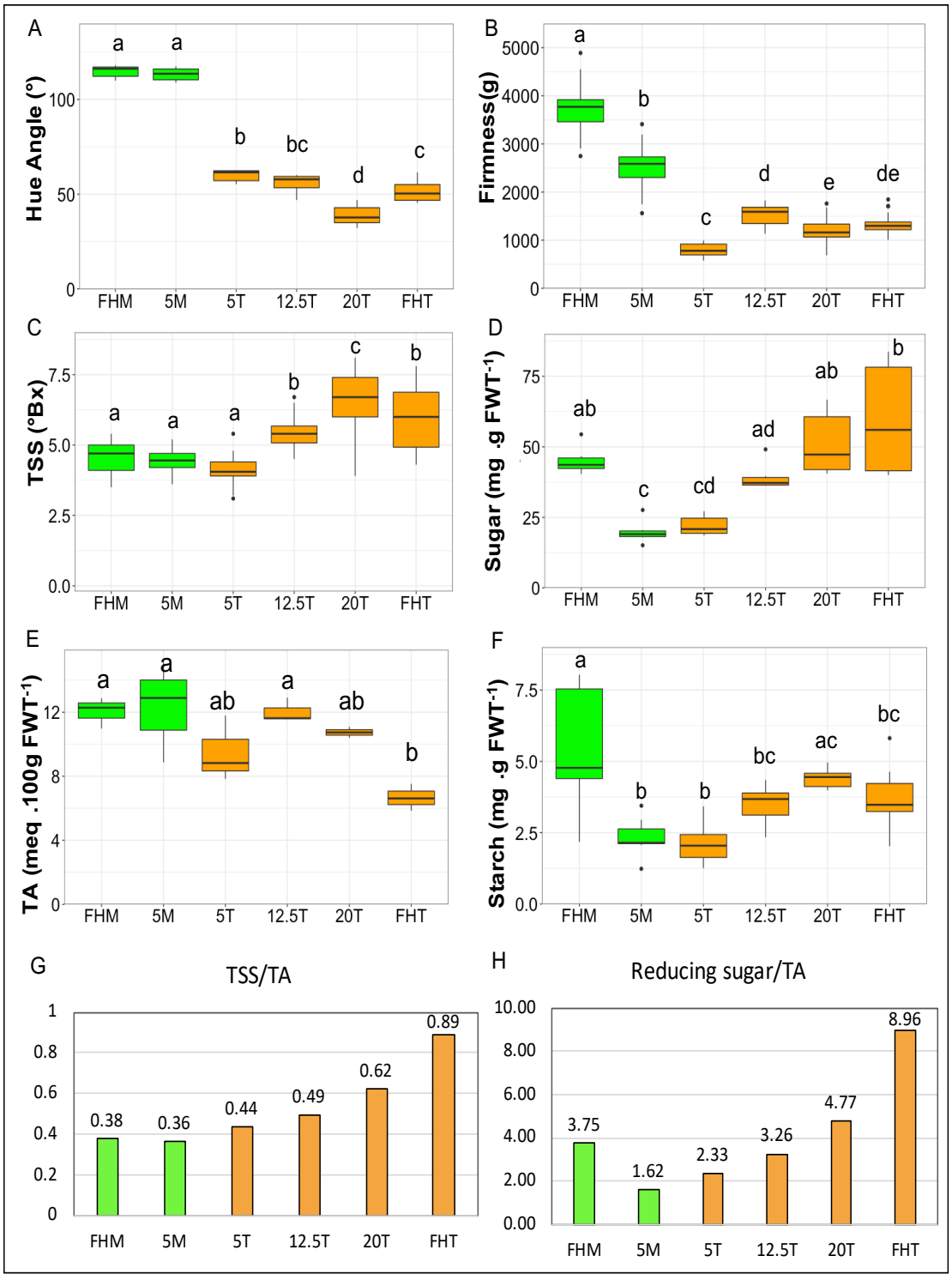

Figure 8. Fruit quality assessment. Six groups, including 'FHM', '5M', '5T', '12.5T', '20T' and 'FHT', were analyzed. Letters above the box indicate significant differences across all groups ($p < 0.05$, CLD). (A) Objective color: Hue angle (°). (B) Firmness (g). (C) Total soluble solids

(TSS) (°Bx). (D) Reducing sugars, represented by mg. sugar g-1 (fresh weight). (E) Titratable acidity (TA), represented by meq. 100g-1 (fresh weight). (F) Starch content represented by mg. starch g-1 (fresh weight). (G) TSS to TA ratio, was calculated using the mean value of each treatment. (H) Reducing sugars to TA ratio, was calculated by the mean value of each treatment.

3.3 Transcriptional analysis of DNA demethylase *SlDML2* and the master ripening regulator *RIN* in fruit ripened under different conditions

Examining the transcriptional levels of *SlDML2* may contribute to an understanding of DNA methylation dynamics due to postharvest handling. The *SlDML2* transcriptional activity was generated by Semi-quantitative RT-PCR (SRT-PCR) and Quantitative Real-Time PCR (RT-qPCR). SRT-PCR can verifiably and efficiently allow for qualitative comparisons of transcripts with relatively high accuracy and at lower costs than RT-qPCR with the necessary optimization (Marone et al., 2001; Romero et al., 2007; Antiabong et al., 2016). The data generated from SRT-PCR was largely in agreement with that from RT-qPCR (Fig. S2).

RIN is a key controller of tomato fruit ripening and quality by regulating the expression of some, but not all ripening genes connected to climacteric ethylene production (Ito et al., 2020; Li et al., 2020). We hypothesized that assessing *RIN* transcriptional levels will help to understand the effect of postharvest handling on fruit ripening process. As shown in Fig. 9B, *RIN* transcripts were not detected in Mature green fruit, but were high, and identical among the different Turning groups. We also found no *SlDML2* transcript in Mature green fruit, only in Turning fruit (see Fig. 9A). Surprisingly, the highest transcript levels of *SlDML2* were in ‘12.5T’ fruit, even higher than ‘20T’ and ‘FHT’.

Analysis of the fruitENCODE database (www.epigenome.cuhk.edu.hk/encode.html) (Lü et al., 2018), indicates that *RIN* is expressed after Mature green, increases and reaches maximal levels at Pink (equivalent to the Turning stage in 'Micro-Tom') (Fig. S4). Our data was identical, i.e. *RIN* expression was low in green fruit, but high in all Turning fruit, regardless of postharvest treatments. This suggests that *RIN* transcriptional levels may be determined more by final ripening stage rather than by the preceding postharvest storage conditions.

In 'Ailsa Craig' fruit, *SlDML2* expression was low relative to *RIN*, but it increased almost 3-fold at the onset of Mature green, increased further during Breaker, and decreased thereafter (Fig. S3). Previous work showed that SlDML2 increases from immature to Breaker stage, peaks at Turning, and decreases at Red ripe in a cherry tomato (Liu et al., 2015). In our current study, Turning fruit ripened at 12.5°C had the highest transcript levels of *SlDML2* along with the longest timespan (12-14 days) in the transition from Mature green to Turning stage. Taken together, it can be deduced that during this long-period of low-temperature storage, *SlDML2* transcripts progressively and gradually increased, which is required for the fruit to ripen before the expression peak that usually occurs at Turning. However, the *SlDML2* transcripts of other Turning fruit ('FHT', '20T' and '5T') are low, because expression was less nuanced due to accelerated ripening. This indicates that chronological factors were important for the expression of this gene.

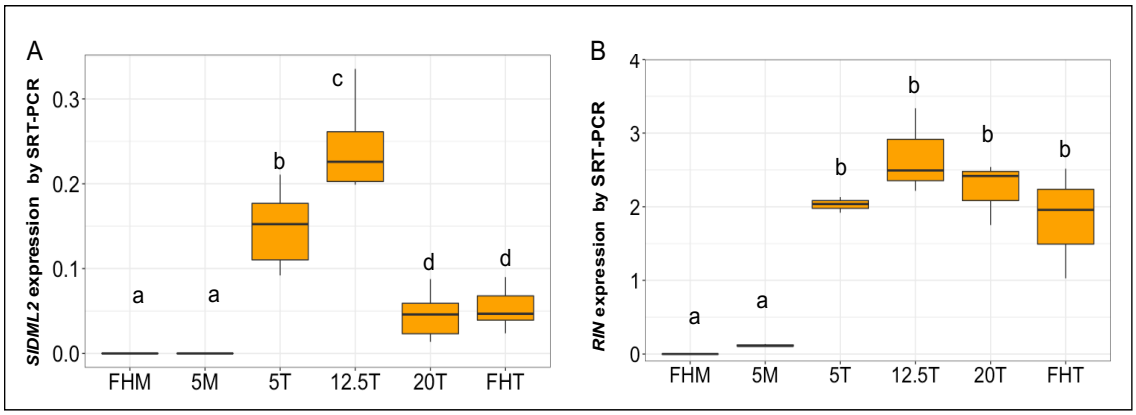

Figure 9. Gene expression levels of *SlDML2* and *RIN* by Semi-quantitative RT-PCR. Three biological replicates were included, in which *SlACT7* was the reference gene. Letters above the box indicate significant differences across all groups ($p < 0.05$, CLD). (A) *SlDML2* relative expression. (B) *RIN* relative expression.

3.4 Multivariate analysis according to DNA methylation and fruit quality

To find patterns that could indicate connections between DNA methylation and fruit quality among the groups of examined tissues, we performed multivariate analyses of the MSAP data using hierarchical clustering analysis (HCA) and principal component analysis (PCA). In the MSAP HCA plot (Fig. 10A), '5T' and '5M' fruit clustered as one group; these fruit interestingly were both chilled, and both showed symptoms of postharvest chilling injury with abnormal firmness (Fig. 8B). The '12.5T' and '20T' fruit clustered into another group, and they were ripened postharvest at non-chilling temperatures. The 'FHT' and 'FHM' clustered as a distinct group, and they were both 'fresh-harvested'. HCA of fruit quality was starkly different to that of the MSAP data, which presented the fruit samples as two clusters that are separated only by ripening stage (Fig. 10B). However, there was some separation among Turning fruit based on ripening temperature.

Similar to the Hierarchical clustering analysis, PCA 2-D and 3-D plots of the MSAP data (Fig. 10C and Fig. S5A) showed that ‘5M’ and ‘5T’ were distinct, and did not group with the other fruit samples, while ‘FHM’ was more similar to the other fruit at the Turning stage. In contrast to the HCA, however, ‘5M’ and ‘5T’ could be easily distinguished from each other on the plot, with the ‘5T’ being closer to, but not clustering with the other samples, which is indicative of the effects of rewarming. Both PCA and HCA indicate that postharvest practices influenced fruit methylation.

PCA of tomato fruit quality (Fig. 10D and Fig. S5B), matched the HCA results i.e., green fruit ‘5M’ and ‘FHM’ formed a distinct cluster away from the Turning fruit. Still on the fruit-quality PCA, ‘5T’ showed some distinction from the other Turning fruit.

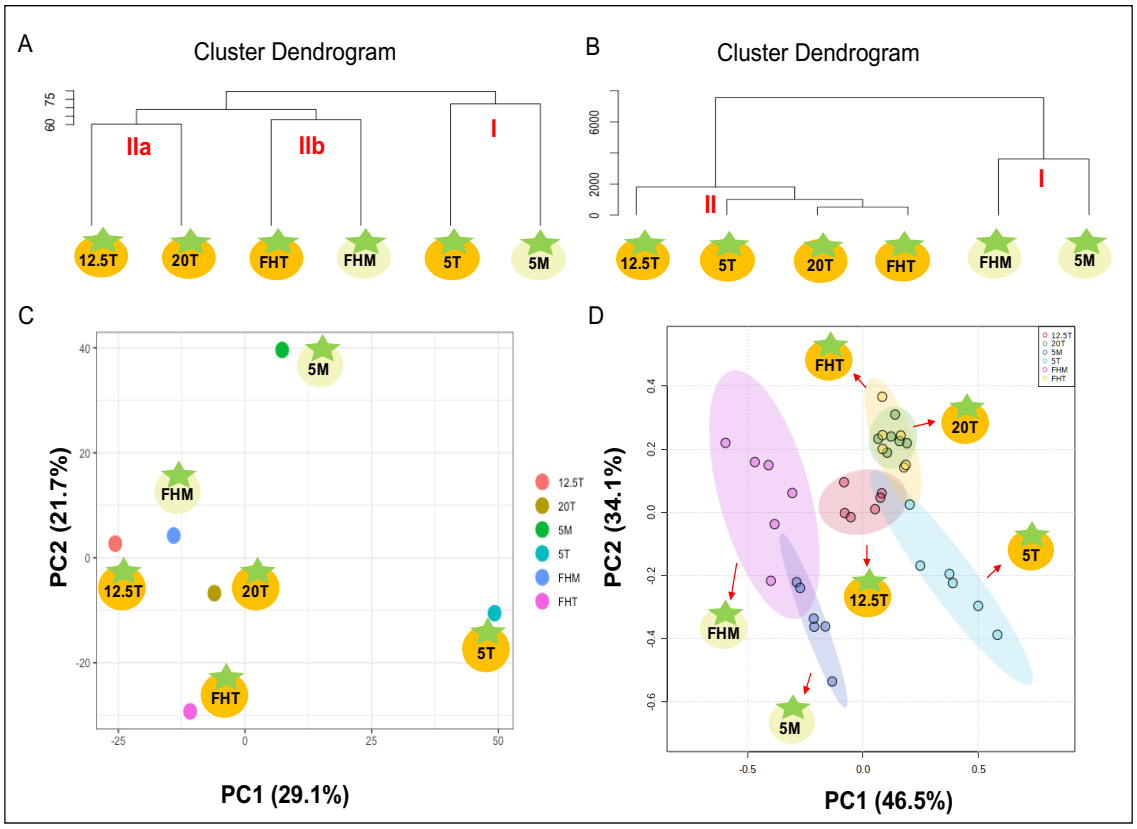

Figure 10. Hierarchical clustering analysis (HCA) of (A) DNA methylation and (B) fruit quality. The calculation for clustering was based on a matrix of site types of (A) methylation status and (B) quality parameters, including color, firmness, TSS, reducing sugar, starch, and TA. Clusters based on the HCA grouping i.e., I, II, and subclusters IIa and b are labeled in red. Principal component analysis (PCA) of (C) DNA methylation and (D) fruit quality. The distance in the PCA plots shows their relationship among the variables. The calculation for clustering was based on the same matrix used in (A) and (B), and the top two PCs, were chosen to generate the 2-D plot.

4. Discussion

DNA methylation has been reported to play a critical role in regulating fruit ripening. In the current work, we focused on potential changes in DNA methylation dynamics due to postharvest handling. Many postharvest strategies are designed to extend fruit shelf life, but often result in loss of fruit quality, and may unintentionally contribute to postharvest waste. While the relationship between DNA methylation and tomato fruit ripening is well understood (Shinozaki et al., 2018), it was not known what effect early harvest and storage temperature, which often disrupt the ripening program, would have on DNA methylation and the expression of the key genes in this process. Our work demonstrated that early harvest and postharvest storage temperatures greatly influenced the speed of fruit ripening, fruit quality and DNA methylation levels, but that the relationship was not linear.

During tomato fruit development and especially during the transition from green fruit to red that occurs during ripening, many genomic demethylation events trigger the expression of ripening-related genes (Giovannoni et al., 2017). We found more demethylation events (26.13%) in Turning fruit compared to Mature green fruit, consistent with data from cv. Alisa Craig (Teyssier et al., 2008; Zhong et al., 2013). This suggests that ripening-induced DNA demethylation is conserved in tomato cvs. 'Micro-Tom' and 'Alisa Craig', even though 'Micro-Tom' has comparatively more methylated regions (Lang et al., 2017).

The chilling-induced inhibition of demethylation detected in this study, potentially explains why postharvest chilling inhibits ripening in Mature green fruit (Biswas et al.,

2016). Similar to chilled red fruit (Zhang et al., 2016), chilling green fruit inhibited the DNA demethylation (presumably of the promoter regions of many ripening-related genes) that occurs during normal ripening. The expression of these genes may be regulated by RIN, but methylation also inhibits RIN's actions, and would delay ripening. Rewarming the chilled Mature green fruit gave rise to DNA demethylation observed in the MSAP analysis, which is a prerequisite for ripening (Lang et al., 2017). However, not all sites influenced by chilling were demethylated during rewarming, explaining why '5T' fruit were of poorer quality compared to other Turning fruit. This is in agreement with the growth rate and methylation pattern of chilled and rewarmed cucumber radicles (Chen et al., 2019).

By incorporating all measurements, the rank in fruit quality at Turning from best to poorest, was: 'FHT' > '20T' > '12.5T' > '5T' (Fig. 8), opposite to changes in cytosine methylation levels generated by MSAP where '20T' < '12.5T' < '5T' (Fig. 6D and Fig. 7D) when compared to 'FHT'. Vine-ripened fruits import nutrients until harvest, while postharvest-ripened fruit are prematurely removed from their source of nutrients. Low-temperature storage further disrupts the ripening program of these harvested fruit. It may be inferred that changes in methylation events are integral to how these anthropogenic factors affect fruit biological processes and influence quality.

The multivariable analysis indicated that DNA methylation is influenced by postharvest handling and fruit ripening stage. Fruit quality correlated strongly with ripening. The exception was '5T' fruit, where there was a distinct DNA methylation state and quality

characteristics, implying a strong regulatory mechanism between chilling, ripening and methylation. Broader analyses of the methylome and transcriptome by whole genome bisulfite-sequencing and RNA-Seq (Al Harrasi et al., 2017; Wang et al., 2009), may provide a more comprehensive picture of how early-harvest and low-temperature storage influence tomato ripening at the molecular level.

We proposed a model connecting postharvest strategies and its induced changes in fruit ripening (Fig. 11). Postharvest handling modulates *SlDML2* expression, which in turn influences fruit global DNA methylation. Changes in methylation status may have consequences for a subset of ripening genes (Lang et al., 2017), even if *RIN* expression remained robust in ripened fruit regardless of storage treatment (Fig. 9B). *RIN* is therefore not a reliable proxy for informing on the endogenous or physiological conditions that influence ripening, only that the stage was attained.

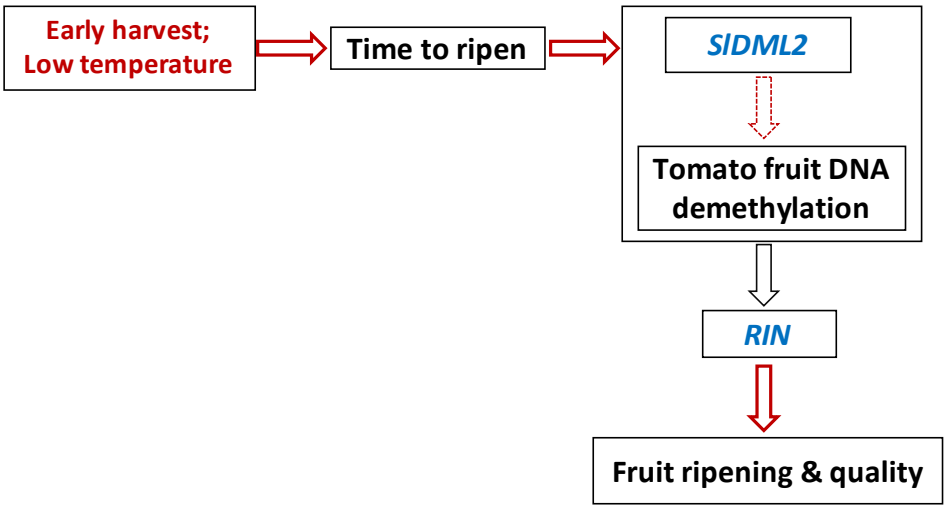

Figure 11. A proposed model showing the relationship between postharvest handling and changes in the tomato fruit development. This model is based on variables (shown as boxes) studied in this work and how they are related. The red arrows indicate positive relationships between variables, and the black arrow indicates no change based on this work. Genes and postharvest conditions are shown in blue and red font respectively. Early harvest and low-temperature storage changed ripening time, resulting in differences in the transcriptional levels of *SlDML2* and changes in global DNA methylation status. However, the relationship between *SlDML2* expression and DNA methylation was not linear (dashed arrow). *RIN* expression remained the same regardless of the different postharvest handling and DNA methylation levels verified in this work but was related to the ripening stage attained by the fruit. Our postharvest practice widened the fruit ripening/developmental window which influenced *SlDML2* expression.

There are many strategies for prolonging the shelf-life of tomato fruit, but they often reduce flavor. The postharvest treatment used in this study negatively influenced fruit sensory attributes and this may be mediated in part through DNA methylation. In support of this, Zhang et al., (2020) recently found that exogenous ethylene stimulated *SlDML2* transcripts and DNA demethylation. There are also additional epigenetic regulatory mechanisms that may indirectly influence tomato fruit ripening and quality, and it would be of interest to determine how they are affected by postharvest methods (Zhou et al., 2019; Lü et al., 2018; Liang et al., 2020). Greater efforts are needed to help to unravel the complex regulatory ripening network in tomato at the epigenetic and transcriptional level. For example, it will be important to explore the effect of anthropogenic postharvest environments on the timing and dynamics of DNA demethylases and DNA methylation.

Such studies may provide novel ways to extend fruit shelf life as well as reduce postharvest loss.

5. Conclusion

Our aim was to understand potential changes in DNA methylation and tomato fruit quality in relation to postharvest handling. We have demonstrated that early-harvest and low-temperature conditions significantly reduced fruit quality such as color, the sugar-to-acid ratio, and firmness. Expression of the *SlDML2* gene is essential for DNA methylation and fruit ripening, here, we showed that its expression was also responsive to postharvest handling. The MSAP data indicated large variations in fruit DNA methylation due to low temperature and early harvest, since the DNA methylation state of fruit at the same ripening stage, but developed under different handling regimes, was varied. The relationship between DNA methylation and fruit quality was not linear, and there are likely to be complex biological mechanisms influenced by DNA methylation that control fruit quality.

Supplementary data

List of Figures and Tables

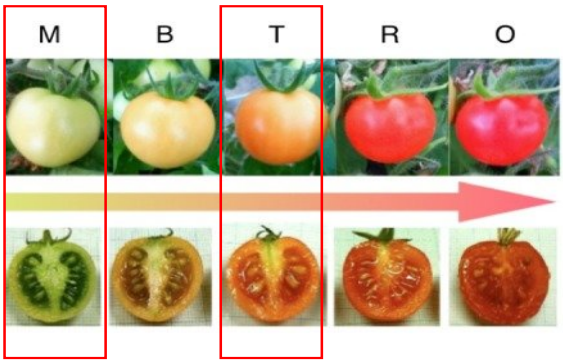

Fig. S1. Fruit developmental stages of cv. Micro Tom (adapted from Takizawa et al., 2014). Mature Green (M); Breaker (B); Turning (T); Ripen(R) and Over Ripe (O). Mature Green and Turning were tested in this work.

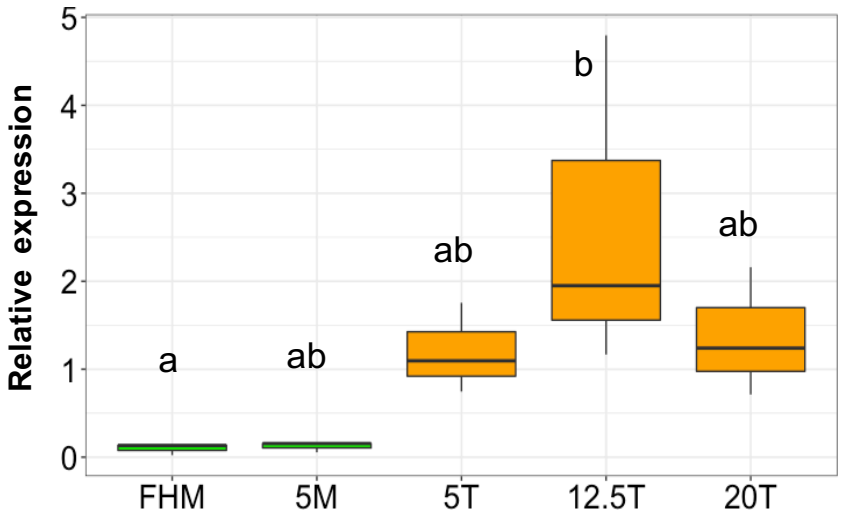

Fig. S2. *SlDML2* relative expression measured by RT-qPCR. 2 $^{\Delta\Delta C_T}$ analysis was used, in which *SlAct7* as reference gene and fresh-harvested Turning fruit ('FHT') were used as the calibrator. Each treatment includes three biological replicates. Letters above the box indicate significant differences across all groups ($p < 0.05$, CLD).

Expression (RPKM)	7 DPA	17DPA	27DPA	37DPA	42DPA	47DPA	Leaf
Colorless non-ripening (Cnr)	43.88	45.48	41.74	49.06	78.54	51.12	NA
green flesh (gf)	NA	NA	20.54	64.86	187.28	188.23	NA
high pigment (hp)	NA	16.57	29.06	48.26	178.43	104.97	NA
non-ripening (nor)	26.59	20.35	28.19	19.53	23.5	28.8	NA
never-ripe (nr)	27.45	21.9	30.96	48.37	242.26	81.44	10.14
ripening inhibitor (rin)	36.7	31.81	31.1	52.47	95.09	122.06	NA
wild-type (Ailsa Craig)	30.08	29.53	11.48	31.69	52.44	39.13	15.35

Mature green Breaker Light red to red ripe

Fig. S3. *SlDML2* expression data of cv. Ailsa Craig from FruitENCODE database (Lü et al., 2018)**.**

Expression (RPKM)	7 DPA	17DPA	27DPA	37DPA	42DPA	47DPA	Leaf
Colorless non-ripening (Cnr)	0.0	15.35	0.0	15.45	210.79	258.03	NA
green flesh (gf)	NA	NA	0.0	11.06	157.65	576.96	NA
high pigment (hp)	NA	0.0	5.4	21.11	605.74	1173.69	NA
non-ripening (nor)	0.0	0.0	2.99	0.0	13.26	14.68	NA
never-ripe (nr)	0.0	0.0	2.76	17.26	614.62	338.6	0.0
ripening inhibitor (rin)	0.0	0.0	1.87	5.35	232.43	663.65	NA
wild-type (Ailsa Craig)	0.0	0.0	0.0	70.5	613.13	614.27	0.0

Mature green Breaker Light red to red ripe

Fig. S4. *RIN* expression data of cv. Ailsa Craig from FruitENCODE database (Lü et al., 2018)**.**

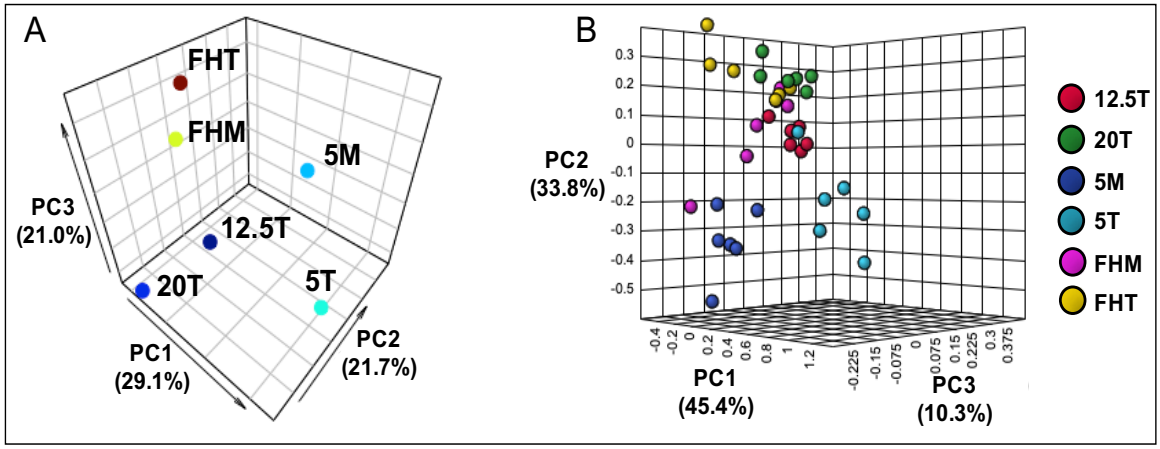

Fig. S5. 3D-PCA representation of (A) DNA methylation and (B) fruit quality. The calculation for clustering was based on a matrix of (A) DNA methylation status and (B) quality parameters that are the same as the matrix used in Fig. 7. The top three PCs were chosen to generate the 3-D plot.

Table S1 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	ATACAGGCCGTCAACTTTGG	445
	DML2-R	CCCTTTGGCATTTATGCTGT	

Table S2 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	

Table S3 Adaptors and primers used in MSAP

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

Table S4 MSAP site types and methylation status

Site type	*Msp*I	*Hpa*II	MSAP Site Type	Methylation State
I	1	1	Demethylated	CCGG GGCC
II	0	1	Hemi-methylated external C	**C**CGG GGCC
			Hemi-methylated both C	**CC**GG GGCC
III	1	0	Fully methylated internal C	C**C**GG GG**C**C
IV	0	0	Fully methylated both C	**CC**GG GG**CC**
			Fully methylated external C	**C**CGG GGC**C**
			Fully methylated external C + Hemi-methylated internal C	**CC**GG GGC**C**
			Hemi-methylated external C + Fully methylated internal C	**CC**GG GG**C**C

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FUTURE WORK

Early harvest and postharvest storage temperatures influences the rate at which tomato fruit ripens, the quality of the fruit, and fruit DNA methylation levels. However, the relationship among these variables is complex. To expand on this topic, and to unravel these integrated processes, a greater effort is needed. The following questions were raised based on the discoveries in Chapter two, and further experiments will be conducted after submitting this thesis.

- The fruit harvested early but stored at different temperatures reached the Turning stage at different times. Ethylene production is necessary for initiating fruit ripening (Iqbal et al., 2017). In climacteric fruit, there is a burst in ethylene production during ripening and, a similar phenomenon is seen in fruit response to chilling stress (Ciardi et al., 1997). To understand the connection between ethylene production and ripening speed due to different postharvest treatments, ethylene production in fruit treated similarly to those described in Chapter two will be assessed. In addition, an increase in the rate of respiration is expected in ripening climacteric fruit and also in response to postharvest chilling stress (Albornoz et al., 2019). Therefore, measurements of respiration will be performed and the relationship between the dynamics of ethylene and carbon dioxide production in relation to fruit ripening speed will be assessed.
- It is established that starch breakdown occurs during fruit ripening. My results also showed that significant fruit starch degradation occurs at 5 °C. In contrast, when fruit is rewarmed after chilling to permit ripening, starch degradation ceases. These data indicate

that chilling stress accelerates fruit starch breakdown. Nevertheless, during the subsequent rewarming phase, when ripening resumes, there is no further degradation. It defies the expectation that ripening resumption should accelerate degradation further. It would be worthwhile to test if starch can act as a biomarker for postharvest chilling stress (PCI) in tomato fruit. This may provide an effective way to detect PCI for industrial application. I have quantitatively analyzed tomato fruit starch under different harvest stages and after different temperatures storage regimes. The correlation among starch content, fruit ripening stages and the degree of PCI will be explored in wild-type and available tomato starch mutants.

- I applied MSAP, which generated limited fragments to assess tissue global DNA methylation state after exposure to different conditions. This method is unable to identify specific sequences that were altered, and it only provides data on a relatively small subset of the genome. A broader analysis of the fruit methylome may provide a more comprehensive picture of tomato ripening altered by postharvest. The gold standard method, i.e., whole-genome bisulfite sequencing (WGBS) will be applied for testing DNA methylation. The WGBS will provide the information on the genes controlling fruit quality, and distinguish among the different types of DNA methylation. The data derived from MSAP and WGBS will be compared.
- The master ripening regulator-*RIN* was assessed in Chapter two, and my results show that *RIN* was regulated more by fruit ripening stage, and not by postharvest handling. It will be interesting to see if other ripening regulators or the RIN downstream genes are affected by postharvest handling, and if changes in their expression are correlated with their methylation state tested by WGBS. Therefore, fruit transcriptomic analysis has been

conducted. A deep analysis of the RNA-Seq experiment provides the possibilities of : (1) understanding how the fruit transcriptome changes due to different postharvest practices; (2) identifying the expression of genes in key ripening pathway that controls fruit biochemical traits; (3) making a correlation network to assess potential interaction among ripening genes induced by postharvest.

To sum up, my aim in this work was to clarify how early-harvest and low-temperature storage influenced tomato fruit DNA methylation state, fruit ripening and quality. The conducted work suggests the association between DNA methylation and fruit quality during the postharvest. Therefore, it's worthwhile to thoroughly understand this connection by more elaborate methods as well as evaluating more fruit ripening and quality parameters. The transcriptomic and methylomics analysis will uncover the expression and DNA methylation state of the genes that determine specific fruit quality due to postharvest treatments. Candidate genes with differential expression and methylation levels will be identified. This is a step towards determining how postharvest handlings alter fruit quality at the molecular level. Furthermore, gene editing could be applied to create a wide range of mutations for studying how these genes affect postharvest properties such as quality and shelf-life. The long-term goal is to help reduce postharvest losses and improve tomato fruit quality.

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