

Relationships between genome methylation, levels of non-coding RNAs, mRNAs and metabolites in ripening tomato fruit

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SUMMARY

Ripening of tomato fruit is a complex tightly orchestrated developmental process that involves multiple physiological and metabolic changes that render fruit attractive, palatable and nutritious. Ripening requires initiation, activation and coordination of key pathways at the transcriptional and post-transcriptional levels that lead to ethylene synthesis and downstream ripening events determining quality. We studied wild-type, *Gr* and *r* mutant fruits at the coding and non-coding transcriptomic, metabolomic and genome methylation levels. Numerous differentially expressed non-coding RNAs were identified and quantified and potential competing endogenous RNA regulation models were constructed. Multiple changes in gene methylation were linked to the ethylene pathway and ripening processes. A combined analysis of changes in genome methylation, long non-coding RNAs, circular RNAs, micro-RNAs and fruit metabolites revealed many differentially expressed genes (DEGs) with differentially methylated regions encoding transcription factors and key enzymes related to ethylene or carotenoid pathways potentially targeted by differentially expressed non-coding RNAs. These included *ACO2* (targeted by MSTRG.59396.1 and miR396b), *CTR1* (targeted by MSTRG.43594.1 and miR171b), *ERF2* (targeted by MSTRG.183681.1), *ERF5* (targeted by miR9470-3p), *PSY1* (targeted by MSTRG.95226.7), *ZISO* (targeted by 12:66127788|66128276) and *NCED* (targeted by MSTRG.181568.2). Understanding the functioning of this intricate genetic regulatory network provides new insights into the underlying integration and relationships between the multiple events that collectively determine the ripe phenotype.

Keywords: ethylene response, carotenoid pathway, non-coding RNAs, genome methylation, tomato fruit.

INTRODUCTION

Fruit ripening is an intricate genetic process driven by the action of diverse factors operating at the level of the epigenome, transcriptome, proteome and metabolome (Osorio *et al.*, 2011; Zhong *et al.*, 2013; Karlova *et al.*, 2014). The activities of numerous metabolic pathways that influence fruit color, flavor, aroma and texture change dramatically

during the ripening process, and this is often associated with the accumulation of transcripts for corresponding genes (Klee and Giovannoni, 2011; Giovannoni *et al.*, 2017; Cai *et al.*, 2018). Important hormonal changes also occur during climacteric fruit ripening, including a decline in auxin signaling and a major increase in ethylene biosynthesis and signal transduction (Klee and Giovannoni, 2011;

Gapper *et al.*, 2013; Su *et al.*, 2015; Shin *et al.*, 2019). Tomato has proved to be an excellent model system for the analysis of ethylene synthesis and action during ripening of fleshy fruits due to its economic importance and many favorable genetic characteristics that have been introduced into near-isogenic experimental tomato lines (Liu *et al.*, 2016; Giovannoni *et al.*, 2017). Numerous ripening-associated tomato regulatory mutants, such as *ripening-inhibitor* (*rin*), *non-ripening* (*nor*) and *colorless non-ripening* (*Cnr*), have been characterized and recent experiments involving CRISPR/Cas9 have revised conclusions about their precise roles in ripening (Ito *et al.*, 2017; Li *et al.*, 2018a,b). In addition, several carotenoid pathway-related mutants have also been identified, such as *high pigment 1* (*hp1*), *high pigment 2* (*hp2*) and *yellow flesh* (*r*), all of which have proved to be great tools for advancing our understanding of the mechanisms of fruit ripening (Fray and Grierson, 1993; Barry *et al.*, 2005; Osorio *et al.*, 2011; Pan *et al.*, 2013; Fu *et al.*, 2016).

Tomato fruit ripening and senescence have been shown to operate at both the transcriptional and post-transcriptional and translational levels (Giovannoni *et al.*, 2017) and microRNAs (miRNAs) have been identified as negative regulators of their target mRNAs, operating by sequence complementarity to cause post-transcriptional degradation of mRNA or translational inhibition (Karlova *et al.*, 2013; Wang *et al.*, 2018a,b; Ma *et al.*, 2020). Deep sequencing and transgenic methods have been employed to explore the functions of these miRNAs during fruit development and ripening in different tomato varieties, including many ripening mutants (Mohorianu *et al.*, 2011; Zuo *et al.*, 2012; Wang *et al.*, 2017a,b), and many miRNAs have been identified and shown to be involved in the fruit ripening process. For example, overexpression of miR156, whose target is the SBP-box gene *CNR*, could drastically alter the ripe phenotype and reduce pigment accumulation (Zhang *et al.*, 2011). MiR1917 modulates the ethylene signaling pathway through *CTR4* splice variants to influence ethylene responses in tomato (Wang *et al.*, 2018a,b). Recently, long non-coding RNAs (lncRNAs) have also been shown to participate in tomato fruit ripening and the ethylene pathway (Li *et al.*, 2018a,b), by interacting with DNA, RNA and proteins to regulate gene expression by DNA methylation, histone modification and chromatin remodeling (Heo *et al.*, 2013; Zhu *et al.*, 2015; Wang *et al.*, 2016; Li *et al.*, 2018a,b). Intriguingly, the newly discovered circular RNAs (circRNAs), which arise from exons (exonic circRNAs), introns (intronic circRNAs) and intergenic regions, which are often generated co-transcriptionally by 'head-to-tail' splicing, have also been found to be involved in fruit ripening, coloration and the ethylene synthesis and response pathways (Ashwal-Fluss *et al.*, 2014; Tan *et al.*, 2017; Wang *et al.*, 2017a,b; Zhou *et al.*, 2018; Yin *et al.*, 2018).

Genomic DNA methylation is a major epigenetic state that has been extensively studied, and changes in methylation have been reported to play important roles in regulating gene expression, transposon silencing, DNA recombination, tissue specificity and stress responses during development (Gehring and Henikoff, 2007; Kawakatsu *et al.*, 2016; Xu *et al.*, 2018; Wang *et al.*, 2018a,b). Accumulating evidence suggests that DNA methylation is a further key regulator of fruit ripening, ethylene synthesis and signal transduction. Cytosine methylation levels across the genome undergo a dramatic reduction in the pericarp cells of fruits during the ripening process (Zhong *et al.*, 2013; Liu *et al.*, 2015a,b; Lang *et al.*, 2017; Lü *et al.*, 2018). Studies on methylome dynamics have revealed extensive changes in the distribution of DNA methylation throughout the genome during tomato fruit development, and demethylation occurs during fruit ripening at promoters of specific genes (Zhong *et al.*, 2013; Gallusci *et al.*, 2016). Application of the DNA methylation inhibitor 5-azacytidine facilitated ripening and RNA interference-mediated downregulation of putative DNA demethylases inhibited fruit ripening in tomato, suggesting that active DNA demethylation plays an important role in regulating the fruit ripening process (Zhong *et al.*, 2013; Liu *et al.*, 2015a,b; Lang *et al.*, 2017).

Although the complete network of components that regulate fruit ripening remains to be determined, cloning of many of the underlying genes and the use of deep sequencing technology and bioinformatics analysis have provided insights into the regulatory mechanisms and hierarchical relationships (Osorio *et al.*, 2011; Giovannoni *et al.*, 2017). In this study, we examined wild-type Ailsa Craig (AC) fruit at different ripening stages at the coding and non-coding transcriptomic, metabolomic and epigenomic levels and compared the results with those from two dominant tomato ripening mutants that are nearly isogenic in the AC background, *Gr* and *r*. The *Gr* gene has been suggested to interact with components of the fruit-specific ethylene response, and ectopic fruit expression of this dominant mutation results in fruit-specific ethylene insensitivity and ripening repression (Barry and Giovannoni, 2006). Phytoene synthase is encoded by the *PSY1* gene, which is mutated in the *r* mutant, and is the major limiting activity for carotenoid flux in the maturing fruit (Bird *et al.*, 1991; Fray and Grierson, 1993; Enfissi *et al.*, 2017).

Numerous differentially expressed (DE) non-coding RNAs present during fruit ripening were identified and the competing endogenous RNAs (ceRNAs) in potential regulation networks involved in fruit ripening were constructed and parsed based on association with ethylene hormone synthesis and action, carotenoid metabolism or both. The combined results of this analysis indicate that DEGs encoding enzymes critical for carotenoid production (*PSY1*, *NSY*, *CrtR-b2*, *LCY1*, *NCED*), ethylene synthesis or signaling

(*ACO2*, *CTR1*) and four ethylene response factors (*ERF2*, *ERF5*, *ERF17*, *ERF114*) have differentially methylated regions (DMRs). These genes are targeted by corresponding miRNAs, lncRNAs and circRNAs as part of an intricate and orchestrated genetic regulatory network that contributes to the regulation of the fruit ripening process.

RESULTS

Multi-omics analysis of ripening WT tomato fruit

We used deep sequencing and bioinformatics analysis of WT Ailsa Craig fruit and *Gr* and *r* mutants of the same age to uncover possible regulatory mechanisms whereby non-coding RNAs and DNA methylation could influence mRNA concentrations related to ethylene responses, carotenoid synthesis and other ripening events during the transformation of mature green fruit to the red ripe stages. Comparing the mature green and red ripe stages of WT fruit, 6486 DE mRNAs (2296 mRNAs were upregulated and 4190 mRNAs were downregulated) were annotated by topGO and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis (Appendix S1 in the online Supporting Information). These included mRNAs encoding several key genes involved in the ethylene biosynthesis, signaling and response pathways: *ACS2/4* (1-aminocyclopropane-1-

carboxylate oxidase 2/4), *ACO5* (1-aminocyclopropane-1-carboxylate oxidase 5), *NR* (Neverripe/Ethylene receptor3), *CTR1* (Constitutive triple response 1), *EIN3/4* (Ethylene insensitive 3/4), *ERF1/2/6*, *PSY1* (Phytoene synthase 1), *ZISO* (15-cis-zeta-carotene isomerase) and *MADS-box TF 23* were downregulated and *ACS3*, *ACO4*, *LCY2* (lycopene- β -cyclase) and the carotenoid accumulation pathway, including *Delta*, *NCED5* (Nine-cis-epoxycarotenoid dioxygenase 5), *CCD1A/4* (Carotenoid cleavage dioxygenases 1A/4) and *ERF1/2/5/12/13* were upregulated, consistent with the previous studies (Appendix S1) (Lee *et al.*, 2012; Shinzaki *et al.*, 2018).

For the WT, 100 miRNAs, 378 lncRNAs and 65 circRNAs were found to be differentially expressed during the fruit ripening process. Of these, 44 miRNAs, 160 lncRNAs and 42 circRNAs were upregulated and 56 miRNAs, 218 lncRNAs and 23 circRNAs were downregulated (Figure 1a, b, Appendix S1). The potential targets of the DE miRNAs, lncRNAs and circRNAs were analyzed by Gene Ontology (GO) and KEGG pathway analysis for WT green and red-ripe fruit. Numerous target genes were identified according to sequence homology which were potentially known to be involved in the fruit ripening process. These included genes in the glutamate metabolic pathway, the monoterpene biosynthetic pathway, pectin catabolic processes,

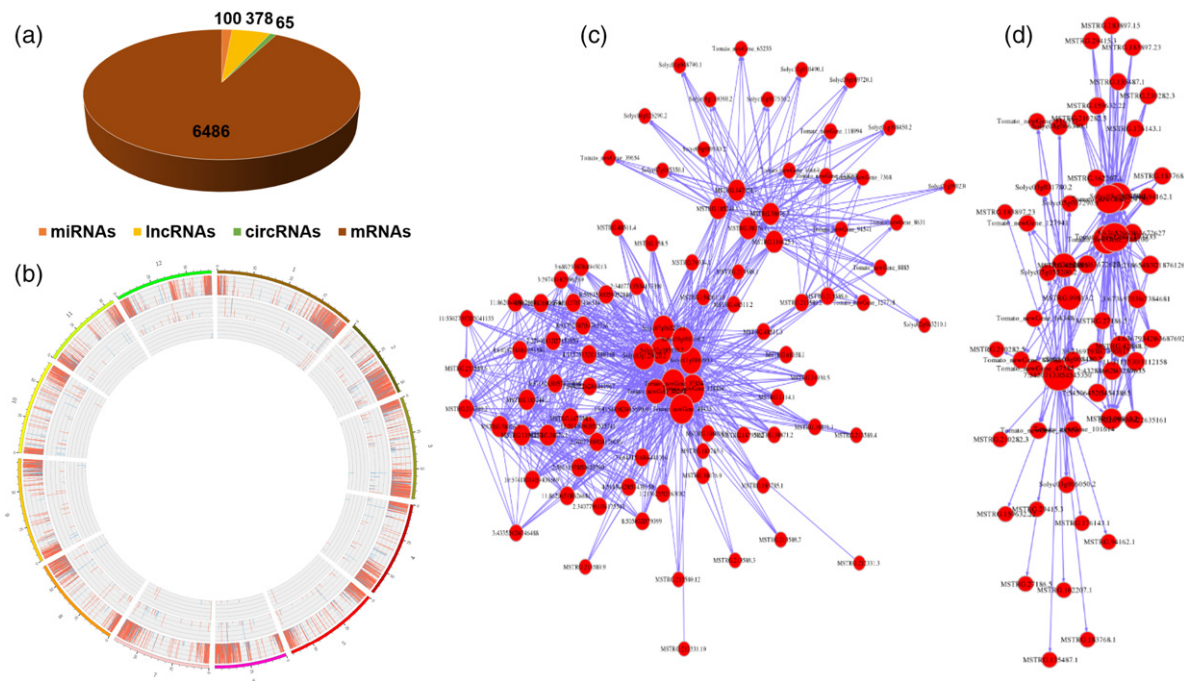


Figure 1. The distribution of differentially expressed (DE) non-coding RNAs and mRNAs and competing endogenous RNAs (ceRNA) networks in wild-type (WT; Ailsa Craig) tomato fruits.

(a) Numbers of DE circular RNAs (circRNAs), long non-coding RNAs (lncRNAs) and mRNAs between the mature green and red ripe stages.

(b) The distributions of DE circRNAs, lncRNAs, miRNAs and mRNAs on different chromosomes.

(c), (d) The ceRNA networks of circRNAs, lncRNAs, mRNAs and miRNAs during fruit ripening process in WT fruit.

plant hormone signal transduction, starch and sucrose metabolism, biosynthesis of amino acids and the glycerophospholipid metabolism pathway (Appendix S1). Also, miRNAs, lncRNAs and circRNAs were identified with potential targets such as *ACS4* (*ACC synthase*), *ACO2* (*ACC oxidase*), *CTR1* (*Constitutive triple response*), *ERF5*, *ERF13* and *MADS-box transcription factors* (TFs), which are involved in ethylene biosynthesis and signal transduction (Table 1). Other miRNAs and lncRNAs were found that potentially targeted genes encoding the cell wall-modifying enzymes *CesA* (*cellulose synthase*), *PE* (*pectinesterase*), *PG* (*polygalacturonase*) and *PL* (*pectate lyase*) (Appendix S1), which are involved in changes in fruit texture (Table 1) (Tucker *et al.*, 2017). Furthermore, other putative targets of miRNAs and lncRNAs included glutamine synthetase and glutamate decarboxylase, which are involved in tomato flavor formation (Table 1). Intriguingly, one putative circRNA (12:66127788|66128276) target was *15-cis-zeta-carotene isomerase* (*ZISO*), which is an important enzyme in tomato pigment accumulation. We also found many targets participating in hormone signaling and synthesis pathways, such as abscisic acid, auxin, jasmonic acid and gibberellic acid (Table 1, Appendix S1). In addition, DE circRNAs,

lncRNAs and mRNAs were identified that can act as ceRNAs of miRNAs during the fruit ripening process, and the ceRNA network was established (Figure 1c,d). Many key non-coding RNAs had putative targets that play key roles in the fruit development and ripening process, including *MADS-box 23*, *ERF5*, *ERF13*, *ERF021*, *PG*, *PL*, *PE*, *CesA* and *glutamine synthetase* (Figure 1c,d, Appendix S1).

Recently, several non-coding RNAs, such as lncRNAs and miRNAs, have been shown to perform their regulatory functions by directing DNA methylation in plants (Song *et al.*, 2015; Lahmy *et al.*, 2016; Deng *et al.*, 2018). To uncover the potential roles of non-coding RNAs in DNA methylation, the epigenomic dynamics and changes in non-coding RNA sequences during fruit ripening were compared. A total of 35 009, 16 891 and 84 162 DMRs of type CG, CHG and CHH were found by comparing data from mature green and red ripe stages of WT fruit, and there were major changes involving genes expressed during ripening (Figure 2a–d). The KEGG pathway analysis indicated that many of these differentially expressed genes (DEGs) were involved in plant hormone signal transduction processes, which are closely related to fruit ripening (Figure 2e–g). The DMRs and DEGs were parsed together,

Table 1 Non-coding RNAs and their targets involved in fruit ripening, the non-coding RNAs (micro-RNAs, long non-coding RNAs and circular RNAs) and their corresponding potential target genes (target ID and description) involved in tomato fruit ripening

Non-coding RNAs	Target ID	Target description
miR172a	<i>Solyc10g084340.1</i>	AP2-like ethylene-responsive transcription factor TOE3
miR396a-5p	<i>Solyc04g054840.1</i>	ETHYLENE INSENSITIVE 3-like
miR477-3p	<i>Solyc04g054840.1</i>	ETHYLENE INSENSITIVE 3-like
miR9470-3p	<i>Solyc03g093550.1</i>	Ethylene-responsive transcription factor 5-like
miR9470-3p	<i>Solyc11g042580.1</i>	Ethylene-responsive transcription factor ERF021
unconservative_3_10278	<i>Solyc01g090310.2</i>	Ethylene-responsive transcription factor 13-like
unconservative_3_10278	<i>Tomato_newGene_100691</i>	MADS-box transcription factor 23-like
unconservative_4_16495	<i>Tomato_newGene_100691</i>	MADS-box transcription factor 23-like
MSTRG.147958.1	<i>Solyc05g050010.2</i>	1-Aminocyclopropane 1-carboxylate synthase
MSTRG.156253.2	<i>Solyc06g036260.2</i>	Beta-carotene hydroxylase
MSTRG.94590.4	<i>Solyc03g007960.2</i>	Beta-carotene hydroxylase
MSTRG.91934.1	<i>Solyc02g089640.2</i>	Cellulose-synthase-like
MSTRG.43572.1	<i>Solyc10g083610.1</i>	Ethylene-inducible CTR1-like protein kinase
MSTRG.20235.2	<i>Solyc01g090300.2</i>	Ethylene-responsive transcription factor 13-like
MSTRG.183681.1	<i>Solyc08g007230.1</i>	Ethylene-responsive transcription factor 2-like
MSTRG.20235.2	<i>Solyc01g090320.2</i>	Ethylene-responsive transcription factor 2-like
MSTRG.43572.1	<i>Solyc10g083560.1</i>	Ethylene-responsive transcription factor ERF024
MSTRG.17850.2	<i>Solyc01g067540.1</i>	Ethylene-responsive transcription factor ERF086
MSTRG.149327.3	<i>Solyc05g054050.2</i>	Glutamate decarboxylase-like
MSTRG.107068.2	<i>Solyc03g083440.2</i>	Glutamate synthase 1
MSTRG.85242.1	<i>Solyc02g062420.2</i>	Green ripe-like 2
MSTRG.92645.13	<i>Solyc02g091550.1</i>	MADS-box transcription factor 23
MSTRG.22786.3	<i>Solyc01g099940.2</i>	Pectinesterase
MSTRG.20572.1	<i>Solyc01g091050.2</i>	Pectinesterase 2
MSTRG.182320.1	<i>Solyc07g064190.1</i>	Pectinesterase 3
MSTRG.86646.1	<i>Solyc02g068410.1</i>	Polygalacturonase QRT3
MSTRG.179295.1	<i>Solyc07g044870.2</i>	Polygalacturonase-like
MSTRG.149779.1	<i>Solyc05g055510.2</i>	Pectate lyase 5
12:66127788 66128276	<i>Solyc12g098710.1</i>	15-Cis-zeta-carotene isomerase
9:69300335 69300656	<i>Solyc09g089580.2</i>	1-Aminocyclopropane-1-carboxylate oxidase

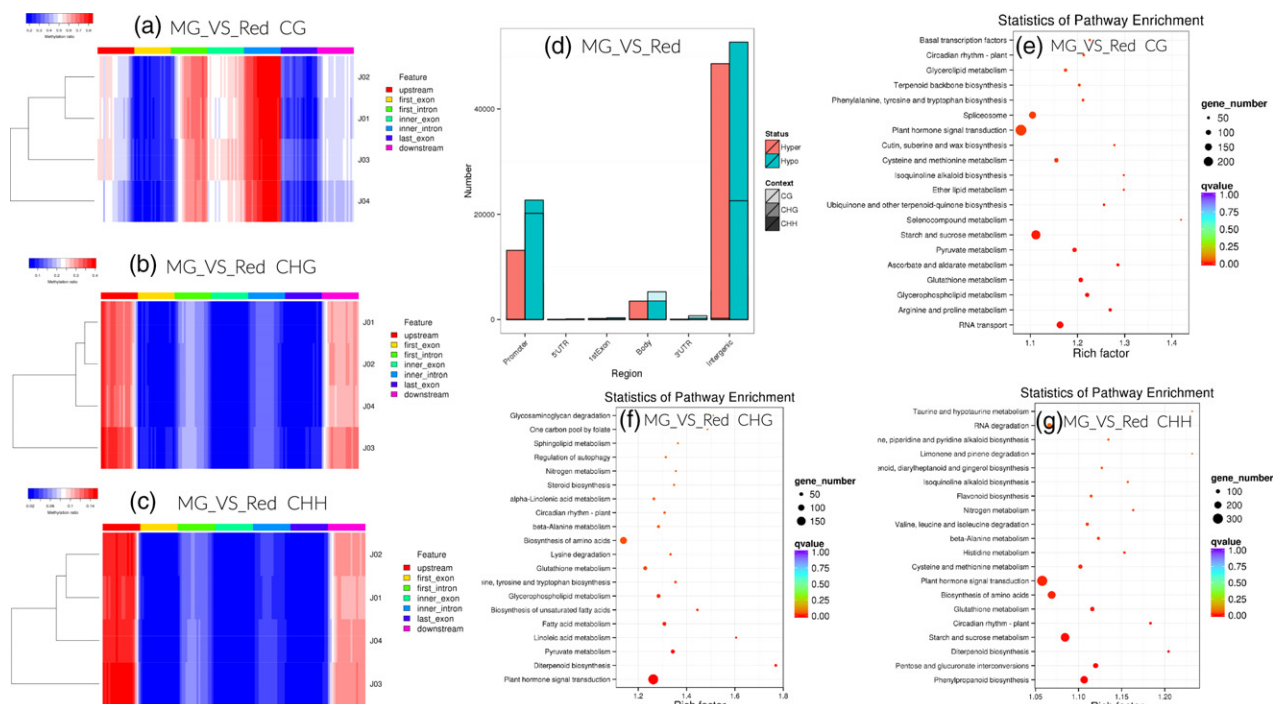


Figure 2. The methylation level: distribution of differentially methylated regions (DMRs) and KEGG pathway analysis of DMRs. (a)–(c) Cluster analysis of DNA methylation levels between mature green and red ripe stages of wild-type (WT) fruit. (d) Annotation of DMRs and their distribution between mature green and red ripe stages of the WT. (e)–(f) KEGG pathway analysis of DMRs between mature green and red ripe stages of WT fruit.

and several DMRs and DEGs were found to be involved in the ethylene synthesis, signaling and response pathway, pigment accumulation and fruit texture (Appendix S2). The combined DMR and DE lncRNA analysis revealed that many ripening-related differentially methylated genes were potentially targeted by lncRNAs, such as *CrtR-b* (carotene beta hydroxylase; targeted by MSTRG.156253.2), *CTR1* (targeted by MSTRG.44159.1), *ERF2* (targeted by MSTRG.183681.1), *ERF3* (targeted by MSTRG.26451.1), *ERF024* (targeted by MSTRG.43594.1), *ERF061* (targeted by MSTRG.201251.2), *ERF086* (targeted by MSTRG.17850.2) and *MADS-box TF23* (targeted by MSTRG.24366.15) (Appendix S2). In addition, the co-joint analysis of DMRs and DE miRNAs also indicated their involvement in the regulation of fruit ripening processes. For example, *ACS8* was targeted by miR159, *ACO2* was targeted by miR396b, *ERF5* and *ERF021* were targeted by miR9470-3p, *PL* was targeted by miR482a and *glutamate synthase 1* was targeted by 9474-5p (Appendix S2).

The LC-MS/MS-based metabolic profiling method was employed in order to understand the multiple metabolite variations that occur during fruit ripening and a total of 361 and 219 different metabolites increased or decreased between mature green and red ripe fruit, respectively (Appendix S3). The combined transcriptome and metabolome analysis showed that the main pathway changes were involved in the biosynthesis of amino acids

(L-tryptophan, L-valine, L-alanine, L-glutamate, L-leucine, L-histidine) and phenylpyruvate, galactose metabolism (sucrose and galactinol) and many metabolites involved in flavonoid biosynthesis (chlorogenic acid, chrysin and naringin) and ascorbate and aldarate (L-arabinose), vitamin B₆ (4-pyridoxic acid) and glutathione metabolic pathways (L-pyroglyutamic acid, L-glutamate and glutathione), which may contribute to flavor accumulation in tomato. The correlation networks of the different metabolites and related pathway genes were constructed and are shown in Appendix S3.

Analysis of differences in ncRNAs and mRNAs between WT and ethylene-insensitive *Gr* mutant fruit

Gr fruit cells show insensitivity to ethylene and have an altered ripening response. We compared the coding and non-coding transcriptomes, epigenomes and metabolomes between WT and *Gr* mutant fruit to explore the differences between the two genotypes and learn more about the molecular basis of ethylene production. We found 34 miRNAs, 171 lncRNAs, 33 circRNAs and 3711 mRNAs that were DE between the WT fruit and the *Gr* mutant; of these, 14 miRNAs, 90 lncRNAs, 10 circRNAs and 2732 mRNAs were upregulated and 20 miRNAs, 81 lncRNAs, 23 circRNAs and 979 mRNAs were downregulated (Figure 3a–c, Appendix S4).

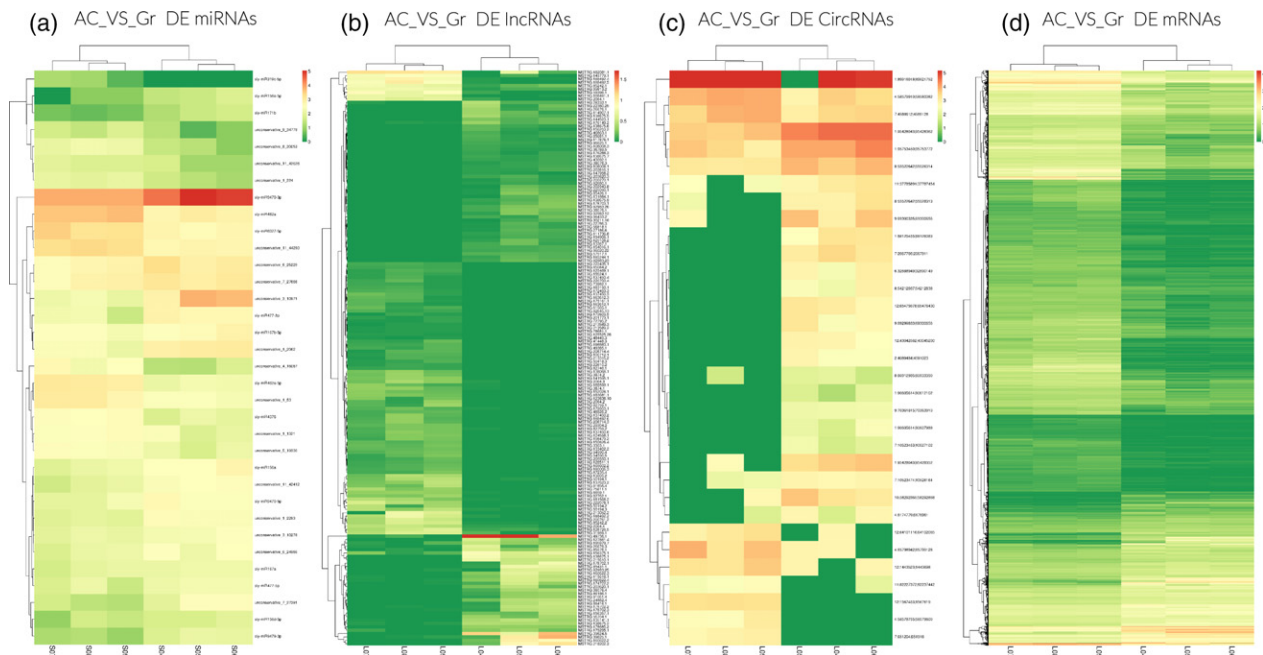


Figure 3. Differences in the differentially expressed (DE) non-coding RNAs and mRNAs between the wild type (WT; Ailsa Craig, AC) and the *Gr* mutant. (a) Differentially expressed micro-RNAs (miRNAs) between the WT and the *Gr* mutant. (b) The differences in DE long non-coding RNAs (lncRNAs) between the WT and the *Gr* mutant. (c) Differentially expressed circular RNAs (circRNAs) between the WT and the *Gr* mutant. (d) Differentially expressed mRNAs between the WT and the *Gr* mutant.

The likely targets of the DE miRNAs, lncRNAs and circRNAs were analyzed using GO and the KEGG pathway and the results suggested involvement of the lncRNAs in the monoterpene biosynthesis and pectin catabolic process, which may be involved in fruit flavor and texture, while the circRNAs were involved in amine metabolism processes. The KEGG pathway results for miRNA targets highlighted differences in gene expression in plant hormone signaling and starch and sucrose metabolism, indicating their independent or joint actions in the fruit ripening process (Appendix S4). We reviewed the targets of the DE non-coding RNAs and found that many were related to the ethylene pathway, such as *ACS4*, *ACO2/5*, *MADS-box TFs*, *ETR2*, *CTR1*, *EIN3*, *ERF2/7* and *ERF109*. Furthermore, several targets of lncRNAs that participate in fruit pigment accumulation were also identified, such as β -carotene hydroxylase (*CrtR-b1* and *CrtR-b2*) and 9-cis-epoxycarotenoid dioxygenase (*NECD*). Additionally, the target of two lncRNAs (*MSTRG.85242.1* and *MSTRG.85242.2*) was *green ripe-like 2*, indicating their involvement in the ethylene signal pathway and carotenoid accumulation (Appendix S4).

Major differences were found between the WT red ripe fruit and the *Gr* mutant fruit; 3711 DE mRNAs were identified, 2732 of which were upregulated and 979 downregulated (Figure 3d; Appendix S4). The topGO results for the DE mRNAs indicated that they were mainly involved in

photosynthesis, oxidation–reduction, and carotenoid, chlorophyll and monoterpene biosynthesis, indicating the specific differences between the WT and the *Gr* mutant. These mRNAs involved in pathways such as carbon metabolism, plant hormone signal transduction, starch and sucrose metabolism and phenylpropanoid biosynthesis were found by KEGG analysis (Figure 3c, Appendix S3). Our analysis of the DE mRNA data showed that many genes involved in the ethylene pathway were upregulated in the WT compared with the *Gr* mutant, such as *GR*, *ACO2/4*, *EIN3*, *EIN4*, *ERF1* and *ERF038/054*, and many genes were downregulated in the *Gr* mutant compared with the WT, such as *ACS2*, *ACO5*, *NR*, *ERF1/2/4/6* and *ERF096/114*. Furthermore, several genes involved in carotenoid accumulation also showed different expression levels, such as *beta-carotene isomerase D27 (Dwarf27)*, *LCY1*, *NECD5*, *CCD4*, *CRTISO* and *ZEP*, which were upregulated, while *ZISO*, *PSY1* and *abscisic acid 8-hydroxylase 1* were downregulated. Additionally, the strigolactone esterase *DAD2*, which is involved in the strigolactone signaling pathway, was also upregulated in the WT compared with the *Gr* mutant (Appendix S4).

Analysis of differences in DNA methylation between WT and *Gr* mutant fruit

It has been found that DNA methylation is involved in regulating ethylene-responsive TFs in tomato fruit (Zhong

et al., 2013), and WT red ripe and the *Gr* mutant were selected for RNA sequencing to explore the relationship between DNA methylation and the function of the ethylene perception and signaling pathway. A total of 57 961, 31 901 and 75 268 DMRs of the CG, CHG and CHH methylation types, respectively, were found between the AC red ripe fruit and the *Gr* mutant fruit (Appendix S5). The DMRs and DEGs were parsed together, and several DMRs and DEGs were identified as being involved in ethylene biosynthesis and signal transduction, such as *ACS2*, *ACO2*, *ACO5*, *MADS-box TFs*, *NR*, *GR*, *EIN3*, *ERF1/2/4/7*, *ERF038*, *ERF054*, *ERF096*, *ERF114* and *ERF119* (Figure 4a–c, Appendix S5). Intriguingly, we also found several DMRs and DE mRNAs to be involved in fruit pigment accumulation, such as *ZISO*, *LCY1*, *CrtL-e-1*, *NECD* and *NSY* (Appendix S5).

The DMRs and DE lncRNAs between the AC red ripe fruit and the *Gr* mutant were also analyzed jointly, and the results suggested that several differentially methylated lncRNAs are involved in targeting the ethylene and carotenoid pathways, such as MSTRG.59396.1 (target *ACO2* and *ERF7*), MSTRG.111174.1 (target *ACO5*), MSTRG.44159.1 (target *CTR1*), MSTRG.181568.2 (target *ETR2*), MSTRG.183681.1 (target *ERF2*), MSTRG.60188.1 (target *ERF017*), MSTRG.43594.1 (target *ERF024*), MSTRG.201251.2 (target *ERF061*), MSTRG.39825.1 (target *ERF109*), MSTRG.111733.1 (target *ERF114*), MSTRG.94590.4 (target *CrtR-b2*), MSTRG.162893.1 (target *NSY*) and MSTRG.181568.2 (target *NCED*) (Figure 4d–f; Appendix S5). In some cases, there was a relationship between DMRs and DE miRNAs, for example related to ethylene and fruit color, such as *miR171b* (target *CTR1*), *miR9470-3p* (target *ERF5* and

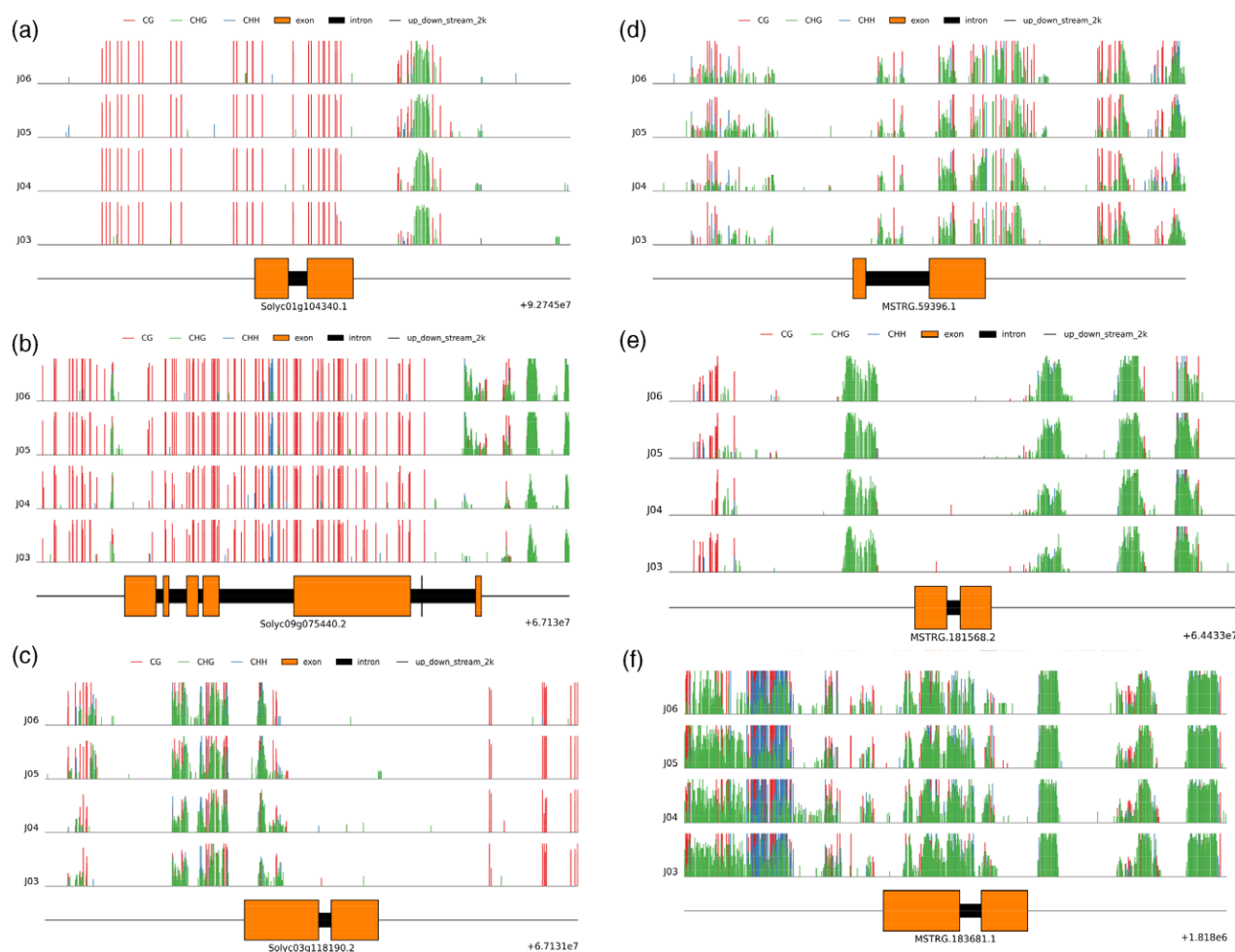


Figure 4. Combined analysis of the differences in differentially methylated regions (DMRs), differentially expressed genes (DEGs) and DMRs and differentially expressed (DE) long non-coding (lncRNAs) associated with ethylene synthesis and signaling in fruit of the wild type (WT) and the *Gr* mutant. (a)–(c) Comparison of DMRs and DEGs for green ripe (*GR*, *Solyc01g104340.1*), ethylene receptor never ripe (*NR*, *Solyc09g075440.2*) and ethylene-responsive transcription factor 114 (*ERF114*, *Solyc03g118190.2*) between WT fruit and the *Gr* mutant. (d)–(f), Analysis of DMRs and DE lncRNA MSTRG.59396.1 (the target is *Solyc12g005940.1*, *ACO2*), MSTRG.181568.2 (the target is *Solyc07g056580.2*, *ETR2*) and MSTRG.183681.1 (the target is *Solyc08g007230.1*, *ERF2*) between WT fruit and the *Gr* mutant.

ERF021), miR477-3p (target *EIN3-like*) and miR5300 (target *ZISO*) (Appendix S5).

Analysis of differences in metabolites between WT and *Gr* mutant fruit

Differences in metabolites between the red ripe WT fruit and the *Gr* mutant fruit were measured by metabolic profiling. A total of 289 different metabolites (85 upregulated and 204 downregulated in the WT compared with the *Gr* mutant) and 204 (51 upregulated and 153 downregulated in the WT compared with the *Gr* mutant) were found in positive or negative mode, respectively. The transcriptome and metabolome analysis were combined, and 34 and 28 different metabolites belonging to different KEGG pathways were identified. Compared with the *Gr* mutant, six different metabolites belong to six pathways were upregulated and 28 different metabolites belong to 28 pathways were downregulated in positive mode in the WT. Furthermore, four different metabolites belonging to four pathways were upregulated and 24 different metabolites belong to 24 pathways were downregulated in negative mode in the WT. Several differentially accumulated metabolites were mainly involved in the biosynthesis of amino acids, 2-oxocarboxylic acid metabolism, phenylpropanoid biosynthesis, phenylalanine metabolism, ascorbate and aldarate metabolism and flavonoid biosynthesis, many of which were related to flavor compounds (Appendix S6).

Non-coding RNAs and mRNA differences between WT and *r* mutant fruit deficient in carotenoids

PSY1 is a key biosynthetic enzyme responsible for the synthesis of tomato fruit carotenoids (Bird *et al.*, 1991; Fray and Grierson, 1993; Pan *et al.*, 2013). To further our understanding of carotenoid formation in tomato fruit, we characterized and compared the coding and non-coding transcriptomes, epigenomes and metabolomes between the WT and *r* mutant to explore carotenoid regulation.

We found 24 miRNAs, 13 lncRNAs, 15 circRNAs and 113 mRNAs that were differentially expressed between the WT and *r* mutant fruit, including 16 miRNAs, 6 lncRNAs, 6 circRNAs, and 89 mRNAs upregulated in the WT compared with the *r* mutant and 8 miRNAs, 7 lncRNAs, 9 circRNAs and 24 mRNAs downregulated compared with the mutant (Appendix S7). The potential targets of the DE miRNAs, lncRNAs and circRNAs were analyzed and, surprisingly, many ncRNAs were found to be involved in the ethylene pathway, such as lncRNA MSTRG.89359.1, which potentially targets *ACO4* that encodes a key enzyme in ethylene biosynthesis, and a circRNA (9:69299833/69300656) that targets *ACO3* (Appendix S7).

The lncRNA and mRNA GO analysis revealed that several lncRNA targets and their mRNAs were also involved in monoterpenoid biosynthetic, oxidation–reduction, pectin

catabolism and polyamine catabolism processes. Further KEGG pathway analysis results showed that many DE miRNA and lncRNA targets were involved in plant hormone signal transduction pathways. Specifically, the likely targets of miR172a, miR482e-5p and miR9472-5p were *AP2-ERF-TOE3*, *ERF-WRI1* and *SBP 7*, respectively, which play important roles during the fruit ripening process.

Analysis of differences in DNA methylation between WT and *r* mutant fruit

To explore the functions of DNA methylation, AC WT red ripe fruit and *r* mutant fruit were chosen for sequencing and further analysis (Figure 5a). A total of 36 355, 26 257 and 52 002 DMRs of the CG, CHG and CHH methylation types, respectively, were found between the WT red ripe and *r* mutant fruit. The KEGG pathway analysis showed that numerous DMRs were related to genes involved in plant hormone signal transduction, phenylpropanoid biosynthesis, starch and sucrose metabolism, carbon metabolism, biosynthesis of amino acids and phenylalanine metabolism processes (Appendix S8). The DMRs and DEGs were also analyzed jointly, and several DMRs and DEGs were characterized as being involved in the carotenoid pathway, such as *ZISO*, *Ctr-b2*, *LCY*, *NECD* and *abscisic acid 8-hydroxylase 1* (Figure 5b–d, Appendix S8). In addition, we also found many DMRs and DE mRNAs involved in the ethylene synthesis and signaling pathways, such as *ACO5*, *NR*, *ERF1*, *ERF2a*, *ERF 4* and *ERF119* (Appendix S8). The comparative analysis of DMR and DE lncRNAs and DMR and DE miRNAs between the WT and *r* mutant fruit suggested that their specific changes are involved in ethylene and carotenoid pathways, such as MSTRG.59396.1 (target *ACO2* and *ERF7*), MSTRG.69293.1 (target *ERF-RAP2-12*), MSTRG.162893.1 (target *NSY*), MSTRG.116865.5 (target *LCY*), MSTRG.95226.7 (target *PSY1*), miR172a (target *AP2-TOE3*), miR5300 (target *ZISO*), miR396a-5p and miR477-3p (target *EIN3-like*) (Appendix S8).

Analysis of differences in metabolites between WT and *r* mutant fruit

To uncover the full range of metabolites associated with the altered ripening phenotypes we compared all pericarp metabolites in WT red ripe and *r* mutant fruit. This identified 140 metabolites (67 upregulated and 73 downregulated in the WT compared with the *r* mutant) and 111 (59 upregulated and 52 downregulated in the WT compared with the *r* mutant) that were significantly different in positive or negative mode, respectively (Figure 6a,b, Appendix S9). The transcriptome and metabolome analyses were combined, and 24 and 16 different metabolites belonging to different KEGG pathways were identified in positive and negative mode, respectively. Compared with the *r* mutant, three different metabolites belonging to three

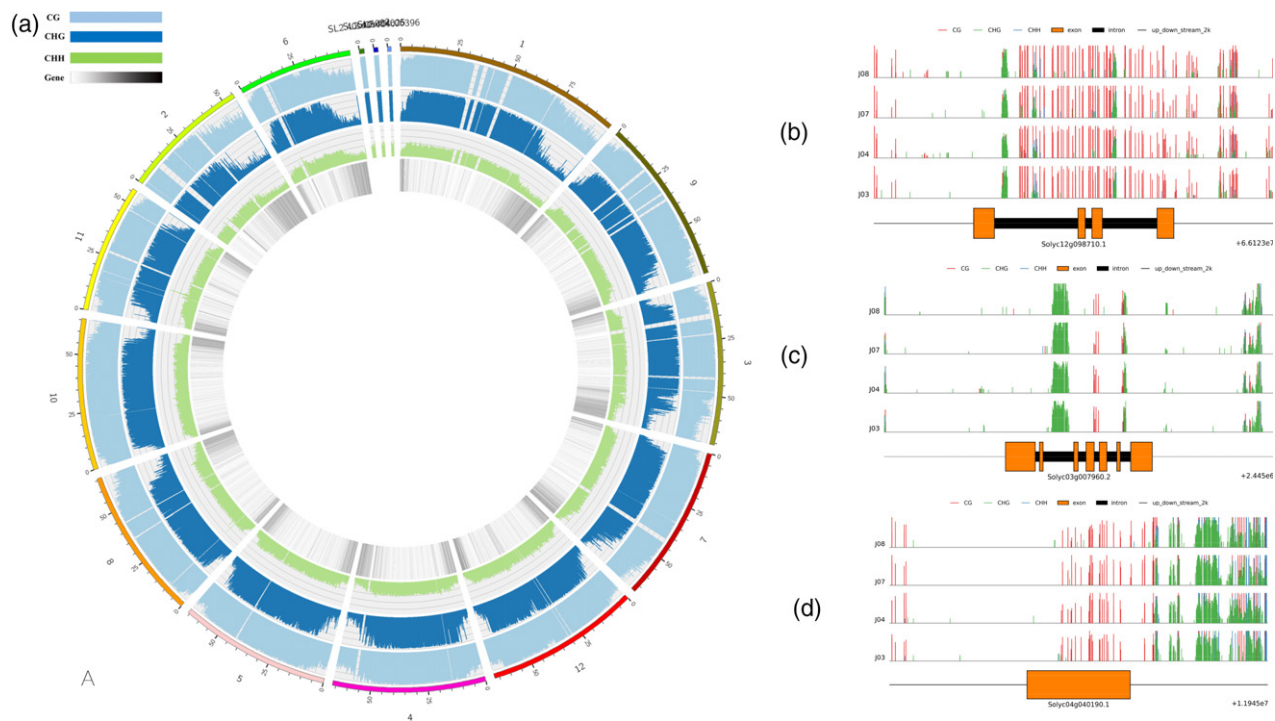


Figure 5. Whole-genome DNA methylation distribution in the *r* mutant and differentially methylated regions (DMRs) and differentially expressed genes (DEGs) between the wild type (WT) and the *r* mutant.

(a) The whole-genome DNA methylation distribution in the *r* mutant.

(b)–(d) The comparison of DMRs and DEGs of *15-cis-zeta-carotene isomerase* (*ZISO*, *Soly12g098710.1*), *beta-carotene hydroxylase* (*Crtr-b2*, *Soly03g007960.2*) and *nine-cis-epoxycarotenoid dioxygenase* (*NCED*, *Soly07g056570.1*) in WT and *r* mutant fruit.

pathways were upregulated and 21 different metabolites belonging to 21 pathways were downregulated in positive mode in the WT. Nevertheless, five metabolites from five different pathways were upregulated and 11 different metabolites from 11 pathways were downregulated in negative mode in the WT. Several differentially accumulated metabolites were identified as being involved in multiple metabolic pathways, including biosynthesis of amino acids, 2-oxocarboxylic acid metabolism and ascorbate and aldarate metabolism. The key pathways were involved in phenylpropanoid biosynthesis and phenylalanine metabolism, cyanoamino acid metabolism and flavonoid biosynthesis, revealing their specific roles in tomato fruit flavor and color formation (Figure 6c,d, Appendix S9).

DISCUSSION

Fruit ripening is driven by an intricate regulatory network, but our understanding of the components is incomplete and the topology and internal interactions of this network are far from understood (Grierson, 2013; Liu *et al.*, 2016; Li *et al.*, 2019). Tomato is a good model system for investigating the mechanistic basis of fruit ripening because of the availability of an excellent annotated genome and a range of well-characterized single gene mutants (Karlova *et al.*, 2014; Qiu *et al.*, 2016). A wide range of studies have

shed light on the biochemical changes underlying processes such as softening, color changes and the regulation of ripening-related signal transduction systems and downstream metabolic networks (Osorio *et al.*, 2011; Grierson, 2013; Seymour *et al.*, 2013). To further elucidate the roles and synergistic interactions of different regulatory components during the fruit ripening process, WT fruit and *Gr* and *r* mutant fruits were chosen for multi-omics comparisons by deep sequencing and bioinformatics analysis. Integration of the results of the analysis of mRNAs, non-coding RNAs, genome methylation and metabolomics together with bioinformatics analysis provides a fuller picture of the key players and coordinated regulation dynamics at different levels during the fruit ripening process. These changes and differences between the WT fruit and mutants revealed likely coordinated regulatory interactions between DNA methylation, non-coding RNAs and the production of transcripts affecting metabolites and provide a valuable resource for further studies on fleshy fruit biology.

Epigenetic modifications of DNA play important roles in regulating gene expression and appear to decrease by 20–30% across the genome in pericarp tissues during tomato fruit ripening (Teyssier *et al.*, 2008; Giovannoni *et al.*, 2017). The DNA methylation parsing in tomato has revealed dynamic changes in 5-methylcytosine (5mC)

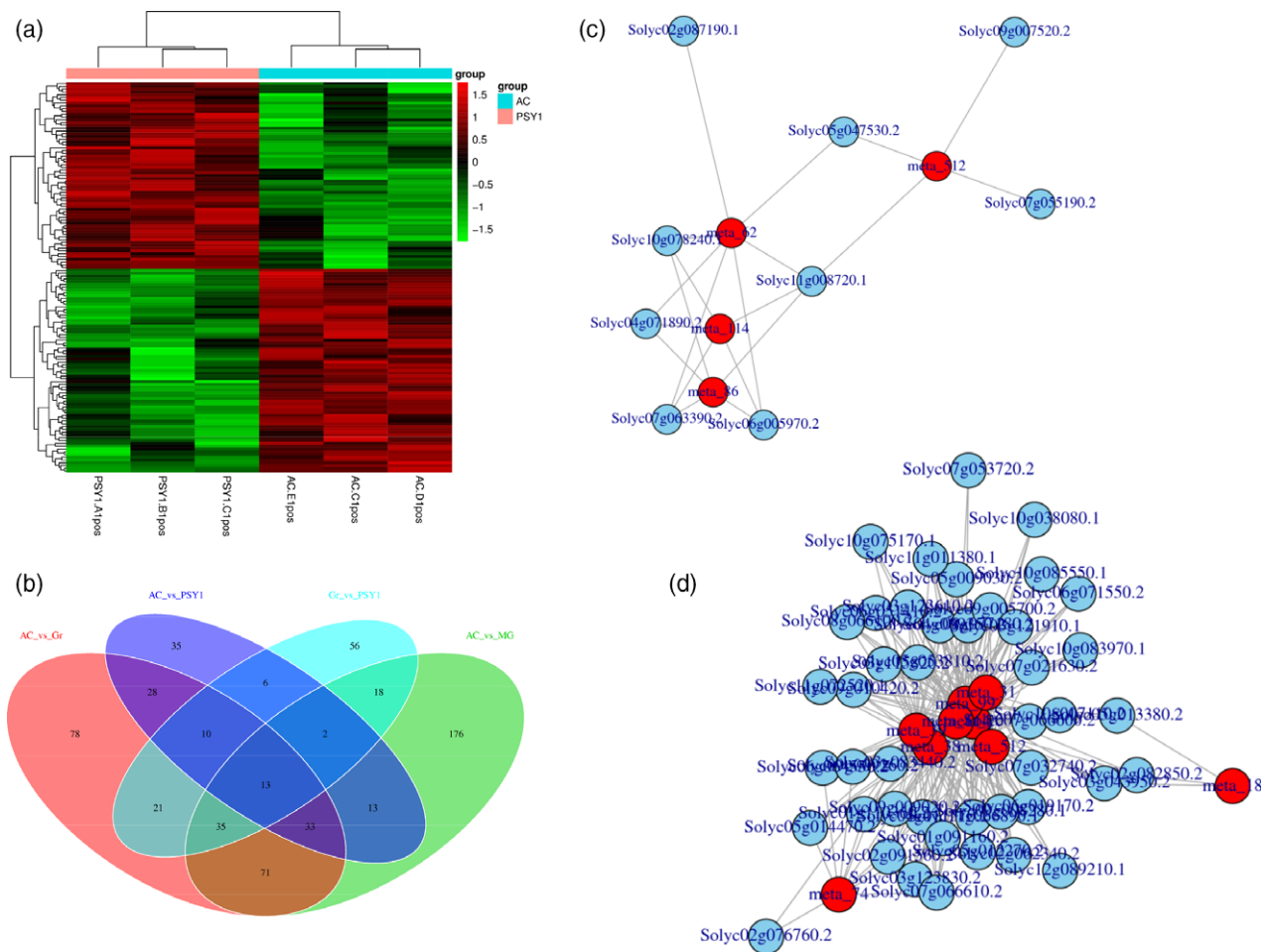


Figure 6. Differential metabolites between red ripe wild-type (WT; Ailsa Craig, AC) fruit and fruit of the *r* mutant and KEGG pathway network analysis.

(a), (b) The differential metabolites between AC and the *r* mutant in positive mode.

(c), (d) Combined KEGG pathway analysis of the differential metabolites and DEGs (c, phenylpropanoid biosynthesis pathway; d, biosynthesis of amino acid pathway).

distribution during fruit development and suggested a loss of 5mC in the promoters of more than 200 ripening-related genes involved in carotenoid accumulation (*PSY1*, *ZISO*), fruit texture (*PG*, *PME*), ethylene biosynthesis (*ACO1*, *ACS2*) and perception (*NR*, *ETR4*), and many transcription factors such as *RIN*, *NOR*, *CNR* and *TAGL1* that are known to play a role in ripening (Zhong *et al.*, 2013; Gallusci *et al.*, 2016; Ito *et al.*, 2017; Li *et al.*, 2018). We identified numerous ripening-related DMRs and DEGs in the three different comparison groups related to fruit texture (*PAE*, *PG*, *PE*, *PL*, β -galactosidase, β -glucosidase) and flavor formation (*glutamate synthase 1*, *flavonoid 3'-monooxygenase*), pigment accumulation (*PSY1*, *DS*, *ZISO*, *CRTISO*, *CCD1A*, *CCD4*, *NCED5*, *ZEP* and *NSY*), ethylene biosynthesis and signal transduction (*ACS2*, *ACO2*, *ACO5*, *MADS-box TFs*, *NR*, *GR*, *EIN3*, *EIN4* and *ERF TFs*). Additionally, several DEGs with DMRs showed specific expression, such as *ERF4*, *NR* and *ZISO* (targeted by 12:66127788|66128276), were downregulated in the *Gr* mutant but upregulated in

the *r* mutant. *AP2-ERF-TOE3* (targeted by miR172a) was upregulated in the *Gr* mutant, *CrtR-b2* (targeted by MSTRG.94590.4) was upregulated and *MADS-box TF23* (targeted by MSTRG.92645.13) was downregulated in the *r* mutant, indicating significant regulatory changes in carotenoid accumulation at the epigenetic level (Figure 7).

In tomato, ripening has been shown to be regulated by several non-coding RNAs in conjunction with ethylene and other plant hormones (Kumar *et al.*, 2014). Due to the rapid development of high-throughput sequencing technology, thousands of non-coding RNAs have been identified, but their regulatory functions are largely unknown, especially those potentially involved in fruit ripening (Mohorianu *et al.*, 2011; Zhu *et al.*, 2015; Tan *et al.*, 2017). We identified hundreds of non-coding RNAs involved in fruit ripening (Table 1, Appendices S1, S4 and S7). Of particular interest were MSTRG.17850.2 (potentially target *ERF086*), MSTRG.156253.2 (target *CrtR-b1*), MSTRG.22786.3 and MSTRG.20572.1 (target *pectinesterase*), which were

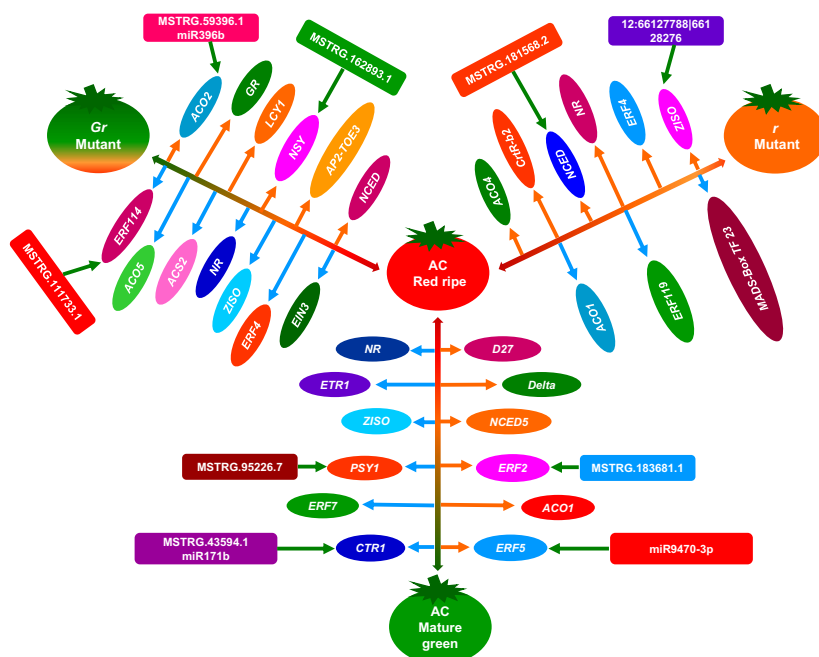


Figure 7. Regulatory model of ethylene response and carotenoid pathway regulation during fruit ripening.

The analysis of differentially methylated regions and differentially expressed genes comparing wild-type (WT) fruit and the *Gr* mutant and WT and the *n* mutant highlighted differences in several key genes involved in ethylene biosynthesis and signal transduction and the carotenoid pathway.

specifically expressed in WT red ripe stage fruit. Several non-coding RNAs, including MSTRG.172493.3 (target *ACO5*), MSTRG.92645.13 (target *MADS-box TF23*), MSTRG.183681.1 (target *ERF2-like*) and MSTRG.201773.1 (target *pectate lyase 4*) were specifically expressed in the *Gr* mutant, suggesting their specific functions in ethylene synthesis and signaling pathways and fruit ripening (Appendices S1, S3 and S5). Intriguingly, many different non-coding RNAs potentially have the same targets, such as MSTRG.59396.1 and miR396b (target *ACO2*), miR396a-5p and miR477-3p (target *EIN3-like*), MSTRG.43594.1 and miR171b (target *CTR1-like*), MSTRG.20235.2 and unconservative_3_10278 (target *ERF13-like*), MSTRG.107068.2 and miR9474-5p (target *glutamate synthase 1*), MSTRG.86646.1 and miR162 (target *PG*). Additionally, a ceRNA model were constructed and numerous important genes such as *ACO2*, *MADS-box 23*, *ERF5*, *ERF13*, *ERF021*, *PG*, *PL*, *PE* and *CesA* are likely to be affected by their synergistic regulations during fruit ripening (Appendices S1, S3 and S5).

Several non-coding RNAs including lncRNAs or miRNAs have been verified to conduct their regulatory functions by directing DNA methylation in plants (Shivaprasad *et al.*, 2012; Ariel *et al.*, 2015; Au *et al.*, 2017; Deng *et al.*, 2018). We identified differentially methylated lncRNA coding sequences by the combined analysis of DE lncRNAs and DMRs. Changes in several differentially methylated lncRNA coding regions were only found in specific comparison groups. For example, MSTRG.156253.2 (target *CrtR-b1*), MSTRG.17850.2 (target *ERF086*) and MSTRG.26451.1 (target *ERF3-like*) were only found in the WT mature green versus red ripe group and MSTRG.111174.1 (target *ACO5-like*), MSTRG.181568.2 (target *ETR2*), MSTRG.60188.1

(target *ERF017*), MSTRG.111733.1 (target *ERF114*) and MSTRG.94590.4 (target *CrtR-b2*) were only found in the WT versus *Gr* mutant group. Differences in the levels of MSTRG.116865.5 (target *LCY1*) and MSTRG.95226.7 (target *PSY1*) were only found in the WT versus *r* mutant group. Many differentially methylated lncRNA coding genes were found in both the WT versus *Gr* and WT versus *r* mutant groups, for example MSTRG.59396.1 (target *ACO2* and *ERF7*). In addition, the DE miRNA and DMR analysis results also suggested the important roles of miRNAs, such as miR171b (target *CTR1-like*) where differences were only found in the WT versus *Gr* mutant group. Differences in miR9470-3p (target *ERF5* and *ERF021*) and unconservative_3_10278 (target *ERF13*) were found in both the WT mature green versus red ripe and WT versus *Gr* mutant comparisons, whereas differences in miR172a (target *AP2-ERF-TOE3*), miR396a-5p and miR477-3p (*EIN3-like*) were both found in the WT versus *Gr* and WT versus *r* mutant groups, which suggests their importance for the ethylene and carotenoid pathways.

Tomato fruit quality is determined by many physiological and biochemical changes to physical and chemical properties and metabolite concentrations. The dynamic changes in sugars, acids and volatiles make an important contribution to taste and aroma, which together generate the flavor formed during ripening that consumers respond to (Carrari *et al.*, 2006; Osorio *et al.*, 2011; Zhu *et al.*, 2018). Genetic manipulation of the ethylene and carotenoid pathways are of special interest since these pathways affect initiation of fruit ripening and pigment accumulation (Davies and Grierson, 1989; Llorente *et al.*, 2016; Klee and Tieman, 2018; Oeller *et al.*, 1991; Picton *et al.*, 1993). The metabolic

profiling and transcriptome analysis results for WT and *Gr* mutant and WT and *r* mutant fruit indicate that disruption of the ethylene response and carotenoid pathway can result in changes in biosynthesis of amino acids, flavonoid, phenylpropanoids, and ascorbate and aldarate metabolism pathways, and lead to the variations in fruit color and flavor (Appendices S3, S6 and S9).

Transcriptome and epigenome changes are involved in a complex and comprehensive regulatory network that regulates ripening. Analysis of the ceRNA networks model revealed the potential for intricate interactions of lncRNAs, circRNAs and mRNAs acting with miRNAs during the fleshy fruit ripening process. Several non-coding RNAs identified in this work have potential targets that play key roles in fruit ripening. These include *ACO2*, *MADS-box 23*, *ERF5*, *ERF13*, *ERF021*, *PG*, *PL*, *PE*, *CesA* and *glutamine synthetase*, and putative coordinated regulation networks were constructed (Appendix S1).

EXPERIMENTAL PROCEDURES

Sample collection and preparation

Wild-type tomato (WT) (*Solanum lycopersicum* cultivar Ailsa Craig) and the corresponding Green ripe (*Gr*) and yellow flesh (*r*) mutants incorporated into the AC background were grown under standard greenhouse conditions (12 h supplemental lighting at 26°C and 12 h at 20°C). Tomato fruit pericarp tissues of AC were harvested at 39 days (mature green) and 52 days (red ripe) and the corresponding *Gr* and *r* mutants were harvested at 52 days after anthesis. The pericarp samples (with three replicates per group) were collected, frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

Library preparation and deep sequencing

The RNA samples were extracted with an RNA Extraction Kit (RN40, Aidlab Biotechnologies, <http://www.aidlab.cn/>). The RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, <https://www.agilent.com/>) to ensure the use of samples of the appropriate quality for sequencing. The libraries for sRNA, lncRNA and circRNA sequencing were constructed as described previously (Zuo *et al.*, 2018). The PCR products were purified (AMPure XP system, Beckman Coulter, <https://www.beckmancoulter.com/>) and the library quality was assessed on the Agilent Bioanalyzer 2100 system (Agilent Technologies).

Identification of non-coding RNA and differential expression analysis

The transcriptome was assembled using StringTie based on the reads mapped to the reference genome. The assembled transcripts were annotated using the gff compare program. The unknown transcripts were used to screen for putative lncRNAs, and the CIRI tools were used to identify circRNAs. The related parameters and methods for identifying of conserved and novel miRNAs, lncRNAs and circRNA were as in previous studies (Zuo *et al.*, 2018). Differential expression analysis of three libraries (WT, *Gr* and *r* mutants) was performed using the DESeq R package, with DESeq statistical routines for determining differential digital

expression and lncRNA, circRNA, and miRNA expression data using a model based on the negative binomial distribution. The resulting *P*-values were adjusted to control the false discovery rate (FDR; Benjamini and Hochberg, 1995). Genes, lncRNAs, and circRNAs with an adjusted *P*-value < 0.01 and an absolute value of log₂ (fold change) > 1 found by DESeq were designated as DE. Micro-RNAs with an adjusted *P* < 0.05 found by DESeq were designated as DE (Zuo *et al.*, 2019; supplied by Beijing Biomarker Technologies).

Gene function annotation and GO and KEGG pathway enrichment analysis

Gene function was annotated using the following databases: Nr [National Center for Biotechnology Information (NCBI) non-redundant protein sequences; <ftp://ftp.ncbi.nih.gov/blast/db/FASTA/>]; Pfam (Protein family; <http://pfam.xfam.org/>); KOG/COG (Clusters of Orthologous Groups of proteins; <http://www.ncbi.nlm.nih.gov/KOG/>); Swiss-Prot (a manually annotated and reviewed protein sequence database; <http://www.uniprot.org/>); KEGG (<http://www.genome.jp/kegg/>); and GO (<http://www.geneontology.org/>). The GO enrichment analysis of the DEGs was implemented by the Goseq R packages based on Wallenius non-central hypergeometric distribution. We used KOBAS software to test the statistical enrichment of DEGs in KEGG pathways (Mao *et al.*, 2005; Zuo *et al.*, 2019; supplied by Beijing Biomarker Technologies).

The ceRNA network analysis of non-coding RNAs

A hypergeometric test was executed for each ceRNA pair separately, using the following four parameters: (i) *N*, the total number of miRNAs used to predict targets; (ii) *K*, the number of miRNAs that interact with the chosen gene of interest; (iii) *n*, the number of miRNAs that interact with the candidate ceRNA of the chosen gene; and (iv) *c*, the common miRNA number between these two genes. The test calculates the *P*-value by using the following formula:

$$P = \sum_{i=c}^{\min(K,n)} \frac{\binom{K}{i} \binom{N-K}{n-i}}{\binom{N}{n}}.$$

All *P*-values were subject to FDR correction. The following criteria were applied for ceRNAs: (i) number of miRNAs that interact with the candidate ceRNA ≥ 5 and (ii) FDR < 0.05 (Li *et al.*, 2014; Zuo *et al.*, 2018, 2019; supplied by Beijing Biomarker Technologies).

Methylation level and DMR detection

The bisulfite sequencing (BS-seq) libraries were constructed according to previous studies (Zuo *et al.*, 2018) and the PCR-enriched libraries were purified and subjected to high-throughput Illumina sequencing (BioMarker, Beijing Biomarker Technologies, Beijing, China, <http://en.biomarker.com.cn/>). A binomial test was used to determine if the observed methylation frequency was above the background expected from inefficiencies in the bisulfite conversion reaction and sequencing errors. When using this test (FDR < 0.05), the amount of methylation at a given site is typically expressed as the ratio of reads with methylation to the total number of reads covering the position (≥2×). We refer to this site-specific metric as the methylation level of the site (Schultz *et al.*, 2012).

We used ComMet (v.1.1) to detect DMRs, in a two-step method. First, differentially methylated cytosines (DMCs) were detected

through a comparison of alignment results between the samples. Then, DMCs at neighboring positions were grouped as contiguous DMRs using certain distance criteria. ComMet detects DMRs based on log-likelihood ratio scores (-threshold 0 for CG, -noncpg -threshold 30 for CHG and CHH). The score for detecting a certain region (no change: NoCh) as a DMR directed to dir (=UP or DOWN) was defined using MOABS, based on a beta-binomial hierarchical model. The regions where the Fisher's exact test *P*-value was less than 0.05 were recognized as DMRs (supplied by Beijing Biomarker Technologies).

Integrated functional analysis of DM genes

Translated gene sequences were compared against various protein databases by BLASTX, including the NCBI non-redundant protein (Nr) database and the Swiss-Prot database, with a cut-off *E*-value of 10^{-5} . Furthermore, we used KOBAS to examine the KEGG pathways for genes. Genes were retrieved based on the best BLAST hit (highest score), along with their protein functional annotation (Wei *et al.*, 2017). For GO terms, the Nr BLAST results were imported into the Blast2 GO program (Conesa *et al.*, 2005) to obtain GO annotations. This analysis mapped all the annotated genes to GO terms in the database and counted the number of genes associated with each term. A Perl script was then used to plot the GO functional classification for the unigenes with a GO term hit to view the distribution of gene functions (Cui *et al.*, 2017). The obtained annotations were enriched and refined using topGO (R package) with the 'elim' method and the Kolmogorov-Smirnov test (Xia *et al.*, 2017). The gene sequences were also aligned to the COG database to predict and classify functions (Tatusov *et al.*, 2000). The KEGG pathways were enriched using the right-sided Fisher's exact test (supplied by Beijing Biomarker Technologies).

Methylation of DNA and lncRNA and miRNA combined analysis

The methylation level from the 2k region upstream of lncRNA to the 2k region downstream of the gene was calculated by measuring CG/CHG/CHH and C. Each sample methylation level was calculated as weighted. The coding NA indicates that the value was null. According to the expression level, the detected lncRNAs were divided into four groups: highest, medium high, medium low and lowest. The weighted methylation level method was used to calculate the methylation levels in the upstream 2k, the gene body and downstream 2k regions. Key genes were identified by observing whether target genes of DE miRNAs had DMRs (Schultz *et al.*, 2012; supplied by Beijing Biomarker Technologies).

Metabolome and transcriptome combined analysis

Analysis was performed using an Agilent 1290 Ultra High-Performance Liquid Chromatography system using a UPLC BEH Amide column (1.7 $\mu\text{m} \times 2.1 \times 100$ mm) from Waters (<https://www.waters.com/>). The AB 5600 Triple time-of-flight mass spectrometer can acquire primary and secondary mass spectrometry data based on the IDA function with Analyst TF 1.7 software (AB Sciex, <https://sciex.com/>). In each data acquisition cycle, the molecular ion with the strongest intensity and value greater than 100 was selected to collect the corresponding secondary mass spectrometry data. The bombardment energy was set at 30 eV with 15 secondary spectra every 50 msec. The electrospray ionization ion source parameters were set as follows: atomization pressure (GS1) 60 psi; auxiliary pressure 60 psi; air curtain pressure 35 psi; temperature 650°C; spray voltage 5000 V (positive ion mode) or -4000 V (negative ion

mode). The original mass spectrometry data were converted to mzXML using ProteoWizard software and XCMS was used for retention time correction, peak identification, peak extraction, peak integration, peak alignment, etc. Minfrac was set to 0, and the cut-off was set to 0.6. The peaks were also identified using a self-written R package and a self-built secondary mass spectrometry database.

The results of the differential metabolite analysis were combined with the results of differential gene expression analysis from the transcriptome and DEGs and metabolites were mapped to the KEGG pathway maps to better understand the relationship between gene transcripts and metabolite levels.

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AUTHOR CONTRIBUTIONS

JJG, JZ and LTC conceived and designed the research. JZ and DG wrote the article. LTC, YW, LG, XZ, BZ, YL and QW participated in the related experiments and analyzed the data. All authors read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing commercial interests in relation to this work.

DATA AVAILABILITY STATEMENT

Sequence data from this article can be found in the NCBI database under the accession numbers (SRA11461626–SRA11461649 and SRA11468335–SRA11468342).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Appendix S1. The differences in non-coding RNAs between wild-type mature green and red ripe fruit.

Appendix S2. The differentially methylated regions and differentially expressed genes in wild-type mature green and red ripe fruit.

Appendix S3. The combined analysis of transcriptome and metabolomics differences between wild-type mature green and red ripe fruit.

Appendix S4. The differences in non-coding RNAs between wild-type and *Gr* mutant ripe fruit.

Appendix S5. The differences in differentially methylated regions and differentially expressed genes between ripe wild-type and *Gr* mutant ripe fruit.

Appendix S6. The combined analysis of transcriptome and metabolomics differences between ripe wild-type and *Gr* mutant ripe fruit.

Appendix S7. The differences in non-coding RNAs between ripe wild-type and *r* mutant ripe fruit.

Appendix S8. The differences in differentially methylated regions and differentially expressed genes between ripe wild-type and *r* mutant ripe fruit.

Appendix S9. The combined analysis of transcriptome and metabolomics of ripe wild-type and *r* mutant ripe fruit.

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