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Gene regulation in climacteric fruit ripening

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Abstract

Seed dispersion and consequent plant propagation depend on the success of fruit ripening. Thus, ripening is a highly regulated developmental process aiming to maximize fruit organoleptic traits to attract herbivores. During ripening, the developing fruit experiences dramatic modifications, including color change, flavor improvement, and loss of firmness that are remarkably coordinated. Dynamic interactions between multiple hormones, transcription factors, and epigenetic modifications establish the complex regulatory network that controls the expression levels of ripening-related genes. Tomato, as a climacteric fruit, displays a burst of respiration once the seeds mature, followed by an increase in ethylene that regulates ripening. The accepted paradigm of the ripening transcriptional regulation has been recently challenged by the generation of true-null mutants of the previously considered master regulators of ripening. In addition to hormonal and transcriptional control, epigenetic shifts regulate the ripening process. Future research will contribute to better understanding the factors regulating fruit ripening.

Addresses

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Keywords

Fruit ripening, Transcriptional regulation, Ethylene, Plant hormones, Epigenetic modifications, Tomato.

Introduction

Angiosperm plants produce fruits that fulfill two key functions: the protection of developing seeds by the immature fruit and the dispersal improvement of mature seeds by the ripe fruit. According to these purposes, fruits experience physiological and metabolic modifications during their development, which encompasses three main phases: fruit set, growth, and ripening. Tomato (*Solanum lycopersicum*) has become the major fleshy fruit model to study development and ripening for several reasons, straightforward diploid genetics, autogamy, short life cycle, efficient transformation and greenhouse propagation, accessibility to germplasm resources including mutant lines, availability of a high-quality reference genome, and several RNA-seq approaches that have provided gene expression data at the genome-wide scale during the entire ripening process with specific tissue resolution [1–6].

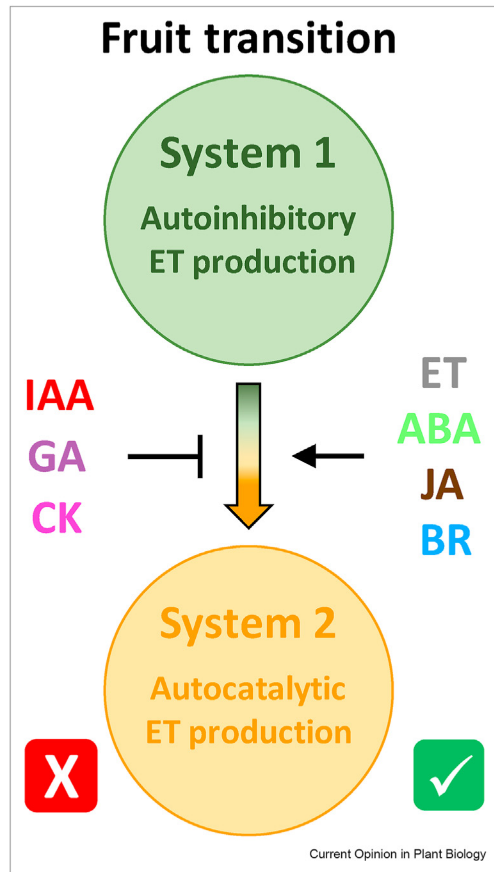
Fruit ripening requires the fine-synchronization of various independent processes that cause change of color triggered by chlorophyll degradation and pigments accumulation, flavor improvement as a result of sugars, acids, and volatile compounds production, and fruit softening promoted by cell wall remodeling. Fruit ripening comprises all these specific processes that are spatiotemporally regulated and exceptionally organized by an interacting set of plant hormones, transcriptional regulators, and epigenomic modifications that ultimately define fruit quality. Control of ripening is a dynamic process coordinated by the effects of multiple hormones at many different levels, including chromatin accessibility, transcription, translation, and post-translational modifications.

This review summarizes the current understanding of the most important aspects that regulate fruit ripening, such as the key impact of ethylene production and response, the redefined roles of previously considered Transcription Factor (TF) master regulators, and the effect of major epigenetic modifications at the DNA and histone levels.

Hormonal regulation of the fruit ripening

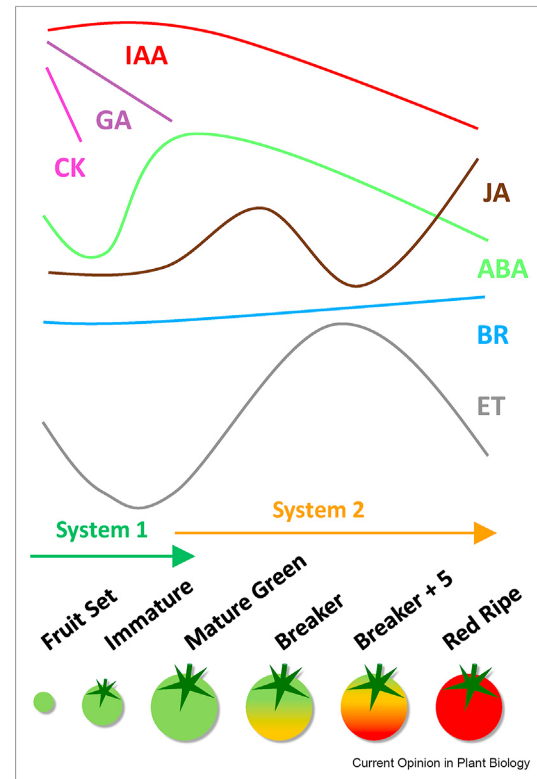
The orchestrated activities of auxin (IAA), gibberellic acid (GA), and cytokinin (CK) are key to modulate fruit set [7–9]. Fruit growth is mainly regulated by IAA and CK [10–13]. Ethylene (ET) plays a central role in initiating and governing fruit ripening. Additional hormones control particular features of the ripening process. Abscisic acid (ABA) is a major fruit ripening and senescence regulator [14–18]. IAA promotes the growth-to-ripening shift [19–22]. Methyl-jasmonate (MeJA) and brassinosteroids (BRs) also contribute to specific aspects of fruit ripening (Figure 1).

Figure 1



Hormone effect on fruit transition. At the onset of ripening, the transition from System 1 to System 2 is promoted by ethylene, abscisic acid, jasmonate, and brassinosteroids. Conversely auxin, gibberellic acid, and cytokinin repress the shift. The check mark (green square) denotes promotion of the transition by the hormones present on the right side of the figure. The cross mark (red square) signifies repression of the transition by the hormones present on the left side of the figure. Hormones are denoted by the following color pallet: ABA is light green, BR is blue, CK is pink, ET is grey, GA is purple, IAA is red, and JA is brown. Black arrowhead represents positive regulation, and black blunt arrow represents negative regulation.

Figure 2



Hormone levels during fruit set, growth, and ripening. GA and CK levels decline after fruit set. IAA accumulation in the fruit is reduced in concert with the transition to fruit maturation. Exogenous applications of GA, CK, or IAA at the immature stage inhibit fruit ripening progression. The transition to System 2 and the boost of ET biosynthesis are preceded by the highest ABA contents in the fruit. Similarly, JA exhibits a peak before the rapid accumulation of ethylene. Together with ABA and ET, the second JA wave might be associated with the fine-tuning of fruit quality parameters. Exogenous treatments with ABA, JA, ET, or BR stimulate fruit ripening. Hormone levels are displayed as relative values and denoted by the following color pallet: ABA is light green, BR is blue, CK is pink, ET is grey, GA is purple, IAA is red, and JA is brown.

In tomato, fruit ripening is tightly regulated by different hormonal signaling pathways, yet as a climacteric fruit, tomato requires ET for ripening [20–28]. The levels of ET synthesis, sensitivity, and response are determined by the fruit developmental stage. During vegetative growth and immature stages, the fruit ET production is limited to low-basal levels and regulated in an auto-inhibitory manner. During this phase, known as System 1, treatments with exogenous ET have negative effects on ripening. Fruits experience a burst of respiration accompanied by a large increase in the fruit endogenous ET synthesis, exhibiting more than a 100-fold rise in ET concentration during this transition phase to System 2, characterized by autocatalytic ET production (Figures 1 and 2) [28–30]. This transition triggers the onset of ripening at the mature green stage, once seed maturation is complete and the locule surrounding the seeds liquefied. Interestingly, parthenocarpic fruits (with no seeds) undergo a similar series of events, suggesting that signals coming from the seeds are not required for the onset of ripening. The well-established association, at the physiological level, between the sequential increase of respiration and ethylene production is the most important step to initiate the ripening process. However, at the molecular level, the players controlling the transition from System 1 to System 2 remain unidentified. The interaction between IAA and ET is crucial for this transition [31,32]. Application of exogenous IAA on immature fruits causes a delay in the transition to the ET autocatalytic production phase having an obvious effect on the fruit color changes, preserving high levels of xanthophylls and chlorophyll, and repressing the

production of pigment compounds, including carotenoids and anthocyanins [33]. At this stage, IAA and ET display clear antagonistic effects on ripening (Figure 1) [17,18,34].

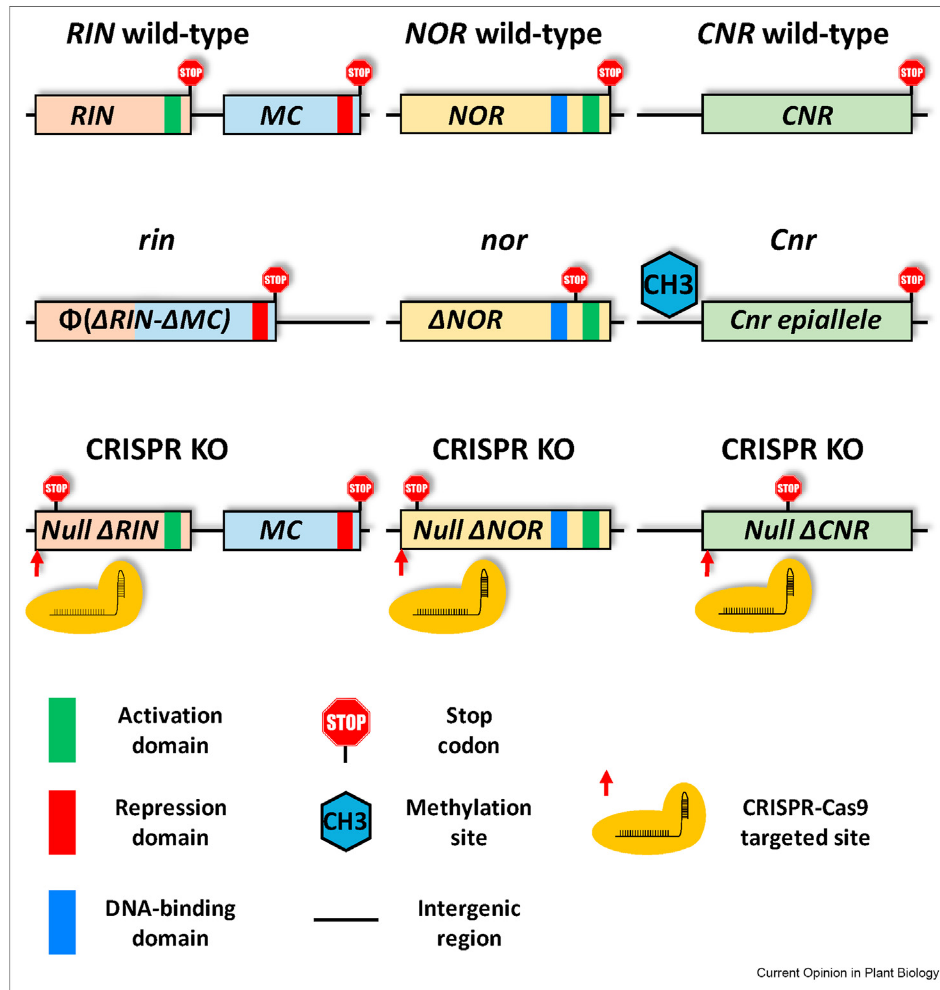
Ethylene induces the expression of cell wall—modifying enzymes, pectin methyl esterase, pectate lyase (PL), and polygalacturonase (PG) that catalyze pectin depolymerization resulting in gradual fruit softening [34—36]. ABA also affects various aspects of fruit ripening. Silencing of the *SlNCED1* gene, encoding a 9-*cis*-epoxycarotenoid dioxygenase involved in ABA biosynthesis, compromises ABA production causing the transcriptional downregulation of different ripening-related cell wall enzymes, including the aforementioned *PL* and *PG*. Low ABA levels slow down softening and therefore extend fruit shelf-life [37]. The climacteric respiration boost and the transition to System 2 of ET production are preceded by the highest ABA levels in the fruit. ABA is also able to stimulate ripening by inducing ethylene biosynthesis [37]. Conversely, exogenous application of GA delays ripening [38—41]. Consistently, reduction of endogenous GA levels in the fruit through overexpression of GA2OX1, a gibberellin 2-oxidase key for GA catabolism, triggers early ripening. ET biosynthesis is promoted in GA-deficient fruits [28]. As the fruit ripens, endogenous MeJA accumulates [42], contributing, in collaboration with ET and ABA, to regulate the production of sugars, acids, pigments, and volatile organic compounds that define fruit quality [43]. BRs are also synthesized during ripening (Figure 2). Overexpression of *CYP90B3,* a cytochrome P450 monooxygenase that catalyzes the rate-limiting step of BRs biosynthesis, promotes BRs accumulation, which was positively correlated with fruit softening and elevated levels of soluble sugars, carotenoids, and volatile compounds. BRs seem to work in cooperation with ethylene to stimulate fruit ripening [44].

Transcriptional regulation of the fruit ripening

At the onset of ripening, ET triggers major ripening-associated changes, including extensive modifications of gene transcription caused by several TFs that promote fruit softening, production of sugars, acids, pigments, and volatile compounds. Various of these TFs controlling fruit ripening have been identified to date. Spontaneous occurring and gene-edited mutations in some of these TFs, and/or transgenic approaches upregulating or downregulating their expression have revealed the involvement of particular TFs in the tomato ripening process. Among the traditional mutants, *rin* (*ripening-inhibitor*), *nor* (*nonripening*), and *Cnr* (*colourless nonripening*) have been widely studied because of their strong phenotypes. These mutants' fruits are unable to transition from System 1 to System 2 of ET production after mature green stage exhibiting a clear arrest of the ripening initiation that severely impacts color, flavor, and texture of the fruit [45—47]. *RIN* codes for a member of the SEPALLATA4 (SEP4) group of MADS-box transcription factor genes. *NOR* is a member of the NAC domain transcription factor gene family, whereas *CNR* encodes a transcription factor of the SQUAMOSA PROMOTER BINDING PROTEIN (SPBP) family. For a long time, these TFs have been accepted as classic master regulators of the ripening process [48—50]. However, the activity of these TFs as master regulators have been reconsidered after the characterization of recently CRISPR-Cas9-generated null mutant alleles (CRISPR KO) of these genes and the comprehensive re-examination of the traditional spontaneous mutants (*rin*, *nor*, and *Cnr*), which actually harbor gain-of-function mutations coding for dominant repressor TFs. The new *RIN* and *NOR* CRISPR KO mutant lines showed partial induction of ripening, displaying milder phenotypes than the traditional mutants, including the development of orange fruits rather than the green fruits borne by the original mutant [51—53]. The causal mutation on C*nr* was identified by positional cloning and mapped to a region in the *CNR* promoter that was hypermethylated in the *Cnr* mutant blocking the transcription of the *CNR* gene [54]. The fruits of this traditional *Cnr* mutant exhibited a strong nonripening phenotype. In contrast, the fruits from the new *CNR* CRISPR KO alleles showed a slight delay in ripening [55], a much lighter effect than the pleiotropic phenotype displayed by fruits of the original C*nr* (Figure 3).

The traditional mutants exhibited strong nonripening phenotypes because of the now recognized gain-of-function mutations harbored by these classic lines. For example, in the *rin* mutant, the spontaneous deletion of a genomic DNA fragment between *RIN* and the downstream gene *MC* produced a chimeric TF missing the RIN activation-domain and acquiring the EAR motif-like repression-domain from MC (Figure 3). The traditional chimeric *rin* exhibits a dominant-repressor activity able to inhibit the expression of the *RIN* paralogs' (homologous members of the RIN TF-family) target genes. Conversely, CRISPR KO mutations on these previously considered master regulator TFs only partially affected fruit ripening. For example, in the *RIN* KO mutant, RIN-target genes remained expressed due to the activity of *RIN* paralogs that assume some of the RIN roles in establishing TF-complexes able to recognize RIN binding motifs. These observations support the concept of RIN and NOR being required to achieve full ripening [56], but also suggest that RIN, NOR, and CNR activity is partially redundant to the activity of their paralogs. These classic TFs, believed to be master regulators of the ripening process and absolutely required for the transition from System 1 into System 2 phase of ET production, have to be considered members of a robust

Figure 3



Reevaluation of the role of ripening master regulators. In the spontaneous *rin* mutant, a chimeric Φ(*ΔRIN-ΔMC*) gene is generated by the deletion of the genomic DNA between these two genes positioned in tandem on the chromosome. The resulting chimeric gene retains the 5′ end of *RIN* (*ΔRIN*), coding for the first 215 of the total 242 amino acids and the 3′ end of *MACROCALYX* (*ΔMC*), missing the first 62 of the total 219 amino acids. The fruits from the lines harboring the CRISPR-Cas9 null (*ΔRIN*) mutation exhibit a milder phenotype than the fruits from the traditional *rin*. Many genes repressed in the *rin* mutant presented normal expression levels in the CRISPR-Cas9 null *ΔRIN*. These differences might be caused by the presence of the MC repressor domain and the absence of the RIN activator domain in the chimeric TF Φ(*ΔRIN-ΔMC*). Thus, the spontaneous *rin* is a gain-of-function mutation producing a dominant repressor TF instead of a null mutation. The spontaneous *nor* mutation presents a two base pair (bp) deletion in the coding region, that causes a frameshift resulting in a premature stop codon. The truncated protein produced by the *nor* mutant still harbors the DNA-binding domain, but has lost the activation domain. The fruits of the *nor* mutant show a more severe phenotype than the fruits from the CRISPR-Cas9 null that codes for a short peptide missing all DNA-binding domains. As *rin*, *nor* is also a gain-of-function mutation coding for a dominant repressor TF that can bind its target genes but represses their transcription. The spontaneous *Cnr* mutation is located in a 286 bp hypermethylated region in the *CNR* promoter. This epiallele is associated with a reduced expression of *CNR* that causes a pleiotropic phenotype in the fruit. The fruits of the CRISPR-Cas9 null *CNR* lines display a much milder phenotype. As in *rin*, numerous ripening-related genes differentially expressed in *Cnr* are not in the CRISPR-Cas9 null *ΔCNR*.

transcriptional regulation network that possesses high functional redundancy.

ET not only induces the expression of *RIN*, but also other paralogs, members of the MADS-box family of TFs, including *TOMATO AGAMOUS-LIKE 1* (*TAGL1*), *FRUITFULL 1* (*FUL1*), and *FUL2* [25,57,58], as well as, members of the APETALA2/ethylene response factors (AP2/ERFs) family that comprise the transcriptional network regulation of ET-responsive genes harboring GCC-box motifs where ERFs are able to bind [59]. Although ERFs were initially identified as ET-responding TFs, some ERFs can also be activated by IAA, or by both hormones ET and IAA. ABA and BRs can also act on the ET signal transduction and transcriptional pathways to stimulate fruit ripening progress [60–63]. As mentioned earlier, ET induces the expression of *RIN* that directly targets *SAUR69*, which

in turn alters IAA transport and reinforces ET production and sensitivity during the fruit shift to the breaker stages [4,5,32,64]. Both ET and IAA are able to upregulate the expression of *ET RESPONSE FACTOR.B3* (*SlERF.B3*), a TF that modulates ET responses and fruit ripening [65]. *SlERF.B3* integrates ethylene and auxin signals by directly binding to the promoter and inducing the expression of *SlIAA27* [61].

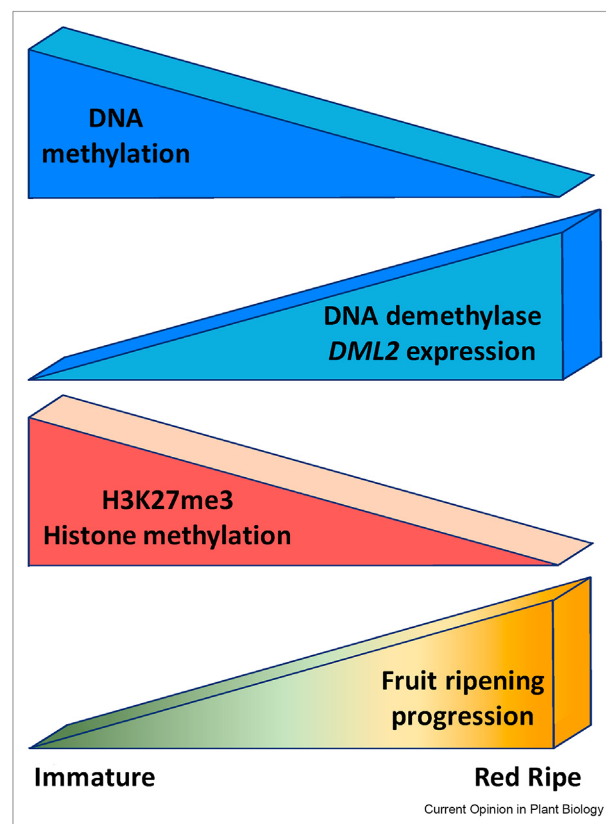
Expression of *RIN* and *NOR* has been recently reported to be upregulated in fruits of the GRAS4 TF overexpression lines. GRAS4 is induced during ripening and is able to directly bind *ACO1* and *ACO3* promoters to induce their transcription and consequently, promote ethylene biosynthesis that in turn triggers the *PHYTOENE SYNTHASE 1* (*PSY1*) upregulation and the accumulation of carotenoids. GRAS4 also directly targets the promoter of *MADS1*, a negative regulator of tomato fruit ripening, but in this case, GRAS4 represses the expression of *MADS1*. Thus, GRAS4 performs diverse regulatory activities that globally promote ripening [66]. In addition, NOR can also undergo post-translational modifications that are pivotal to regulate its activity. Oxidized NOR possesses low DNA-binding capacity that compromises its transcriptional regulatory abilities. E4 and MsrB2, two met sulfoxide reductases, are able to reduce the oxidized-NOR improving its DNA-binding and transcriptional regulatory capacities on ripening-related genes [67].

In tomato, 2026 genes are annotated as TFs and 516 of them are expressed in the ripening fruit [55], suggesting that the TF regulatory network modulating the ripening process is more intricate and robust than anticipated. Thus, fruit ripening regulation represents an intricate system that integrates diverse hormonal cues on a complex transcriptional network where the ability of TFs to reach and interact with target DNA regions lastly determine the expression levels of ripening-related genes.

Epigenetic regulation of the fruit ripening

As mentioned earlier, in addition to hormonal and transcriptional control, epigenetic modifications also regulate ripening progression by modulating chromatin accessibility and binding of key TFs to DNA. The major effects are caused by the DNA methylation levels and histone modifications. A series of findings revealed that DNA methylation levels were gradually being reduced as the fruit ripening process advanced. Immature tomato fruits treated with 5-azacitidine, an inhibitor of cytosine DNA METHYLTRANSFERASE (MET) activity, caused a whole-genome hypomethylation affecting the expression of specific ripening-related genes, such as key TFs, genes involved in cell wall remodeling, and ethylene and carotenoid production that triggered premature ripening [65,68]. The homeostasis of DNA methylation is not only modulated by methylation, but also demethylation [69,70]. In tomato, among the four *DNA DEMETHYLASE* (*DML*) genes, *DML2* is sharply induced at the onset of ripening [25,69]. Consistently, the downregulation of *DML2* affects the methylation status of key ripening genes resulting in delayed fruit ripening [25,69]. In wild-type fruits, the promoter of *CNR* is demethylated during ripening. In *Cnr* mutant fruits, however, the promoter region remains methylated blocking the transcription of *CNR* causing the classic nonripening fruit phenotype of *Cnr* mutant. The CHROMOMETHYLASE3 (CMT3) contributes to maintain high methylation levels on the *Cnr* mutant promoter. Knocking down the expression of *CMT3* allows *Cnr* mutant fruits to ripen [71]. In addition to DNA methylation levels, RNA methylation has been identified as an important regulator of transcript stability. During tomato fruit ripening, mRNA N6-methyladenosine (m6A) methylation displays similar

Figure 4



Progression of epigenetic modifications during fruit ripening. DNA methylation experiences a global decrease. DNA demethylation is induced with the expression of *DML2*, a DNA demethylase, drastically induced at the onset of ripening. Genome-wide DNA hypomethylation promotes ripening. Before ripening, histone trimethylation H3K27me3, a repressive epigenetic modification, marks ripening-related genes suppressing their expression. As the fruit ripens, the H3K27me3 marks are removed from these genes, linking the loss of H3K27me3 with the upregulation of these genes and ripening stimulation.

patterns as the DNA methylation. In tomato, ALKBH2, a m6A RNA demethylase, controls the ripening-associated modifications of transcript methylation. ALKBH2 binds and regulates the stability of *DML2* mRNAs [72]. This mechanism establishes a dynamic relationship between mRNA and DNA methylation levels that ultimately promotes ripening [73].

Epigenetic modifications experienced by histones have relevant effects on ripening. The trimethylation of histone H3 at Lys27 (H3K27me3), a repressive epigenetic mark, plays a key role in different developmental processes [74]. fruitENCODE data have revealed an association between the loss of the H3K27me3 mark in particular genes and the progress of ripening. This association is conserved among the 147 histone modification profiles analyzed [6]. During vegetative growth and System 1 of ET production, H3K27me3 was detected on ripening-related TFs and genes involved in ET production repressing their expression and thus blocking ripening (Figure 4). As the fruit ripens, the H3K27me3 marks are progressively lost, suggesting that the release of key ripening genes from these repressing epigenetic marks could be required to promote ripening [6,75]. A wide range of epigenetic modifications is involved in the expression regulation of ripening-related genes [73].

Concluding remarks

Large-data approaches examining fruit transcriptomes, proteomes, and metabolomes together with the characterization of specific molecular mechanisms have undoubtedly improved our perception of the ripening process. However, we are only starting to understand the global regulatory network including hormonal interactions that coordinate transcriptional and epigenetic factors that ultimately modulate gene expression. Ripening is composed of discrete processes that take place in different tissues with a highly refine synchronization that is determined by the fruit ripening stage.

Future efforts on the implementation of new methodologies to carefully dissect the ripening process will certainly provide innovative outcomes. The utilization of single-cell RNA-seq combined with the available tissue-specific transcriptomic data can provide high-resolution information throughout the ripening process of individual cells' transcriptomes that can unveil novel cell/tissue-specific regulatory networks. This high-resolution transcriptomic data can aid in defining the primary tissues and genes responsible to trigger the transition to the fruit ripening phase. Technologies for studying TFs are on the rise. To understand the robust and complex TF ripening regulatory network, an increase on TF ChiP-Seq assays performed in ripening fruit tissues is required to better define TF binding motifs, generate a comprehensive TF target-genes database, and identify connections between downstream genes being multitargeted by redundant TF groups. Similarly, optimization of the ribosome footprinting technology in ripening fruits complemented with proteomics data can uncover a totally unexplored layer of gene expression regulation at the translational level. The development of new molecular tools to visualize in real-time sites of hormone synthesis and response is key to establish with high resolution the spatiotemporal distribution of hormones that regulate particular aspects of the ripening process. The ease of genome editing provided by CRISPR-Cas tools is revolutionizing the use of reverse-genetic approaches in tomato fruit ripening. Cutting-edge metabolomics techniques can evaluate the subcellular distribution of metabolites and clearly determine the metabolic shifts that take place during ripening. Further research is needed to ascertain the factors regulating the production and accumulation of flavor-related secondary metabolites throughout the ripening process. Additional work on other climacteric fruit species is required to test the conservation of the regulatory mechanisms reported in this review and mostly characterized in tomato.

The identification of additional 'regulatory pieces' in the 'fruit ripening puzzle' is required to build a holistic view of the process and advance toward the objective of establishing straightforward breeding programs to obtain elite varieties that boast not only improved traits such as flavor and quality but also acquired features to cope with the climate change conditions our planet is facing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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* * of outstanding interest

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