Ethylene Control of Fruit Ripening: Revisiting the Complex Network of Transcriptional Regulation[1]

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The plant hormone ethylene plays a key role in climacteric fruit ripening. Studies on components of ethylene signaling have revealed a linear transduction pathway leading to the activation of ethylene response factors. However, the means by which ethylene selects the ripening-related genes and interacts with other signaling pathways to regulate the ripening process are still to be elucidated. Using tomato (*Solanum lycopersicum*) as a reference species, the present review aims to revisit the mechanisms by which ethylene regulates fruit ripening by taking advantage of new tools available to perform in silico studies at the genome-wide scale, leading to a global view on the expression pattern of ethylene biosynthesis and response genes throughout ripening. Overall, it provides new insights on the transcriptional network by which this hormone coordinates the ripening process and emphasizes the interplay between ethylene and ripening-associated developmental factors and the link between epigenetic regulation and ethylene during fruit ripening.

As a developmental process, fruit ripening is coordinated by a complex network of endogenous and exogenous cues. Indeed, the making of a fruit is a genetically regulated process unique to plants involving three distinct stages: fruit set, development, and ripening. Fruit development is characterized by a series of developmental transitions tightly coordinated by a network of interacting genes and signaling pathways. Among these, ripening has received the greatest attention from both geneticists and breeders. From the scientific point of view, fruit ripening is seen as a process in which the biochemistry and physiology of the organ are developmentally altered to influence the appearance, texture, flavor, and aroma (Giovannoni, 2004). Since most of the fruit sensory and nutritional quality traits are elaborated at the ripening stage, deciphering the key genetic and molecular factors regulating ripening becomes a major task toward improving overall fruit quality (Carrari and Fernie, 2006). In addition, the control of fruit ripening is also instrumental to maintain the quality attributes of the fruit during the postharvest shelf life.

Based on their mode of ripening, fleshy fruits are divided into two categories, climacteric and nonclimacteric, depending on the presence or absence of the climacteric rise in respiration and of autocatalytic ethylene production (Lelièvre et al., 1997). In climacteric fruit, the plant hormone ethylene is the major cue that controls most aspects of ripening. By contrast, the ripening of nonclimacteric fruit does not strictly depend on ethylene, and the nature of the triggers of ripening in this type of fruit remains yet to be elucidated. Since the upstream components of the ethylene transduction pathway are common to all ethylene responses, the apparent simplicity of the ethylene signaling pathway cannot account for the wide diversity of ethylene responses. A plausible hypothesis is that differential responses to ethylene are directed at the level of ethylene response factor (ERF) transcription factors, which are encoded by one of the largest families of plant transcription factors, and therefore, are most suited to conferring such a large diversity and specificity of ethylene responses.

A rich literature indicates that the alteration of most components of ethylene signaling and responses has an impact on the course of maturation (Grierson, 2013). Nevertheless, the understanding of the control mechanisms underlying the specificity of ethylene action requires the uncovering of the components mediating ethylene responses that are specific to each developmental process. For instance, the identification of ripening-associated transcriptional regulators acting upstream or in concert with ethylene has brought new insights into understanding the ripening control mechanisms. Functional characterization of key ripening-related

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transcriptional regulators, such as *RIPENING-INHIBITOR* (RIN; Vrebalov et al., 2002; Ito et al., 2008), *COLORLESS NONRIPENING* (CNR; Manning et al., 2006), *NON-RIPENING* (NOR; Giovannoni, 2004), *TOMATO AGAMOUS-LIKE1* (TAGL1; Itkin et al., 2009; Vrebalov et al., 2009; Giménez et al., 2010), *Homeodomain-leucine zipper homeobox protein* (LeHB-1; Lin et al., 2008c), *MADS-boxS*1 (MADS1; Dong et al., 2013), *APETALA2a* (AP2a; Karlova et al., 2011), SlERF6 (Lee et al., 2012), and SlERF.B3 (Liu et al., 2014), indicates that transcription factors play key roles in relaying ripening-inducing signals and controlling ethylene biosynthesis and signaling. Taking advantage of the newly generated tools and resources on the tomato species, the present review aims to revisit the role of ethylene in fruit ripening by integrating the latest advances on the transcriptional network by which this hormone orchestrates the ripening process. Because most of our knowledge on the role of ethylene in fleshy fruit ripening has been achieved using tomato (*Solanum lycopersicum*), we will mainly focus on this reference species. In addition, several publicly accessible databases, such as the Tomato Expression Database (Fei et al., 2006) and the TomExpress online tool (http://gbf.toulouse.inra.fr/tomexpress), are used to explore the expression of relevant ripening-related genes.

ETHYLENE BIOSYNTHESIS AND PERCEPTION IN TOMATO FRUIT RIPENING

The involvement of ethylene in fruit ripening was initially reported a long time ago (Burg and Burg, 1962), and since then, direct evidences have accumulated to demonstrate that ethylene mediates fruit ripening at the physiological, biochemical, and molecular levels. Altering ethylene at the level of its biosynthesis, perception, signal transduction, or gene transcription was shown to impact fruit ripening (Hamilton et al., 1990; Oeller et al., 1991; Lanahan et al., 1994; Tieman et al., 2001; Lee et al., 2012; Liu et al., 2014). According to the currently accepted model (Fig. 1), ethylene signaling relies on a linear transduction pathway where the hormone is perceived by a specific receptor, which initiates a signaling cascade by releasing the block exerted by CTR1 on EIN2. This activates a transcriptional cascade, involving EIN3/EIL1 as the primary transcription factor and then ERFs, which in turn regulate genes underlying ripening-related traits, such as color, firmness, aroma, taste, and postharvest shelf life (Solano and Ecker, 1998; Ju et al., 2012; Chang et al., 2013).

Ethylene Biosynthesis Is Instrumental to Climacteric Fruit Ripening

In higher plants, ethylene biosynthesis (Fig. 1) originates from *S*-adenosyl-Met and comprises two steps catalyzed by ACS and ACO, the latter converting ACC into ethylene (Yang and Hoffman, 1984). The genome-wide search for ACS and ACO genes performed using tBLASTn as the program and SlACS1A and SlACO1 as

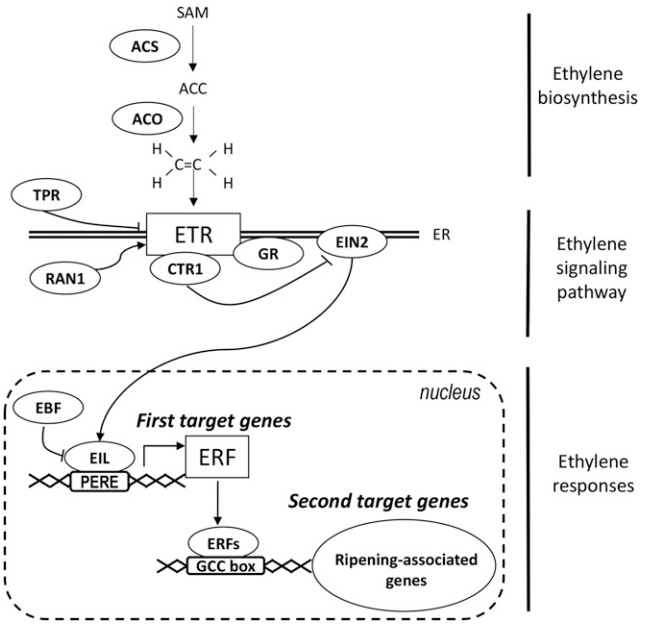

Figure 1. Simplified scheme showing ethylene synthesis and response in tomato. Ethylene synthesis results from the activity of 1-amino-cyclopropane-1-carboxylic acid (ACC) synthase (ACS) and 1-amino-cyclopropane-1-carboxylic acid oxidase (ACO), which transform *S*-adenosyl-L-Met (SAM) into ACC and convert ACC into ethylene, respectively. Ethylene is perceived by the receptor proteins (ETR), located in the endoplasmic reticulum (ER). RAN1 delivers the copper cofactor required for ethylene binding. GR is probably associated with the receptor and mediates the receptor signal output. It is suggested that TPR binds to ethylene receptors and leads to receptor degradation. The receptors are negative regulators of ethylene signaling, and in the absence of ethylene, the receptors activate Constitutive Triple-Response1 (CTR1), which suppresses the ethylene response via inactivation of Ethylene Insensitive2 (EIN2). The transcription factors EIN3/Ethylene Insensitive3-Like1 (EIL1) undergo a degradation process mediated by the Ethylene Insensitive3-binding F-box (EBF) proteins. In the absence of EIL, transcription of ethylene response genes is shut off. Ethylene binding to the receptors induces their inactivation, and by consequence, switches off CTR1 phosphorylation activity. Active EIN2 stabilizes EIL transcription factors, which can activate the expression of target genes, including those encoding the *ERF* transcription factors via binding to primary ethylene response elements (PEREs; Solano et al., 1998). ERFs, in turn, modulate the transcription of ethylene-regulated genes through binding to GCC-box type cis-elements present in their target promoters. Arrowheads represent positive regulatory interactions, and bar heads represent negative regulation.

query identified 14 sequences corresponding to putative ACS and 6 to ACO in the most recent tomato genome sequence (Tomato Genome Consortium, 2012). For the ACS and ACO described here, InterProScan analysis confirmed the presence of specific domains characteristic of these proteins, and the Kyoto Encyclopedia of Genes and Genomes orthology analysis validated the presence of the enzymatic domains, EC:4.4.1.14 and EC:1.14.17.4, characteristic of ACS and ACO, respectively. Even though the ACS proteins have not been biochemically characterized, phylogenetic analysis clustered the 14 putative ACS proteins in the same branch as Arabidopsis (*Arabidopsis thaliana*) ACSs

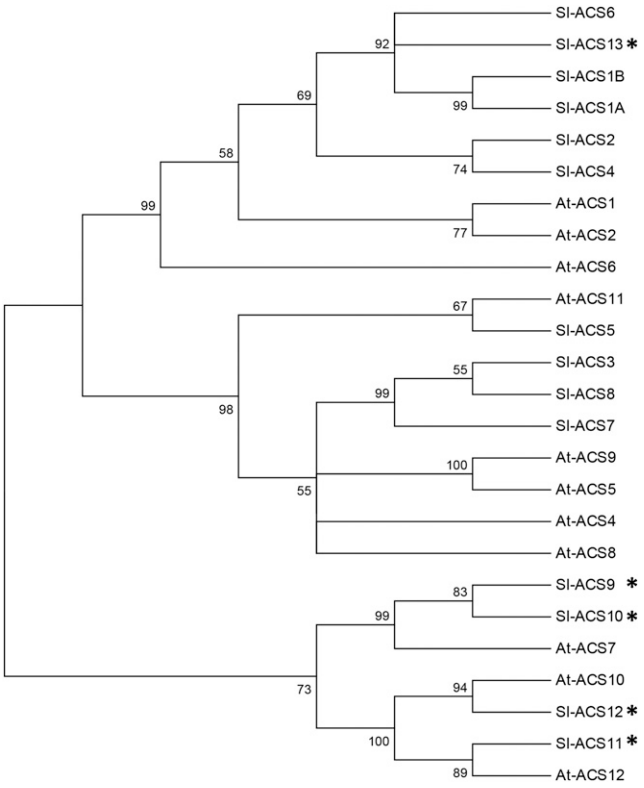

Figure 2. Phylogenetic tree of tomato and Arabidopsis ACS. The phylogenetic tree was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 3.82205137 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are the number of amino acid substitutions per site. The analysis involved 25 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 138 positions in the final data set. Phylogenetic trees were conducted in MEGA7. *, New tomato ACS genes identified in the current study.

(Fig. 2). Moreover, the putative tomato ACS genes were further checked for the presence of the pyridoxal 5′-phosphate-binding site, a necessary feature of ACS enzymes (Jakubowicz, 2002), showing that this domain is well conserved in the five new ACS genes identified (ACS9–13), allowing them to be assigned to the ACS group. All genes related to ethylene biosynthesis, perception, or signaling are listed in Supplemental Table S1, providing the correspondence between gene names, Solyc numbers, and, when relevant, other names cited in the literature. The implementation of the newly available TomExpress pipeline (http://gbf.toulouse.inra.fr/tomexpress) allowed in silico mining of the expression pattern of all *ACS* genes in the tomato based on the publicly available RNA-seq data sets (Fig. 3A). While confirming that *ACS2* and *ACS4* are the main family members expressed during ripening (Oeller et al., 1991; Theologis et al., 1992), the new expression study also confirmed that *ACS1A* transcript accumulation peaks at the breaker stage (Barry et al., 2000), suggesting its potential contribution to the climacteric ethylene production, although its expression level is quantitatively lower than *ACS2* and *ACS4*. Moreover, among the new *ACS* genes, *ACS11* and *12* also display a significant up-regulation during fruit ripening, whereas *ACS1B, 5, 7, 8, 9, 10,* and *13* transcripts are almost undetectable in tomato fruit (Fig. 3A).

Genome-wide analysis confirmed the presence of six *ACO* genes in the tomato genome (Seymour et al., 2013), and mining their expression with TomExpress pipeline (Fig. 3B) indicated that *ACO1* and *ACO2* (Supplemental Table S1) display the most striking ripening-regulated pattern of expression peaking at the breaker stage, whereas *ACO4* expression undergoes a steady but slight increase throughout ripening (Barry et al., 1996; Nakatsuka et al., 1998; Van de Poel et al., 2012). The present expression analysis confirms previous studies pointing to *ACO1* and *ACO4* as the main ACO genes supporting ripening-associated ethylene production (Nakatsuka et al., 1998). The transcript level of *ACO3, ACO5,* and *ACO6* remains very low, suggesting that their contribution to climacteric ethylene production is negligible. Two systems of ethylene biosynthesis have been proposed in climacteric fruits (McMurchie et al., 1972). System 1 is responsible for producing basal ethylene levels that are detected in all tissues, including those of nonclimacteric fruit (Fig. 4). System 1 is known to be ethylene autoinhibitory and is reported to function during fruit growth, whereas system 2 operates during the climacteric ripening and is autocatalytic (Fig. 4). System 1 relies on *ACS1A* and *ACS6,* both being negatively regulated by ethylene, whereas the up-regulation of *ACS2* and *ACS4* through a positive feedback by ethylene is responsible for the activation of system 2 (Nakatsuka et al., 1998; Barry et al., 2000). *ACO1* and *ACO4* are both expressed at low levels in immature green fruit where system 1 is operating, but their transcripts accumulate with the climacteric rise of ethylene production and are therefore responsible for the transition to system 2 (Fig. 4). Moreover, *ACO4* maintains a sustained expression during fruit ripening (Nakatsuka et al., 1998). The expression of ethylene biosynthesis genes was shown to be regulated by developmental regulators, such as RIN and LeHB-1, which modulate the expression of *ACS2* and *ACO1* through direct binding to their promoter (Lin et al., 2008c; Fujisawa et al., 2013). It is therefore possible that system 2 ethylene production is not the only mechanism contributing to the autocatalytic regulation of climacteric ethylene. Recent data showed that ethylene biosynthesis displays a tissue-specific and developmental differentiation throughout tomato fruit growth, indicating that it is organized and regulated in a well-defined tissue-specific way (Van de Poel et al., 2014).

Altered Ethylene Perception Impairs Fruit Ripening

The ethylene receptors have been studied in detail in tomato, where six genes have been initially described,

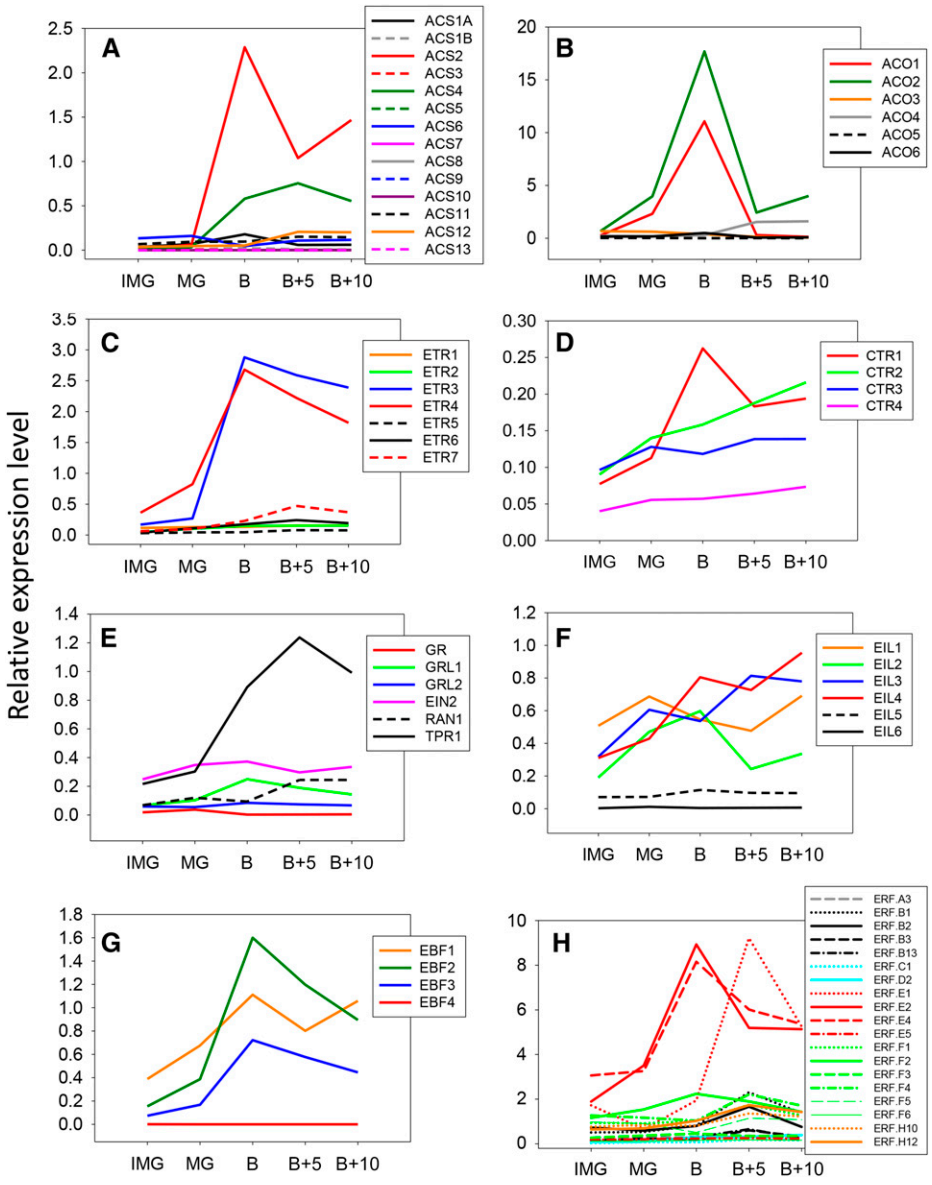

Figure 3. Expression data of ethylene biosynthesis and signaling genes during fruit ripening extracted from public databases and processed using the TomExpress platform. Five fruit developmental stages have been studied: immature green (IMG), mature green (MG), breaker (B), 5 d after breaker (B+5), and 10 d after breaker (B+10). Expression patterns for the following gene families are presented: ACS (A), ACO (B), ETR (C), CTR (D), ETR partners (E), EIL (F), EBF (G), and ERF (H). For each gene, the plot represents normalized counts per base for RNA-seq data released from transcriptome analyses in multiple tomato cultivars.

named LeETR1 to LeETR6 (Wilkinson et al., 1995; Lashbrook et al., 1998; Tieman and Klee, 1999; Klee and Tieman, 2002; Gapper et al., 2013). The genome-wide search identified LeETR7 as a new member of the tomato ethylene receptor family. Phylogenetic analysis validated its similarity with other receptors, and its expression pattern was established using the TomExpress pipeline (Fig. 3C; Supplemental Figs. S1 and S2). Like in other plant species, two subfamilies of ethylene receptors are present in tomato. LeETR1, LeETR2, and LeETR3 (also named NR for never ripe) belong to subfamily I, and harbor three transmembrane domains (Supplemental Fig. S1) and His kinase and Histidine kinase-like ATPase (HATPase_c) domains predicted by the SMART online tool (http://smart.embl-heidelberg.de/). LeETR1 and LeETR2, but not LeETR3, have a receiver domain at the C-terminal position containing a phosphoacceptor described as important in eukaryotic two-component systems (Schaller et al., 2011). Subfamily II gathers four receptors, LeETR4 to LeETR7, containing four transmembrane domains as confirmed by the TMpred online tool (http://www.ch.embnet.org/software/TMPRED_form.html). In the *Nr* mutant, a point mutation, leading to a substitution of Pro to Leu in the N-terminal ethylene binding pocket, results in impaired fruit ripening (Lanahan et al., 1994; Wilkinson et al., 1995). While confirming *LeETR3* and *LeETR4* as the main receptor genes expressed at the inception of tomato fruit ripening (Kevany et al., 2007; Klee and Giovannoni, 2011), the TomExpress tool revealed that *LeETR7* also displays a ripening-regulated expression, being the third most highly abundant receptor transcript during ripening (Fig. 3C).

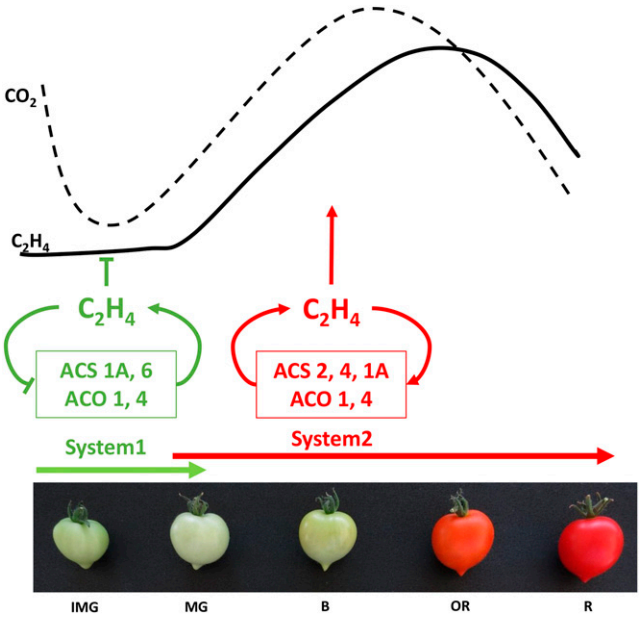

Figure 4. Two different systems of ethylene production operate during fruit development and ripening. At immature stages, ethylene biosynthesis is mediated by system 1, whereas system 2 takes over during ripening and is characterized by autocatalytic ethylene production. The main genes involved in system 1 are *ACS6* and *ACS1A,* both genes being down-regulated by ethylene. From mature green stage onward, system 2 ethylene production is driven mainly by *ACS2* and *ACS4,* the expression of which is stimulated by ethylene. *ACS1A* transcripts show a transient increase at the onset of ripening, suggesting that this gene may be important in regulating the transition from system 1 to system 2. *ACO1* and *ACO4* transcript levels are low in immature green stages, but undergo sharp increase at the climacteric peak when system 2 ethylene production is operating. IMG, Immature green; MG, mature green; B, breaker; OR, orange; R, red. Arrowheads represent positive regulatory interactions, and bar heads represent negative regulation.

It has been reported that receptor proteins are regulated by GREEN-RIPE (GR), a small protein made of around 240 amino acids, and the *Gr* mutant was described to display impaired fruit ripening (Barry and Giovannoni, 2006). A genome-wide search identified three *GR* genes in the tomato (*GR, Green-Ripe Like1* [*GRL1*], and *GRL2*). *GRL1* is the closest ortholog of the Arabidopsis *REVERSION TO ENTHYLENE SENSITIVITY1* (*RTE1*) gene (Resnick et al., 2006). The *rte1* mutants were able to restore ethylene sensitivity in the *etr1-2* mutant, suggesting that RTE1 and GR homologs may act at the receptor levels (Resnick et al., 2006). *GRL1* is the most expressed homolog during fruit development, and its transcript levels peak at the breaker stage (Fig. 3E). This is consistent with the work by Ma et al. (2012) who were the first to report the expression of GR/GRL1/GRL2 in fruit development, showing that GRL1 displays ripening-related expression. It was also suggested that GR and GRL1 may confer a subfunctionalization of the receptors by mediating different responses to ethylene (Ma et al., 2012). Nevertheless, overexpression of *GRL1* or *GRL2* does not seem to impact fruit ripening (Klee and Giovannoni, 2011).

Two other proteins, Response to Antagonist1 (RAN1) and tetratricopeptide repeat1 (TRP1), play important roles at the receptor levels. SlRAN1 is the ortholog to the Arabidopsis AtRAN1 that delivers the copper ion, essential for ethylene binding activity (Binder et al., 2010). *SlRAN1* shows continuous low expression levels with a slight rise at late ripening stages (Fig. 3E). SlTPR1, known to bind the ethylene receptors, has been suggested to lead to receptor degradation (Lin et al., 2008b). Interestingly, *SlTPR1* expression is high in the late ripening stages (Fig. 3E) when LeETR3 and LeETR4, its potential targets, are also highly expressed (Fig. 3C).

CTR1 and Fruit Ripening

The Mitogene-activated protein kinase kinase kinase, known as CTR1, acts directly downstream of the ethylene receptors. The *ctr1* loss-of-function mutations result in the constitutive activation of ethylene response in seedlings and adult plants, indicating that the encoded protein acts as a negative regulator of ethylene signaling (Lin et al., 2008a; Klee and Giovannoni, 2011). So far, four CTR1 homologs (*SlCTR1*, *SlCTR2*, *SlCTR3*, and *SlCTR4*) have been identified in the tomato, three of which can completely (*SlCTR3*) or partially (*SlCTR1* and *SlCTR4*) complement the Arabidopsis *ctr1-8* mutation (Leclercq et al., 2002; Adams-Phillips et al., 2004; Lin et al., 2008a), suggesting a conserved function for tomato CTR proteins. All tomato CTRs display ability to interact with one or more ethylene receptors in yeast two-hybrid systems (Zhong et al., 2008). Tomato and *CTR1, 2, 3*, and 4 show differential expressions in various plant tissues (Adams-Phillips et al., 2004; Lin et al., 2008a), and the ethylene-responsive *CTR1* (Zegzouti et al., 1999; Leclercq et al., 2002) displays a ripening-related expression pattern. Our present study indicates that *SlCTR1* displays a typical ripening-regulated expression, whereas *SlCTR2* shows a steady increase in its expression during ripening (Fig. 3D) and was up-regulated in ripening-impaired mutants *Nr* and *rin* (Lin et al., 2008a), suggesting its putative role in the ripening process. The suppression of *SlCTR1* via Virus-induced gene silencing (VIGS) strategy was reported to promote tomato fruit ripening, consistent with CTR being a negative regulator of climacteric ripening (Fu et al., 2005).

EIN2, Another Component of Ethylene Signaling Influencing Ripening

In Arabidopsis, EIN2 is required for all ethylene responses, and based on genetic analyses, EIN2 acts downstream of the receptor/CTR1 complex to positively regulate ethylene responses. It constitutes a critical step in the signal transduction pathway and acts between CTR1 and the EIN3/EIL transcription factors (Alonso et al., 1999; Guo and Ecker, 2003). Although the expression of *EIN2* in tomato is ethylene independent

and does not exhibit substantial changes during fruit growth and ripening (Fig. 3E), its down-regulation by a cosuppression mechanism or via VIGS strategy resulted in ethylene insensitivity and ripening inhibition associated with reduced expression of ethylene- and ripening-related genes (Fu et al., 2005; Hu et al., 2010), suggesting that *LeEIN2* is a positive regulator of ethylene-mediated responses during fruit ripening.

Posttranslational Regulation of Ethylene Perception Proteins and Fruit Ripening

Ethylene receptors are negative regulators of ethylene signaling, and it is therefore rather intriguing that the corresponding genes undergo dramatic up-regulation during fruit ripening (Fig. 3C). Pioneering studies addressing the evolution of ethylene receptor proteins during tomato fruit ripening showed that the levels of receptor transcripts are not correlated with the amount of receptor proteins, thus suggesting that the posttranslational regulation of ethylene perception is an essential mechanism (Kevany et al., 2007). Indeed, exogenous ethylene treatment of immature fruits results in enhanced accumulation of *ETR* transcripts concomitant with a decrease in the corresponding encoded proteins, and the use of the MG132, an inhibitor of proteasome, suggested that ETR protein degradation was mediated by the proteasome (Kevany et al., 2007). Moreover, these authors developed the hypothesis of a relationship between the phosphorylation status of the receptor proteins and their degradation. In support of this hypothesis, it was reported that the amount of LeETR3 and LeETR4 receptor proteins increases at the onset of ripening, and that the phosphorylation level of some N-terminal residues plays a critical role in switching on or off the downstream ethylene signal transduction (Kamiyoshihara et al., 2012). The phosphorylation status of LeETR4 was shown to decrease over the transition from immature green to breaker stage, and exogenous ethylene induces dephosphorylation of the receptor protein. Taken together, these studies (Kevany et al., 2007; Kamiyoshihara et al., 2012) suggest that, during fruit ripening, ethylene signaling is modulated at the level of the receptor proteins either quantitatively by tuning their amount or by adjusting their phosphorylation status.

TRANSCRIPTIONAL CASCADE LEADING TO THE ACTIVATION OF ETHYLENE-RESPONSIVE GENES

EIL Proteins in Fruit Ripening

Ethylene regulates ripening-related genes through a transcriptional cascade that comprises primary (EIL) and secondary response factors (ERFs). Four tomato EIL genes (*SlEIL1, SlEIL2, SlEIL3*, and *SlEIL4*) were initially described (Tieman et al., 2001; Yokotani et al., 2003), and mining the most updated tomato genome sequence identified two additional genes (named here as *SlEIL5* and *SlEIL6*) based on the presence of the typical domains characteristic of Arabidopsis EIL proteins, including the acidic and basic domains as well as the Pro-rich domain. Tomato *SlEIL1, SlEIL2, SlEIL3,* and *SlEIL4* genes exhibit a ripening-associated pattern of expression, with *SlEIL1* and *SlEIL2* transcripts accumulating at the onset of ripening and declining at later stages, whereas those corresponding to *SlEIL3* and *SlEIL4* show a steady increase throughout ripening (Fig. 3F). Notably, the expression of *SlEIL5* and *SlEIL6* is not regulated during fruit ripening, which may suggest distinct roles among EILs. Down-regulation of *SlEIL* genes in transgenic tomato plants altered fruit ripening (Tieman et al., 2001), and overexpression of *SlEIL1* in the tomato *Nr* mutant partially restored normal fruit ripening and stimulated the expression of some ethylene-responsive genes, supporting the role of EILs in ethylene-mediated fruit ripening (Chen et al., 2004). Moreover, down-regulation of *SlEIL* genes resulted in limited increase in *SlACS2* and *SlACS4* expression (Yokotani et al., 2009), suggesting that EILs might be essential for the activation of genes involved in autocatalytic ethylene production. A new phosphorylation region, named EIN3/EIL phosphorylation region1, has been shown to be essential for the transcriptional activity of tomato *SlEIL1* and dimerization of SlEIL1 proteins (Li et al., 2012). In Arabidopsis, EIL proteins are known to be regulated by EBFs at the posttranslational level (Guo and Ecker, 2003). Two tomato homologs of these F-box proteins, EBF1 and EBF2, have been shown to regulate ethylene signaling and fruit ripening through mediating the degradation of EIN3/EIL proteins (Yang et al., 2010). Mining the annotated tomato genome sequence identified two new EBF proteins (SlEBF3 and SlEBF4) based on the presence of conserved F-box domains and Leu-rich repeats. *SlEBF1, SlEBF2,* and *SlEBF3* exhibit a typical ripening-associated expression pattern with a peak of transcript accumulation at the onset of ripening (Fig. 3G), suggesting that EBFs may actively contribute to the control of ripening-associated ethylene signaling.

ERFs and the Regulation of Fruit Ripening

The ethylene signaling cascade ends with transcriptional activation of the transcription factors termed ERFs. ERFs belong to the AP2/ERF superfamily shown to regulate the expression of ethylene-responsive genes through direct binding to their promoter regions (Ohme-Takagi and Shinshi, 1995; Pirrello et al., 2012). ERFs represent one of the largest plant multigene families of transcription factors, which makes these components suited to channel the ethylene signaling toward specific responses through recruiting the appropriate ethylene-responsive genes. Taking advantage of the recently released annotated tomato genome sequence (Tomato Genome Consortium, 2012), 146 genes were postulated to encode proteins containing the AP2/ERF domain, of which 77 belong to the ERF

subfamily (Pirrello et al., 2012). Although our knowledge of the specific functions assigned to tomato ERFs is still scarce, in recent years, an increasing number of studies showed that ERF proteins play an important role in fruit ripening. Most of the tomato *ERF* genes identified so far are ethylene inducible and show ripening-related expression (Pirrello et al., 2012; Liu et al., 2014). Comprehensive expression analysis using the TomExpress online tool revealed that 55 out of 77 ERF family genes exhibit a ripening-associated pattern of expression, with 27 being up-regulated during ripening, whereas the remaining 28 are down-regulated, which suggests that different ERFs may have contrasting roles during fruit ripening (M. Liu, B. Lima Gomes, E. Purgatto, L.E.P. Peres, E. Maza, M. Zouine, J.P. Roustan, M. Bouzayen, and J. Pirrello, unpublished data). *SlERF.E1*, *SlERF.E2*, and *SlERF.E4* exhibit the highest level of expression during ripening (Fig. 3H) and show dramatic down-regulation in *rin*, *nor*, and *Nr* tomato ripening mutants (M. Liu, B. Lima Gomes, E. Purgatto, L.E.P. Peres, E. Maza, M. Zouine, J.P. Roustan, M. Bouzayen, and J. Pirrello, unpublished data), suggesting that members of subclass E may have the most prominent role in regulating the ripening process. Interestingly, these three ERFs are among the 23 ERFs identified by chromatin immunoprecipitation on chip (ChIP-chip) and chromatin immunoprecipitation coupled to sequencing (ChIP-seq) approaches as potential direct targets of the RIN key ripening regulator (Fujisawa et al., 2013; Zhong et al., 2013). Altogether, these data are consistent with the assumption that *ERF* genes are important components of ethylene- and RIN/NOR-dependent ripening and suggest that ERFs may represent the link between ethylene signaling and developmental regulation of fruit ripening. Further supporting the active role of ERFs in fruit ripening, overexpressing *SlERF.H1* (Supplemental Table S1) resulted in constitutive ethylene response and accelerated tomato fruit ripening (Li et al., 2007). A ripening-related pattern of expression has also been shown for *SlERF.E1 (LeERF2)* and *SlERF.A3* in tomato fruit (Tournier et al., 2003; Chen et al., 2008; Supplemental Table S1). Moreover, a systems biology approach identified SlERF.E4 as a negative regulator of ethylene and carotenoid biosynthesis in fruit ripening (Lee et al., 2012). More recently, the use of a dominant repression strategy revealed that SlERF.B3 is involved in the control of fruit ripening by regulation of climacteric ethylene production and carotenoid accumulation (Liu et al., 2013, 2014). In other climacteric fruits, such as apple (*Malus domestica*), banana (*Musa* spp.), plum (*Prunus salicina*), and papaya (*Carica papaya*), although direct evidence showing the involvement of ERF family genes in fruit ripening is lacking, some ERFs were reported to exhibit a ripening-associated expression pattern (Wang et al., 2007; El-Sharkawy et al., 2009; Li et al., 2013). In concert with the master regulator RIN, ERFs regulate autocatalytic ethylene biosynthesis in climacteric fruit ripening, and can directly modulate the expression of ripening-related genes involved in various metabolic pathways activated during fruit ripening.

TRANSCRIPTION FACTORS REGULATING FRUIT RIPENING IN CONCERT WITH ETHYLENE

It is widely accepted that climacteric fruit ripening involves a complex interplay between ethylene and ripening-associated developmental regulators (Fig. 5). Indeed, the cloning of genes responsible for impaired-ripening mutations in the tomato, including *RIN*, *NOR*, and *CNR*, represents a major breakthrough in deciphering the transcriptional control underlying fruit ripening. Fruits produced by *rin*, *nor*, and *Cnr* mutants exhibit inhibited ripening that cannot be rescued by exogenous ethylene treatment (Klee and Giovannoni, 2011; Karlova et al., 2014). The *RIN* gene encodes a

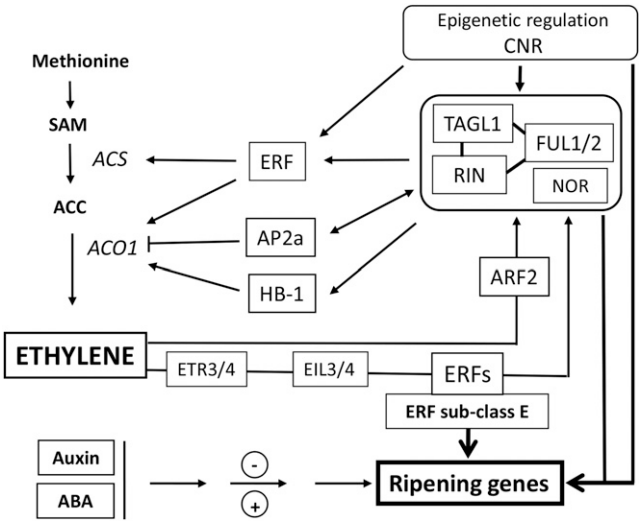

Figure 5. Schematic overview of the multifactor regulatory network involved in ethylene biosynthesis and signaling during fruit development and ripening. RIN, TAGL1, and FUL1/2 are linked since they probably function as complexes of varying composition. The ripening master regulator NOR is placed in the same box. Along with CNR, these factors are master regulators of climacteric ripening. CNR affects the expression of RIN, LeHB1, SlAP2a, and SlTAGL1. FUL1 and FUL2 can potentially regulate ethylene biosynthesis, perception, and signaling genes. RIN promotes ripening via direct regulation of some transcription factors, such as ERFs. *SlERF.B1* and *SlERF.E1* are hypermethylated in *cnr* and *rin* mutants. ERFs regulate ethylene production in tomato by interaction with the promoters of *ACO*. Another transcription factor, *LeHB-1*, can bind in vivo to the promoter of *ACO*. The putative transcription factor *Sl-AP2a* was described as a negative regulator of fruit ripening and ethylene production. In addition, the control of ethylene biosynthesis can be regulated by RIN through direct interaction with the promoters of *ACS2*, *ACS4*, and *ACO1*. The ethylene biosynthesis pathway is controlled by a feedback mechanism, where ethylene regulates the expression of RIN. Moreover, there is evidence that ARFs also contribute to this complex feedback mechanism. ERF-type transcription factors are involved in fruit ripening through the control of ethylene and carotenoid biosynthesis pathways in tomato. Other hormones, such as Auxin and abscisic acid (ABA), also play a role in tuning fruit ripening. In particular, ARF2 was reported to be an essential component of the regulatory network controlling fruit ripening in tomato. Arrowheads represent positive regulatory interactions, and bar heads represent negative regulation. SAM, *S*-adenosyl-L-Met.

MADS-box transcription factor, and molecular studies showed that RIN protein can directly bind to the promoters of *ACS2*, *ACS4,* and *ACO1* ethylene biosynthesis genes, *NR* and *ETR4* ethylene receptor genes, and *ERF* genes (Fujisawa et al., 2013; Zhong et al., 2013; M. Liu, B. Lima Gomes, E. Purgatto, L.E.P. Peres, E. Maza, M. Zouine, J.P. Roustan, M. Bouzayen, and J. Pirrello, unpublished data). These data provide convincing evidence for a link between the RIN-mediated transcriptional regulation and ethylene during fruit ripening. On the other hand, ethylene was shown to regulate the expression of *RIN*, suggesting an active interplay between RIN and ethylene signaling (Fujisawa et al., 2013). The *Cnr* mutant is due to an epigenetic change that alters the methylation of a gene encoding a putative SQUAMOSA promoter-binding (SBP) protein, which results in pleiotropic ripening inhibition and inhibited expression of ethylene-associated genes, including *ACO1*, *E8*, and *NR* (Manning et al., 2006; Osorio et al., 2011). Ethylene biosynthesis is impaired in the tomato *nor* mutant, and it was recently shown that *nor* has a more global effect on ethylene-related gene expression than *rin* (Osorio et al., 2011). LeHB-1, another transcription factor, can bind the *LeACO1* promoter, and silencing of *LeHB-1* via VIGS strategy results in down-regulation of *LeACO1* expression associated with delayed fruit ripening (Lin et al., 2008c). The *TAGL1* gene, which is highly expressed during fruit ripening, was reported to act as a positive regulator of fruit ripening, and *TAGL1* knock-down fruits produce lower amounts of ethylene with a reduced expression of *LeACS2*, suggesting that TAGL1 controls fruit ripening by regulating ethylene biosynthesis (Itkin et al., 2009; Vrebalov et al., 2009). The putative transcription factor *SlAP2a*, a member of the AP2/ERF superfamily gene, was described as a negative regulator of fruit ripening and ethylene production and signaling since its down-regulation leads to higher levels of ethylene and fast ripening (Chung et al., 2010; Karlova et al., 2011). Likewise, SlMADS1 is a negative regulator of fruit ripening, and its down-regulation via RNA interference strategy results in early ripening and increased ethylene production (Dong et al., 2013). More recently, *SlNAC1* (for tomato *NAM, ATAF1/2, CUC2*), a new tomato NAC domain protein whose expression increases in ripening fruit, was described as a negative regulator of ripening. Its overexpression resulted in altered carotenoid pathway and decreased ethylene synthesis mainly due to the reduced expression of system 2 ethylene biosynthetic genes (Ma et al., 2014). These data indicate that both positive and negative ripening regulators are involved in the control of fruit ripening, at least partially in an ethylene-dependent pathway. Interestingly, although the transcription factors Fruitfull1 (FUL1) and FUL2 were initially reported to impact fruit ripening in an ethylene-independent manner (Bemer et al., 2012), recent evidences support the involvement of FUL1/FUL2 in the regulation of ethylene biosynthesis during fruit ripening (Fujisawa et al., 2014; Shima et al., 2014; Wang et al., 2014).

EPIGENETIC REGULATION OF ETHYLENE-REGULATED FRUIT RIPENING

Deciphering the basis of the tomato *Cnr* epimutation provided the initial clue on the epigenetic control of fruit ripening by demonstrating that the impaired ripening phenotype is due to hypermethylated cytosines in the promoter of *SQUAMOSA Promoter Binding* Protein-like (*LeSPL*)-*CNR*, a gene encoding the SBP-box transcription factor (Manning et al., 2006). Subsequently, it was shown that demethylation is essential for climacteric ethylene production, and that treatment of immature fruit with an inhibitor of methyltransferases results in early ripening, indicating that DNA methylation impacts the transition from system 1 to system 2 of ethylene production (Zhong et al., 2013). Demethylation is critical to the binding of RIN protein to the promoter of ripening genes (Zhong et al., 2013), and repression of a DEMETER-like DNA demethylase in tomato results in DNA hypermethylation, ripening inhibition, and a dramatic decrease in climacteric ethylene production (Liu et al., 2015). Furthermore, the hypermethylated *cnr* mutant can be rescued by down-regulating the tomato Chromomethylase3 gene, a plant-specific CHROMOMETHYLASE (Chen et al., 2015).

Global methylation level at the 5′ end of genes gradually declines during fruit development while remaining high in the tomato ripening-deficient *Cnr* and *rin* mutants. The RIN binding sites in *ACS4* and *ACO1* genes undergo decreased methylation during tomato fruit ripening; by contrast, these sites remain hypermethylated in *cnr* and *rin* mutants (Fig. 5). Likewise, the ethylene response components, *SlERF.B1* and *SlERF.E1,* are hypermethylated in *cnr* and *rin* mutants compared with the wild type (Zhong et al., 2013). These data suggest that regulation of the ethylene pathway through RIN is strongly controlled by the methylation status of target genes.

ETHYLENE AND OTHER PHYTOHORMONES IN FRUIT RIPENING

It has long been considered that other plant hormones besides ethylene are likely required for climacteric fruit ripening (Dostal and Leopold, 1967; Frenkel and Dyck, 1973; Mizrahi et al., 1975; Fan et al., 1998). ABA is known to promote ripening, whereas auxin seems to have an antagonistic effect (Frenkel and Dyck, 1973; Mizrahi et al., 1975; Zhang et al., 2009; Su et al., 2015). The expression of *ACS2*, *ACS4*, and *ACO1* genes is induced by exogenous ABA, revealing an ABA/ethylene interplay operating at the level of ethylene biosynthesis (Chernys and Zeevaart, 2000; Jiang et al., 2000; Zhang et al., 2009). Down-regulation of the key ABA biosynthesis enzyme 9-cis-epoxycarotenoid dioxygenase1 in tomato fruit resulted in altered firmness and color but surprisingly higher ethylene production, indicating the complexity of the ABA/ethylene interplay during ripening (Sun et al., 2012). In tomato, ABA might also be perceived through an ethylene-independent

pathway that is mediated by tomato Zinc Finger Transcription Factor (Weng et al., 2015).

The expression of ethylene biosynthesis and signaling genes is regulated by auxin in tomato and other fleshy fruits, such as peach (*Prunus persica*; Gillaspy et al., 1993; Jones et al., 2002; Trainotti et al., 2007; Pirrello et al., 2012). The auxin inhibitor p-Chlorophenoxyisobutyric acid mimics ACC treatment, confirming the antagonistic action of the two hormones during fruit ripening, and auxin delays tomato ripening by affecting a set of key factors, such as RIN, ethylene, and ABA (Su et al., 2015). Consistent with the role of auxin in fruit ripening, tomato fruit firmness was shown to be partly regulated by tomato *Auxin Response Factor4* (*SlARF4*), a transcription factor known to mediate auxin responses (Jones et al., 2002; Guillon et al., 2008; Sagar et al., 2013). More recently, SlARF2, a tomato auxin response factor, was described as an essential component of the regulatory network controlling fruit ripening. Indeed, tomato fruits underexpressing *SlARF2* exhibited dramatic ripening defects associated with reduced climacteric ethylene production and dramatic down-regulation of the key ripening regulators *RIN*, *CNR*, and *NOR* (Hao et al., 2015). These data highlight the complex interplay between ethylene and other hormone-signaling components during fruit ripening. Further sustaining the idea of an interplay between ethylene and auxin during fruit ripening is the ethylene-induced expression of *PIN-FORMED1* auxin transporter and the requirement of high auxin levels to produce large amounts of system 2 ethylene in peaches (Trainotti et al., 2007; Tatsuki et al., 2013).

CONCLUSION

During the last decade, the implementation of advanced high-throughput technologies in genomics, metabolomics, and proteomics threw new light on the mechanisms by which ethylene regulates the ripening process. Although these studies confirmed ethylene as the main hormone regulating climacteric ripening, they provided evidence supporting the intervention of a complex network of interacting signaling pathways (Fig. 5). Indeed, it is now clear that hormonal and developmental factors act in concert to tune the whole set of ripening-associated pathways. The emerging idea is that fruit development and ripening are complex multilevel processes depending on the coordinated action of master regulators, including multiple hormone signaling, microRNAs, epigenetic maintenance, and epigenetic modifying genes. Future challenges will consist of unraveling the molecular mechanisms underlying the specificity of ethylene responses during plant development and fruit ripening. It is particularly important to uncover how the ethylene perception system evolves at the protein level and to address the functional significance of individual ERF genes. Deciphering the function of *ERF* genes in both ethylene-dependent and ethylene-independent processes during ripening and identifying the target genes of individual ERFs will be instrumental to better clarify their specific contribution to fruit ripening. Moreover, deciphering the ethylene receptor subfunctionalization and assigning specific roles to ERF members will open new avenues toward engineering fruit development and ripening via targeted approaches, especially when aiming to enhance some desirable traits and metabolic pathways and to reduce unwanted ones.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phylogenetic tree of tomato ETRs.

Supplemental Figure S2. Expression pattern of ETR genes during fruit ripening.

Supplemental Table S1. Correspondence between common names for the genes and their Solyc numbers.

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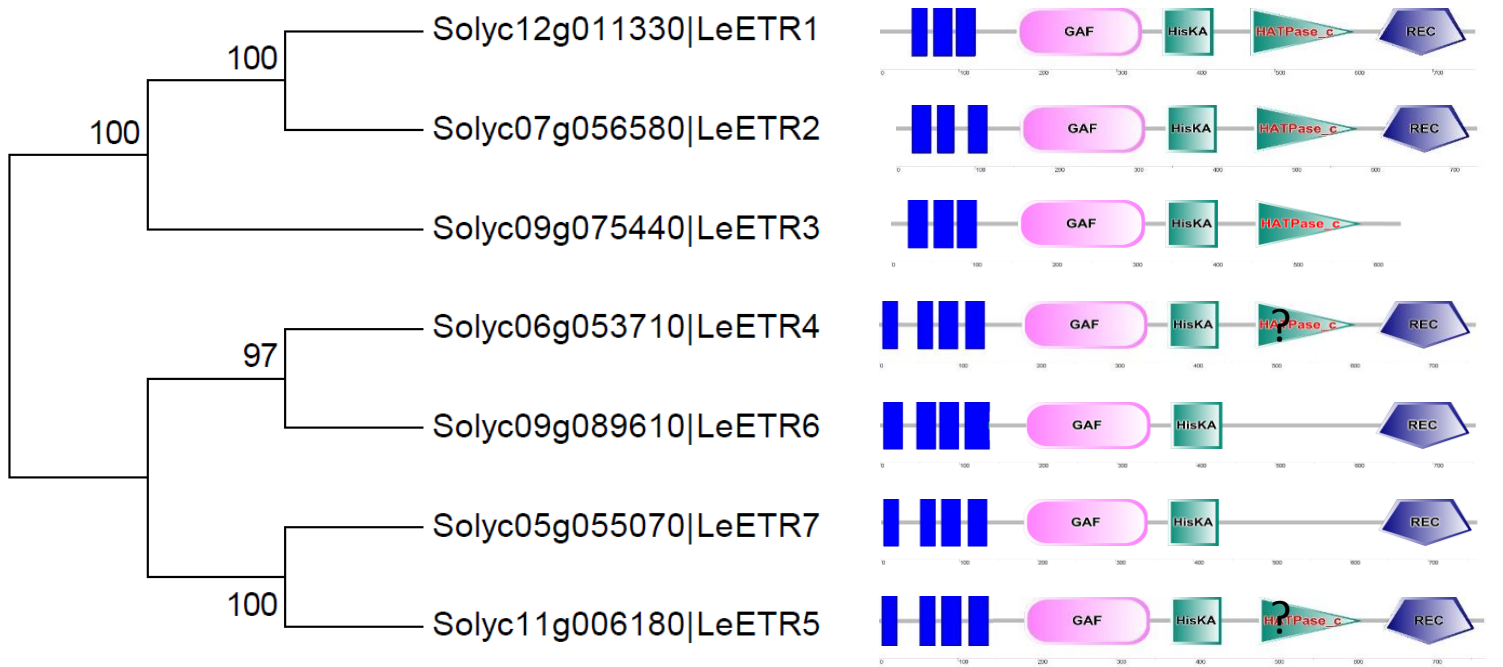

Figure S1. Phylogenetic tree of Tomato ETR**.** Phylogenetic trees were constructed with the whole protein sequences using neighbour joining method. Structural domains predicted by the SMART online tool (http://smart.embl-heidelberg.de/) were represented on for each ETR.

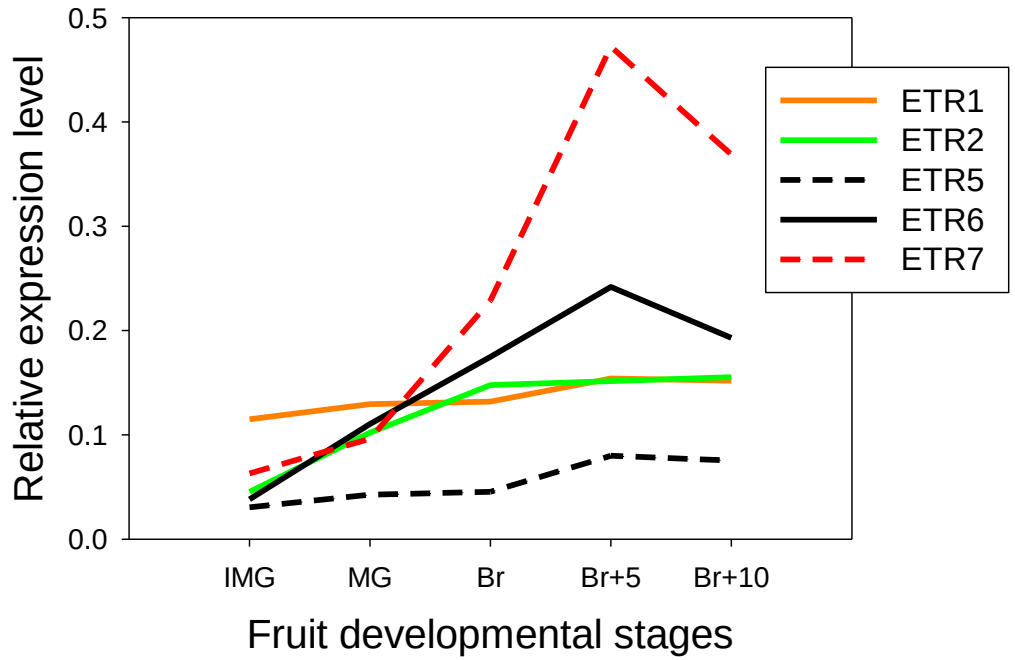

Figure S2. Expression data of ETR1, ETR2, ETR5, ETR6, ETR7 during fruit ripening extracted using TomExpress platform. Five fruit developmental stages have been studied: Immature Green (IMG), Mature Green (MG), Breaker (B), 5 days after Breaker (B+5), 10 days after Breaker (B+10). For each gene, the plot represents normalized counts per base for RNA-Seq data released from transcriptome analyses in multiple tomato cultivars.

Supplemental Table S1. Correspondence between common names and their Solyc numbers. Others names proposed by some literature are also listed. ACC, 1-aminocyclopropane-1-carboxylic acid; ETR, Ethylene receptors; CTR, constitutive triple-response; EIN2, Ethylene insensitive2; EIL, Ethylene insensitive3-Like; EBF, EIN3-binding F-box; ERF, Ethylene response factor.

Unified nomenclature	***Solyc number***	***Other names***
ACC synthase (ACS)		
ACS1A	Solyc08g081550	
ACS1B	Solyc08g081540	
ACS2	Solyc01g095080	
ACS3	Solyc02g091990	
ACS4	Solyc05g050010	
ACS5	Solyc04g077410	
ACS6	Solyc08g008100	
ACS7	Solyc02g063540	
ACS8	Solyc03g043890	
ACS9	Solyc07g026900	
ACS10	Solyc12g008740	
ACS11	Solyc03g007070	
ACS12	Solyc08g079750	
ACS13	Solyc12g056180	
ACC oxidase (ACO)		
ACO1	Solyc07g049530	
ACO2	Solyc12g005940	
ACO3	Solyc07g049550	*ACO4* (Van der Poel et al., 2014)
ACO4	Solyc02g081190	
ACO5	Solyc07g026650	
ACO6	Solyc02g036350	
ETR and partners		
RAN1	Solyc02g068490	
GR	Solyc01g104340	
GRL1	Solyc08g065320	
GRL2	Solyc02g062420	
TPR1	Solyc07g006180	
ETR1	Solyc12g011330	
ETR2	Solyc07g056580	
ETR3	Solyc09g075440	
ETR4	Solyc06g053710	
ETR5	Solyc11g006180	
ETR6	Solyc09g089610	

ETR7	Solyc05g055070	
CTR		
CTR1	Solyc10g083610	
CTR2	Solyc01g097980	
CTR3	Solyc09g009090	
CTR4	Solyc10g085570	
EIN2 and EIN-like		
EIN2	Solyc09g007870	
EIL1	Solyc06g073720	
EIL2	Solyc01g009170	
EIL3	Solyc01g096810	
EIL4	Solyc06g073730	
EIL5	Solyc01g014480	
EIL6	Solyc01g006650	
EBF		
EBF1	Solyc08g060810	
EBF2	Solyc12g009560	
EBF3	Solyc07g008250	
EBF4	Solyc06g049010	
ERF		
ERF.A3	Solyc05g052050	*Pti4* (Zhou et al., 1997)
ERF.B1	Solyc05g052040	
ERF.B2	Solyc03g093560	*ERF5* (Pan et al., 2012)
ERF.B3	Solyc05g052030	*LeERF4* (Tournier et al. 2003)
ERF.B13	Solyc08g078190	
ERF.C1	Solyc05g051200	*TERF1/JERF2 (*Huang et al., 2004)
ERF.D2	Solyc12g056590	
ERF.E1	Solyc09g075420	*LeERF2* (Pirrello *et al.,* 2006)
ERF.E2	Solyc06g063070	*JERF1* (Zhang *et al.,* 2004)
ERF.E4	Solyc01g065980	*SlERF6* (Lee *et al.,* 2012)
ERF.E5	Solyc12g049560	
ERF.F1	Solyc10g006130	*SlERF36* (Upadhyay et al., 2013)
ERF.F2	Solyc07g064890	
ERF.F3	Solyc07g049490	
ERF.F4	Solyc07g053740	
ERF.F5	Solyc10g009110	*SlERF3/LeERF3b* (Tournier et al., 2003)
ERF.F6	Solyc12g005960	
ERF.H10	Solyc04g054910	
ERF.H12	Solyc04g072900	