UNIVERSITY OF SÃO PAULO
School of Pharmaceutical Science
Graduate Program in Food Science
Field of study Bromatologie

Ethylene and auxin: new insights into the hormonal regulation of tomato fruit ripening

Bruna Lima Gomes

Thesis for obtaining DOCTORAL degree in Science
Advisor: Prof. Dr. Eduardo Purgatto

São Paulo
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Ethylene and auxin: new insights into the hormonal regulation of tomato fruit ripening

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This thesis is dedicated to my dad.

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ABSTRACT

GOMES, B. L. **Ethylene and auxin: new insights into the hormonal regulation of tomato fruit ripening.** 2016. 83p. Tese (Doutorado) - Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2016.

Our knowledge of the factors mediating ethylene-dependent and -independent ripening of climacteric fruit remains limited. Besides the known importance of ethylene roles, auxin has also been emerged as crucial to regulating ripening. Furthermore, the crosstalk between ethylene and auxin in tomato fruit ripening still awaits clarification. ERFs (Ethylene Responsive Factors) are transcription factors belonging to a large family acting downstream on ethylene signaling that direclty regulate ripening-related metabolisms, but their specific roles are still lacking. We present here a comprehensive expression profiling of tomato *ERFs* in wild-type and tomato ripening-impaired tomato mutants (*Nr*, *rin* and *nor*) indicating that out of the 77 *ERFs* present in the tomato genome, 27 show enhanced expression at the onset of ripening, while 28 display a ripening-associated decrease in expression, suggesting that different ERFs may have contrasting roles in fruit ripening. Members of subclass E, *ERF.E1*, *ERF.E2* and *ERF.E4*, show dramatic down-regulation in the ripening mutants suggesting their expression might be instrumental to fruit ripening. The study illustrates the high complexity of the regulatory network interconnecting RIN and ERFs and identifies subclass E members as the most active ERFs in ethylene- and RIN/NOR-dependent ripening. Additionally, with the aim to shed more light into ethylene and auxin interplay, hormonal treatments were applied to tomato fruits and several ripening aspects were then evaluated such as the volatile profile. Overall, results elicited that auxin delay the onset of ripening further showing epistatic effects over the influence of ethylene. Several ripening-related genes, including components of the ethylene signaling, were affected by auxin suggesting potential crosstalk points between the two hormones. Moreover, ethylene appears as potentially part of the auxin regulation through inducing its conjugation. The modulation of hormone levels in tomato fruit throughout ripening can be useful to help designing approaches that both improve fruit quality and extend shelf life.

Key words: ethylene, auxin, tomato fruit ripening, ripening regulation.

RESUMO

GOMES, B. L. **Etileno e auxina: novas percepções sobre a regulação hormonal do amadurecimento de frutos de tomateiro (*Solanum lycopersicum*).** 2016. 83p. Tese (Doutorado) - Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2016.

O conhecimento acerca dos fatores dependentes e independentes de etileno que regulam o amadurecimento de frutos climatéricos é ainda limitado. Além da importância conhecida do etileno, a auxina também tem sido apontada como crucial para o controle do amadurecimento. Mais ainda, há poucos estudos envolvendo o *crosstalk* entre etileno e auxina em frutos de tomateiro. ERFs (Ethylene Responsive Factors) são fatores de transcrição que atuam nos últimos níveis da via de sinalização de etileno, regulando diretamente metabolismos associados ao amadurecimento. Contudo, seus papéis específicos ainda são desconhecidos. O presente estudo revela o perfil detalhado de expressão de *ERFs* em tomate selvagem e nos mutantes cujo amadurecimento é comprometido (*Nr*, *rin* e *nor*) indicando que dos 77 *ERFs* presentes no genoma, 27 apresentam aumento de expressão no início do amadurecimento, enquanto 28 apresentam redução, sugerindo que diferentes ERFs possivelmente têm papéis distintos na regulação do amadurecimento. Membros da subclasse E, *ERF.E1*, *ERF.E2* e *ERF.E4*, apresentam drástica redução de expressão nos mutantes *Nr, rin* e *nor* apontando que tais fatores devem atuar fortemente no amadurecimento. O estudo ilustra também a complexidade das vias de regulação envolvendo RIN e ERFs e ainda aponta os membros da subslasse E como os mais ativos ERFs atuando nas vias etileno-dependentes e RIN/NOR-dependentes. Indo além, com o objetivo de se aprofundar no *crosstalk* entre etileno e auxina, tratamentos hormonais foram aplicados em frutos de tomateiro e diversos parâmetros do amadurecimento foram avaliados. De uma maneira geral, a auxina retarda o amadurecimento e ainda parece sobrepor os efeitos indutórios do etileno. Genes relacionados ao amadurecimento, incluindo genes relacionados à via de sinalização de etileno, foram afetados pela auxina sugerindo potenciais pontos de *crosstalk* entre os dois hormônios. O etileno ainda parece regular o metabolismo de auxina no fruto via indução de conjugação pela ativação de GH3s. Compreender o papel dos hormônios no controle da maturação é essencial para o desenvolvimento de tecnologias que visam melhorar a qualidade pós-colheita de frutos.

Palavras-chaves: etileno, auxina, amadurecimento de frutos de tomateiro, regulação do amadureicmento.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

SAM	S-adenosyl-methionine
ACC	1-aminocyclopropane-1-carboxylic acid
ACS	ACC synthase
ACO	ACC oxidase
ETR	Ethylene receptor
Nr	Tomato *Never-ripe* mutant
Gr	Tomato *Green-ripe* mutant
CTR	Constitutive Triple Response
MAPKKK	Serine/threonine mitogen-activated protein kinase kinase
EIN	Ethylene Insensitive
RIN	Ripening-Inhibitor
ERF	Ethylene Response Factors
PG2a	Polygalacturonase 2a
EXP1	Expansin 1
LOXC	Lipoxygenase C
ADH2	Alcohol dehydrogenase 2
HPL	Hydroperoxidelyase
CNR	Colorless Non-Ripening
NOR	Non-Ripening
SPB	SQUAMOSA promoter-binding
TAGL1	Tomato Agamous-Like1
AP2a	APETALA2a
VIGS	Virus-induced gene silencing
IAA	Indole-3-acetic acid
ARF	Auxin Response Factor
TPL	Topless protein
DPA	Days post anthesis
MG	Mature green stage
Br	Breaker stage
Br3	3 days post-breaker
Br10	10 days post-breaker

SPME	Solid phase micro-extraction technique
TIP	TIP41-like protein
EXP	Expressed Unknown Protein
DML2	DEMETER-like DNA demethylase
CCD1	Carotenoids cleavage dioxygenase
GH3	Gretchen Hagen protein
PCA	Principal Component Analysis
PC	Principal Component

INDEX

1 INTRODUCTION

Fruit ripening comprises a complex set of metabolic activities that lead to permanent changes in color, texture, aroma and flavor. From the biological point of view, fruits have evolved to be attractive to frugivores in order to enhance seed dispersal. However, fruits became important elements of the human diet and the knowledge behind the conditions of the ripening process is the key for the development of good quality attributes which makes fruits attractive to consumers.

The ripening changes result from a genetic programmation that leads to a wide alteration on gene expression encoding proteins related to several metabolic pathways which ultimately activate the development of fruit traits and physiological responses. A dynamic interplay between multiple phytohormones in conjunction with a set of developmental non-hormonal factors is required for regulating these processes (reviewed in Klee and Giovannoni, 2011 and Karlova et al., 2014).

The plant hormone ethylene is considered the typical ripening promoting hormone, especially in climacteric fruits. Indeed, climacteric fruits show a burst of ethylene production and a rapid rise in respiration at the onset of the ripening. For non-climacteric, however, our knowledge is rather limited and the role of ethylene is less clear. Ethylene is also involved in a wide range of developmental processes and physiological responses such as flowering, organ senescence, abscission, root nodulation, seed germination, programmed cell death, cell expansion, and responses to abiotic stresses and pathogen attacks. In the last decades, tremendous progress has been made towards deciphering the mechanisms by which plants perceive and respond to ethylene (Benavente and Alonso, 2006; Ju et al., 2012).

Tomato (*Solanum lycopersicum*) has been widely accepted as a reference species for Solanaceae genomics research and as a model system for studying fleshy fruit development, due to its advantages over other fleshy fruit species of agronomical interest (Gapper et al., 2013). As a climacteric fruit, tomato ripening has been extensively studied regarding the elements of the ethylene signaling and their role on ripening regulation.

Ethylene biosynthesis pathway comprises two steps: conversion of S-adenosyl-methionine (SAM) into 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC

synthase (ACS) and latter converting ACC into ethylene by ACC oxidase (ACO) (Yang and Hoffman, 1984). Inhibition or delay in fruit ripening by antisense strategy targeting *ACS2* or *ACO1*, isoforms of ACC synthase and ACC oxidase expressed in tomato fruit, provided direct evidence that ethylene biosynthesis is essential for climacteric fruit ripening (Hamilton et al., 1990; Oeller et al., 1991).

As a gaseous hormone, ethylene disperses through the tissues and binds to its specific receptors thus triggering the signal transduction cascade that ultimately activate a large set of genes whose transcription leads to the mentioned development of ripening attributes. Based on genome-wide search, there are seven ethylene receptors in tomato named LeETR1 to LeETR6 (Wilkinson et al., 1995; Lashbrook et al., 1998; Klee and Tieman, 2002; Gapper et al., 2013; Liu et al., 2015). A model currently accepted for ethylene perception is that the receptors act as negative regulators of ethylene signaling and it is therefore rather intriguing once the expression of the corresponding genes show a sharply increase during ripening (Kevany et al., 2007). These studies addressed that the levels of receptor transcripts are not correlated with the amount of receptor proteins thereby suggesting that the post-translational regulation of ethylene perception is an essential mechanism. Moreover, further results reported that the phosphorylation status of receptor proteins in tomato fruit is crucial to switch on or off the downstream ethylene signal transduction (Kamiyoshihara et al., 2012).

Indeed, altered ethylene perception impairs fruit ripening. The tomato Never-ripe (*Nr*) mutant, a mutation in the ethylene receptor now called ETR3 conferring ethylene insensitivity, produced non-ripening fruits (Wilkinson et al., 1995). Another mutant, Green-ripe (*Gr*), a dominant ripening mutation that occurs in a gene encoding another component of ethylene signaling that may act at the receptor level, failed to fully ripen as a consequence of reduced ethylene responsiveness (Barry et al., 2005).

Another key ethylene perception element is *SlCTR1* which is up-regulated during tomato fruit ripening and encodes the CONSTITUTIVE TRIPLE RESPONSE 1, a serine/threonine mitogen-activated protein kinase kinase (MAPKKK) that forms a complex with ethylene receptors. The transcriptional induction of ethylene receptors and CTR genes in response to high levels of ethylene was previously described in tomato (Wilkinson et al 1995, Tieman and Klee 2002, Leclerq et al., 2002), apple (Yang et al., 2013), kiwifruit (Yin et al., 2008) and papaya (Zhu et al., 2014).

The ethylene binding to its receptors leads to the inactivation of CTR1 and therefore switches on the signal transduction. Hence, CTR1 is also considered a negative regulator of ripening. It was demonstrated in *Arabidopsis thaliana* that CTR1 act on the phosphorylation of EIN2, a downstream element of the cascade called ETHYLENE INSENSITIVE, which prevents the migration of C-terminal portion to the nucleus (Chuanli et al., 2012). Once in the nucleus, EIN2 is responsible to the transcriptional activation of EIN3 (ETHYLENE INSENSITIVE 3) and EIL (EIN3-like) which in turn leads to the activation of the transcription factors named ERFs (ETHYLENE RESPONSIVE FACTORS). The transcriptional cascade ends with these ethylene-responsive genes that directly modulate the metabolic events of ripening.

Upstream to the ethylene signaling pathway are the so called developmental factors: genes that encode transcription factors belonging to different families. One member of the MADS-box family, RIN (RIPENING-INHIBITOR; Vrebalov et al., 2002), is thought to be one of the master regulators of the tomato fruit ripening. The transcription factor RIN controls directly several key genes involved in the most important ripening-related pathways by the interaction with the promoter of genes involved in ethylene biosynthesis (*ACS2* and *ACS4*), ethylene perception (*NR* and *ETR4*), ethylene response factors (*ERFs*), cell wall metabolism (polygalacturonase 2a, *PG2a*; expansin 1, *EXP1*), carotenoid accumulation (phytoene synthase 1, *PSY1*), aroma production (lipoxygenase C, *LOXC*; alcohol dehydrogenase 2, *ADH2*; hydroperoxidelyase, *HPL*), among others (Ito et al., 2008; Martel et al., 2011; Fujisawa et al., 2012; Fujisawa et al., 2013; Zhong et al., 2013).

The *rin* mutant is unable to develop the ripe phenotype, even undergoing exogenous ethylene treatment (Vrebalov et al. 2002). RIN is also capable to bind to the promoter of other important developmental factors including the CNR (COLORLESS NON-RIPENING) and NOR (NON-RIPENING) (Martel et al. 2011) and the tomato fruit that harbors mutations in these two genes are also unable to ripen normally.

The *Cnr* mutation induces an epigenetic change that alters the promoter methylation of a SQUAMOSA promoter-binding (SPB) protein, resulting in a pleiotropic ripening inhibition phenotype and inhibited expression of ethylene-associated genes *ACO1, E8* and *NR* (Thompson et al., 1999; Manning et al., 2006). The *nor* mutant causes delay on tomato fruit ripening with a phenotype similar to the *rin* mutant (Giovannoni, 2004) and

the NOR protein is a member of the NAC-domain transcription factor family (Giovannoni, 2007). Recently, a systems biology approach indicated that NOR may have a more global regulation effect on ethylene synthesis/perception genes than RIN in controlling fruit ripening (Osorio et al., 2011).

The identification of these key ripening regulators in the tomato, such as RIN, NOR, CNR and other components such as TAGL1 (TOMATO AGAMOUS-LIKE1; Itkin et al., 2009; Vrebalov et al., 2009), AP2a (APETALA2a; Chung et al., 2010; Karlova et al., 2014) and LeHB-1 (HD-Zip homeobox protein; Lin et al., 2008), provided novel insights into the understanding of the complex mechanisms underlying fruit ripening. TAGL1 is another MADS-box protein that controls fruit ripening by regulating ethylene synthesis by binding to the *ACS2* promoter (Itkin et al., 2009; Vrebalov et al., 2009). Tomato plants down-regulated in TAGL1 produced yellow-orange fruit whereas ectopic expression of TAGL1 in tomato resulted in sepal expansion and lycopene accumulation, supporting the active role of TAGL1 in ripening (Vrebalov et al., 2009). Down-regulation of SlAP2a results in early ripening and ethylene over-producer fruit, suggesting that this member of the AP2/ERF superfamily acts as a negative regulator of fruit ripening and ethylene production (Chung et al., 2010; Karlova et al., 2011). LeHB-1, which binds the promoter of *ACO1* (Lin et al., 2008) and its silencing via virus-induced gene silencing (VIGS) strategy results in down-regulation of LeACO1 expression associated with delayed fruit ripening. Furthermore, Liu et al. (2015) demonstrated a direct cause and effect relationship between active DNA demethylation, mainly mediated by the tomato DML2, and fruit ripening as actively part of the transcriptional control. Taken together, the characterization of these transcriptional regulators indicated that transcription factors play essential roles in modulating transduction signals and regulating ethylene biosynthesis/perception, and hence, in controlling fruit ripening.

Although the emphasis on the ethylene role and the evidences demonstrating its importance for fruit ripening, in the last years a large body of results has been changed the definition of ethylene as the major ripening regulator hormone to one of the hormones actively controlling the dynamic network of ripening, which includes several ethylene-independent factors and the interplay between other hormones signaling components. Within this scenario, auxin emerges as important element acting on that interplay and it is now largely accepted as one of the main regulators of fruit ripening

(Kumar et al., 2014; McAtee et al., 2013; Muday et al., 2012; Seymour et al., 2013). Despite its initial characterization as growth-promoting substance, auxin is required for cell division, expansion and differentiation, fruit set and development, and is involved in gravitropism and apical dominance. On fruit ripening control, studies have shown that auxin has an opposite effect compared with ethylene (Kumar et al., 2014; Su et al., 2015; Li et al., 2016).

Inhibitory effects of auxin were observed for the first time by the removal of achenes from strawberry, which produces auxin, and then ripening was accelerated (Frenkel and Dyck, 1973). In banana, studies have demonstrated that auxin acts retarding the activation of metabolic events of ripening (Purgatto et al., 2001, 2002). In grape berry and mango, reduction of active auxin levels seems to be instrumental for the onset of ripening (Zaharah et al., 2012; Böttcher et al., 2010, 2013.) Interestingly, reports have also pointed auxin as a crucial element for triggering ripening acting on the transition between system I and II of ethylene production, not necessarily displaying an inhibiting role (Grierson, 2013; McAtee et al., 2013; Seymour et al. 2013; Ross et al., 2011). In addition to that, genes related to auxin synthesis, transport and signaling enhance their expression at the onset of ripening in fruits such as tomato and peaches (Pattison and Catalá, 2012; Exposito-Rodríguez et al., 2011; Trainotti et al., 2007). The signaling components and mechanisms underlying auxin regulation, and even its exact role on ripening control especially its interaction with other hormones signals, are still very unclear.

Indole-3-acetic acid (IAA) is one of the most abundant and important auxin in plants synthesized through both tryptophan-dependent and -independent pathways (reviewed by Korasick et al., 2013; Tivendale et al., 2014). The metabolism of auxin consists in converting free IAA active form by conjugation to carbohydrates or amino acids as the inactive forms. The rapidly conversion of storage forms to free active IAA therefore regulate the auxin homeostasis in the cell (Enders and Strader, 2015; Korasick et al., 2013).

In comparison with ethylene, very little is known about the auxin signaling pathway in tomato fruit ripening. Auxin is first perceived by the TIR1/AFB receptors leading to the transcriptional control of auxin-responsive genes. The auxin response is mediated by Auxin Response Factors (ARFs), transcription factors that directly control auxin-

responsive genes through the binding to their promoters. It has been reported that ARFs play in concert with Aux/IAA proteins, that act both as part of the hormone perception complex and as transcriptional repressors, and with Topless proteins (TPLs), that work as co-repressors (Liu and Karmarkar, 2008). In the absence of auxin, Aux/IAA proteins form dimers with ARFs to inhibit their activity by recruiting the TPL co-repressors. In the presence of auxin, Aux/IAAs bind to the SCF-TIR1 complex and get subsequently ubiquitinated and degraded by the 26S proteasome. The ARFs are then released and can regulate the transcription of its target auxin responsive genes (Hayashi, 2012).

The multiple hormone interplay regulating the metabolic pathways involved in fruit ripening can occur at the molecular level through the interaction between components of the hormones signal transduction. A primary crosstalk has been proposed in which genes may have specific sequences on their promoters for binding to different hormone-related transcription factors. A secondary crosstalk relies on the hormone-related factors regulating genes that belong to other hormone transcriptional activity such as signaling, synthesis and transport (Muday et al., 2012).

Regarding the ethylene and auxin crosstalk, recent reports have elucidated antagonistic effects of the two hormones as auxin playing as repressor oppositely as the known role of ethylene as tomato fruit ripening inducer (Su et al., 2015; Li et al., 2016). Nonetheless, the intricate mechanisms by which these hormones interact and the transcription factors involving in activate or repress in a coordinated manner the transcriptional activity of both hormones responsive genes remain largely unclear. Enhancing the knowledge about the main actors of ethylene signaling and further providing new insights into the current view about ethylene/auxin interplay on tomato ripening regulation are the main objectives of the present study.

In this context, studies regarding the ERFs, important components that mediate the diversity and specificity of ethylene responses as described above, were then achieved and comprise the Section 1 of this document. Taking advantage of the recent release of the complete annotated tomato genome sequence (Tomato Genome Consortium, 2012), 77 tomato ERF genes were identified but their physiological significance has been hampered by the functional redundancy among members of this vast gene family. A number of the ERF genes identified in tomato are ethylene inducible and show ripening-related expression pattern which points out to their putative role in fruit ripening

(Sharma et al., 2010; Pirrello et al., 2012; Liu et al., 2014). Consistent with this hypothesis, tomato LeERF1 was reported to mediate ethylene response and its overexpression resulted in constitutive ethylene response and accelerated fruit ripening and softening (Li et al., 2007). Likewise, Sl-ERF6 plays an important role in fruit ripening by integrating ethylene and carotenoid synthesis pathways in tomato (Lee et al., 2012). More recently, the involvement of Sl-ERF.B3 in controlling fruit ripening through the regulation of climacteric ethylene production and carotenoid accumulation was revealed using a dominant repression strategy (Liu et al., 2014). Although recent studies have shown that some ERF members are involved in fruit ripening, whether different ERF family members play specific roles in mediating ethylene-dependent ripening remains largely unknown. A better understanding of the regulatory mechanisms underlying the ethylene action during climacteric fruit ripening requires the deciphering of the physiological function of ERFs and assigning specific roles to different members of this gene family. Building on the recently generated tools and genomics resources in the tomato, the present study aims at identifying the ERFs that are most active in fruit ripening. A large set of RNA-seq data available for multiple tomato cultivars, was mined at the genome-wide scale using the newly developed bioinformatics platform "TomExpress" (http://gbf.toulouse.inra.fr/tomexpress), leading to the identification of a small subset of ERF genes displaying consistent ripening-associated expression pattern. The selected ripening-related ERF genes were then connected the mechanism underlying ethylene- and RIN/NOR-dependent ripening.

As described above, ethylene is assumed to be a "master switch" controlling tomato fruit ripening while auxin is so called a ripening inhibitor. Concerning the gaps on the knowledge regarding the ethylene/auxin crosstalk**,** the following questions were attempted to be addressed in the present study: does auxin delay the initiation of climacteric ethylene production? Does auxin delay ripening through blocking the ethylene perception and/or signaling and if so which components of ethylene signal transduction are involved? Are low levels of auxin essential to triggering ripening? What ripening parameters are affected by exogenous auxin? Are these effects recovered in fruits reaching good quality traits at red stage?

Comprising the Section 2 of this document and with the aim to shed more light into the questions mentioned right before, auxin and ethylene treatments were applied to tomato fruits and several ripening aspects were then evaluated. Overall, results elicited that

auxin delayed the onset of ripening further showing epistatic effects over the influence of ethylene which was confirmed when fruits were exposed to both hormones. Several ripening-related genes were affected by auxin suggesting potential crosstalk points between the two hormones. Further, ethylene seems to be part of the free active IAA regulation through inducing IAA conjugation. Interestingly, ripening parameters such as peel color and volatile profile could be restored apart from the delay caused by auxin, which ultimately indicate that the modulation of IAA levels in tomato fruit throughout ripening can be useful to help designing approaches that both improve fruit quality and extend shelf life.

2 OBJECTIVES

2.1 GENERAL OBJECTIVE

The aim of the thesis research project was to gain more insight on the role of ethylene and auxin regulating tomato fruit ripening and furthermore the possible crosstalk between the two hormones at this plant developmental stage. The results were then separated into two sections: 1, regarding the ethylene role on ripening through studies on tomato ERFs; and 2, regarding the auxin effects on tomato fruit ripening highlighting the ethylene/auxin interplay.

2.2 SPECIFIC OBJETIVES

2.2.1 SECTION 1

- Providing a comprehensive expression profiling of the tomato ERFs in wild-type and tomato ripening-impaired mutants (*Nr, rin* and *nor*) based on *in silico* studies on TomExpress bioinformatics platform followed by RT-PCR analyses.
- The identification of a small subset of ERF genes displaying consistent ripening-associated expression pattern were then connected to the mechanisms underlying ethylene- and RIN/NOR-dependent ripening.

2.2.2 SECTION 2

- Evidencing a better understanding on the crosstalk between auxin and ethylene by verifying the effects of IAA treatments on several components of the ethylene perception and signaling correlating with the evaluation of ripening parameters such as ethylene production, peel color shift and changes in volatile profile.
- Verifying the effects of ethylene on the IAA conjugation and the expression pattern of the related GH3 genes highly expressed during tomato ripening.

3 MATERIAL AND METHODS

3.1 SECTION 1

3.1.1 Plant materials and growth conditions

Tomato (*Solanum lycopersicum* L. cv MicroTom) wild type and *Nr, rin* and *nor* fruit ripening impaired mutants under the MicroTom background were grown under standard greenhouse conditions. Conditions in the culture chamber room were set as following: 14h-day/ 10h-night cycle, 25/20°C day/ night temperature, 80% relative humidity, 250µmol.m^{-2}.s^{-1} intense luminosity. Fruit samples were collected from different fruit development and ripening stages including 20DPA (20 days post-anthesis), MG (mature green stage), Br (breaker stage), Br3 (3 days post-breaker) and Br10 (10 days post-breaker).

3.1.2 Expression data mining and heatmap generation

RNA-Seq data of transcriptome in multiple tomato cultivars were obtained from the TomExpress bioinformatics platform (http://gbf.toulouse.inra.fr/tomexpress) focused on the ERF family genes during vegetative and reproductive development. TomExpress provides a unified approach of tomato gene expressions from released RNA-seq data sets. Expression data represent normalized counts per base and mean values of multiple cultivars for each tissue and stage were input to generate heatmap representations using R package (Version 3.1.2).

3.1.3 RNA isolation and quantitative RT-PCR analyses

Fruits from each developmental and ripening stage were harvested, frozen in liquid nitrogen and stored at -80°C. Total RNA from pericarp of at least five individual fruits at each developmental stage analyzed here was extracted using the Plant RNA Purification Reagent (Invitrogen, Cat. No. 12322-012) according to the manufacturer's instructions. Total RNA was then DNase-treated (Invitrogen, Cat. No. AM1906). First-strand cDNA was reverse transcribed from 2 µg of total RNA using the Omniscript

Reverse Transcription kit (Qiagen, Cat. No. 74904) following the manufacturer's instructions. Gene-specific primers were designed by Primer Express software (PE-Applied Biosystems) and were further checked using BLAST against the tomato whole genome. Quantitative real-time PCR analyses were performed as previously described (Pirrello et al., 2006). The primer sequences used in this study are listed in Table 1.

Table 1 List of the primers used in the study regarding section 1.

Gene Name	Primer Sequence
Sl-Actin	F 5'- TGTCCCTATTTACGAGGGTTATGC-3'
	R 5'-CAGTTAAATCACGACCAGCAAGAT-3'
Sl-ERF.A3	F 5'-TCGTCGGGAAACGGTTCCAT-3'
	R 5'-GACATCCAACTTGCATGACACTTG-3'
Sl-ERF.B1	F 5'-GAATGATGACGGAATTGTAATGAAGA-3'
	R 5'-TTCCACAATCCCAAATTGAAGA-3'
Sl-ERF.B2	F 5'-CCATCTCTGAACGTAGCAATACC-3'
	R 5'-CATGGCCTCTGTCTAACTCC-3'
Sl-ERF.B3	F 5'-CGGAGATAAGAGATCCAAGTCGAA-3'
	R 5'-CTTAAACGCTGCACAATCATAAGC-3'
Sl-ERF.B6	F 5'-GTGAAGAAGTGTAAGGAAGAG-3'
	R 5'-GTGATAATGGAGGGAGGT-3'
Sl-ERF.C1	F 5'-GCATTATCAATGAGGGGTCCTTG-3'
	R 5'-TTAAAACAGCAGCTGGAGATAATCC-3'
Sl-ERF.D2	F 5'-AGCTCAACCAACGTCGTTCCT-3'
	R 5'-GGTTGTTGATCCGTAAAATCTCCTG-3'
Sl-ERF.E1	F 5'-GTTCCTCTCAACCCCAAACG-3'
	R 5'-TTCATCTGCTCACCACCTGTAGA-3'
Sl-ERF.E2	F 5'-ACTTCGTGAGGAAACCCTGAAC-3'
	R 5'-GTTACTAATATAAGTCATGTTGGGCTGAA-3'
Sl-ERF.E4	F 5'-AGGCCAAGGAAGAACAAGTACAGA-3'
	R 5'-CCAAGCCAAACGCGTACAC-3'
Sl-ERF.E5	F 5'-TGAACACTGAACACAAGCC-3'
	R 5'-GGTACATCCATATTTCCTTCTGAG-3'
Sl-ERF.F1	F 5'-ACGAGCTTTCTTCTTTTCTCTCTCTAAA-3'
	R 5'-GAAACTCGATATCCTTCTGTAAAATCTTC-3'

Sl-ERF.F2	F 5'-GACGATTGCCATAGCGACTGTG-3'
	R 5'-CGGCTTTCTGAAAGAGGAAGAAG-3'
Sl-ERF.F3	F 5'-AGTAGTAAGGTGACCCGGATGAAG-3'
	R 5'-CACCGATCATCCACCACAGA-3'
Sl-ERF.F4	F 5'-GAGCTAATGGCTGATTTTTGTATATAAGTTC-3'
	R 5'-AAATGGTAGAAACAGCACGAGAAAG-3'
Sl-ERF.F5	F 5'-TGGAGCGAAAGCGAAAACTAA-3'
	R 5'-GTCTGACTCGGACTCCGATTG-3'
Sl-ERF.F6	F 5'-CCGATGGGGGAAGGTCATAG-3'
	R 5'-CTGATCATCATCGCCACCGC-3'
Sl-ERF.H7	F 5'-TAATTCCAAGCCAAGAAAGTCC-3'
	R 5'-TCATCTGTGGTGATTTCTGAC-3'

3.1.4 ACC treatment

Tomato fruits were harvested at the mature green stage and then injected with a buffer solution contained 10 mM MES, pH 5.6, sorbitol (3% w/v) or 100 μM of ACC in the same buffer solution as described by Su et al., (2015).

3.1.5 Generation of the correlation network of ERFs and ripening-associated genes

The network was generated from the analysis of co-expressions of ERFs and ripening-related genes in the TomExpress platform with a correlation threshold > 0.85. In the TomExpress platform, correlations are calculated with the Spearman correlation coefficient of gene expressions during fruit development and ripening. Such a correlation coefficient allows to aggregate genes that are co-expressed even if the co-expression is not linear.

3.1.6 Transient expression using a single cell system

Protoplasts used for transfection were isolated from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells in accordance with (Leclercq et al., 2005). The reporter construct was generated with native promoters, ERFs (ERF.E1, ERF.E2,

ERF.E4, ERF.F2), fused to GFP. Protoplast co-transfection assays was performed using the reporter plasmids and effector vectors carrying 35S:RIN. GFP expression was analyzed and quantified by flow cytometry (FACS cyflow instrument; PARTEC, Japan, http://www.sysmex-partec.com/) 16h following protoplast transfection. For each sample, 100–1000 protoplasts were gated on forward light scatter; GFP fluorescence per population of cells corresponds to the average fluorescence intensity of the population of cells above the background. The data were analyzed using flomax software (sysmex-partec) and were normalized using an experiment with protoplasts transformed with the reporter vector in combination with the vector used as effector but lacking the RIN coding sequence.

3.1.7 Cloning and plant transformation

A set of genes (*ERF.B3*, *ERF.E1*, *ERF.E2*, *ERF.E4*, *ERF.F2*) were cloned by Golden Braid strategy (GB; https://gbcloning.upv.es/). The first cloning step consisted in the gene domestication by introducing CDS sequences of interest into the GB destination vectors called pUPD. The followed multipartite and binary assemblies were performed in order to generate constructions containing the transcriptional units, the full CaMV (cauliflower mosaic virus) 35S promoters, the terminators TNos (nopaline synthase terminator) and the *NPTII*, a kanamycin resistance gene. All the reactions were performed according to the instructions available on the GB platform. Transgenic plants using the *Nr* and *rin* mutants under the MicroTom background and also the wild type MicroTom were generated by Agrobacterium-mediated transformation according to Bird et al. (1988) with minor changes: 15 days old cotyledons were used for the transformation; the duration of subcultures for shoot formation was 15 days; the kanamycin concentration was 100 $mg.L^{-1}$.

3.2 SECTION 2

3.2.1 Fruits and hormonal treatments

Tomato plants (Solanum lycopersicum cv. MicroTom) were grown under standard greenhouse conditions. Fruit samples were harvested at mature green stage and randomly separated into four groups according to hormonal treatments: IAA (indole-3-acetic acid treatment); ETHYLENE (ethylene treatment); ETHYLENE + IAA (a combination of both hormones); CTRL (control samples). During the experiments, fruits were left to ripen spontaneously in 323 L chamber at 22°C, for a 16 hour-day/8 hour-night cycle and at 80% relative humidity. The IAA solutions were prepared at 100 µM in 10 mM MES buffer at pH 5.6 + 3% sorbitol and injected through the calyx end as described by Su et al. (2015). Ethylene treatments were performed using a gaseous hormone at 10 µL.L-1 for 12 hours. Fruits from the IAA group received IAA solution, while fruits from the ETHYLENE + IAA group were exposed to gaseous ethylene before being infiltrated by IAA solution. In order to maintain the injection method consistent across all samples, fruits from ETHYLENE and CTRL groups were injected with a buffer solution only. For further analyses, at least five fruits from each experimental condition were collected at different points, covering all ripening stages: 6h (six hours after injection), D1, D2, D4, D10 and D14 (D means day of experiment). Samples were then frozen in liquid nitrogen and stored at -80°C.

3.2.2 Ethylene production

At least five fruits from each group were individually placed in airtight glass containers and left at 25°C. After 1 hour, samples of 1 mL were collected from the headspace with a gastight syringe through a rubber septum for ethylene analysis. The determination of ethylene was performed by gas chromatography (Agilent Technologies, model HP-6890). A flame ionization detector was employed and the column used was an HP-Plot Q (30 m x 0.53 mm x 40 µm, Agilent Technologies). Injector and detector temperatures were both set at 250°C and the oven temperature was set at 30°C. The injections were performed in pulsed splitless mode. Helium was used as carrier gas (1 mL.min-1).

3.2.3 Peel color characterization

Peel colour was measured using the HunterLab ColorQuest XE instrument (Hunter Associates Laboratories) in terms of L (lightness), A (redness and greenness), and B (yellowness and blueness). The hue value was accessed to represent colour variations and was defined as described by Fabi et al. (2007). At least five representative measures were taken from each experimental group.

3.2.4 Analysis of volatile compounds

The headspace volatile production of tomato fruit was determined by solid phase micro-extraction technique (SPME). A pool of at least five fresh fruits was homogenized with a sodium chloride solution 30% (Merck). Aliquots of 10 g were placed in vials and frozen at -20°C. Samples were thawed under agitation at 40°C just before analysis. The headspace equilibrium time was 10 min and adsorption time was 50 min. The SPME fibre (PDMS/DVB/CAR 50μm, Supelco Co.) was injected directly into a Hewlett–Packard 6890 (Agilent Technologies Inc., Santa Clara, USA) gas chromatograph mass spectrometer (GC-MS) and held for 10 min for desorption of volatile compounds. The injector temperature was 200°C. Components were then separated using a capillary column Supelcowax 10 (30 m x 0.25 mm x 0.25 μm) and the oven temperature was programmed to increase from 40°C to 150°C at 2°C/min. These conditions were previously optimized and were selected according to the higher number of peaks and the greater total area of the chromatogram. Volatile compounds were identified using the NIST library (NIST98, version 2.0, Gaithersburg, USA), and by further confirming the results with the retention indexes. All analyses were carried out in triplicate. Principal component analysis (PCA Biplot) was applied to highlight differences among samples by using XLSTAT software.

3.2.5 IAA determination

Free IAA determination was assessed by the method proposed by Ludwig-Müller et al. (2008), with modifications. 500 mg of fruit tissue was used for extraction by isopropanol and acetic acid (95:5), with 2 μg [13C6]-IAA (Cambridge Isotopes,

Andover, MA, USA) added as internal standard (IS), under incubation at 4°C for 2 hours. After centrifugation at 4°C and 13000 x g for 10 minutes, the supernatant was concentrated to a volume of 50 µL using a vacuum concentrator. The pH was adjusted between 2.5 to 3.5 by adding 1M HCL and 500 µL of ethyl acetate, and followed by centrifugation at 4°C and 13000 x g for 5 minutes. The organic phase was collected and the procedure was repeated 2 times in the aqueous phase. Samples were dried by vacuum concentration and then re-suspended in 100 µL of methanol. After being dried again by vacuum, 25 µL of pyridine and 25 µL of MTBSTFA (Sigma-Aldrich – 375934) were added, followed by incubation at 92°C for 1 hour.

GC-MS-SIM was used for quantification in a gas chromatograph (HP-6890, Hewlett-Packard, Palo Alto, CA, USA) coupled to a mass selective detector (HP-5973). One microliter of each technical sample was automatically injected (ALS 7693, Agilent, Santa Clara, CA, USA) on Splitless mode, with He flux at 0.83/min through a DB-5ms column (30 m x 0.25 mm x 0.50 µm). The monitored ions for endogenous IAA (m/z 130 and 232) and labelled internal standard (m/z 136 and 238) were used in the extracted chromatograms and the concentration was obtained by the ratios of chromatogram areas (Sample/IS).

Conjugated forms were assessed by solid phase extraction according to the method adapted from Chen et al. (1988). In 15 ml glass centrifuge tubes, 2 mL of extraction solution (65% isopropanol in imidazole buffer 0,2 M pH 7,0), 500 mg of fruit tissues and 2 µg [13C6]-IAA (Cambridge Isotopes, Andover, MA, USA) as IS was added, followed by centrifugation at room temperature and 13000 x g for 10 minutes. Isopropanol was evaporated (around 65% of the total volume) and 500 µL of 1M NaOH was added to the remaining solution, which was then incubated at room temperature for 1 hour for the hydrolysis of IAA-ester fraction; 500 µL from the remaining solution was transferred to a reaction vial to which 500 µL of 7M NaOH was added, before it was incubated at 100°C for 3 hours, corresponding to the hydrolysis of IAA-amide fraction.

The resins used for sample clean-up were C18 (Supelclean – LC – C18) and aminopropyl (Supelclean – LC – NH2). For both resins, 500 mg was used. Before elution, the pH of both fractions (ester and amide) was adjusted to 2.5. After the sample loading at C18 cartridge, the resin was washed with ultrapure water and the IAA fraction eluted with 2 mL of acetonitrile. This solution was diluted with 18 mL of the

imidazole buffer 0.02M (pH 7.0) and then applied to the NH2 cartridge. The sample was percolated by gravity and the resin was sequence-washed with 2 mL of hexane, 2 mL of ethyl acetate, 2 mL of acetonitrile and 2 mL of methanol. Finally, 3 mL of 2% acetic acid on methanol was added, and the partially purified IAA fraction was collected. Samples were completely dried using a vacuum concentrator and re-suspended with 100 µL of methanol.

3.2.6 Carotenoids analyses

Representative samples for each experimental group were used for carotenoids extractions. The frozen tissues were powdered and analyses were performed according to the method developed by Sérino et al. (2009).

3.2.7 RNA isolation and quantitative RT-PCR analyses

Total RNAs from at least five individual fruits at each experimental stage analysed here were extracted using the Concert Plant RNA Reagent (Invitrogen) and treated with the Ambion DNA-free DNase Treatment & Removal Reagents kit (Invitrogen) for genomic DNA removal following the manufacturer's instructions. RNAs were verified by agarose gel electrophoresis. For the cDNA synthesis, 1 µg of RNA, measured spectrophotometrically, was used in reverse transcription reactions according to the instructions given in the ImProm-II Reverse Transcription System kit (Promega). A total 10 µL of Real Time-PCR reactions were set using the Power SYBR Green Master Mix kit (Applied Biosystems) and reactions were performed in QuantStudio 7 Flex (Applied Biosystems), which was programmed to 95°C for 5 min, 60 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. The TIP41-like protein (TIP) and expressed unknown protein (EXP) genes were used as the internal controls. All the primers used for amplifications are listed in Table 2.

Table 2 List of the primers used in the study regarding section 2.

Gene Name	Primer Sequence
TIP	F 5'- ATGGAGTTTTTGAGTCTTCTGC-3'
	R 5'-GCTGCGTTTCTGGCTTAGG-3'
EXP	F 5'- GCTAAGAACGCTGGACCTAATG-3'
	R 5'-TGGGTGTGCCTTTCTGAATG -3'
RIN	F 5'-CCCAAACTTCATCAGATTCACAG-3'
	R 5'-AATTGTCCCAAATCCTCACCT-3'
NOR	F 5'-TAATGATGGGGTCGTCTTTCG-3'
	R 5'-ATTTTACAGGGCTAACTATTTTTTGC-3'
CNR	F 5'-GCCAAATCAAGCAATGATGA-3'
	R 5'-TCGCAACCATACAGACCATT-3'
DML2	F 5'-AGTACTCATGCCAAAGCCAAA-3'
	R 5'-CCTATCTTCTTTTTACCGACTGGA-3'
AP2a	F 5'-CTGCTCGGAGTCTGAACC-3'
	R 5'-AACGGACCACAATCTTGAC-3'
ERF.E1	F 5'-GTTCCTCTCAACCCCAAACG-3'
	R 5'-TTCATCTGCTCACCACCTGTAGA-3'
ERF.E2	F 5'-ACTTCGTGAGGAAACCCTGAAC-3'
	R 5'-GTTACTAATATAAGTCATGTTGGGCTGAA-3'
ERF.E4	F 5'-AGGCCAAGGAAGAACAAGTACAGA-3'
	R 5'-CCAAGCCAAACGCGTACAC-3'
ACO1	F 5'-GCCAAAGAGCCAAGATTTGA-3'
	R 5'-TTTTTAATTGAATTGGGATCTAAGC-3'
ACO4	F 5'-GGAGCCTAGGTTTGAAGCAA-3'
	R 5'-AAACAAATTCCCCCTTGAAAA-3'
ACS1a	F 5'-AAGGTTTATGGAGAAAGTGAGAGG-3'
	R 5'-AAAGGCATCACCAGGATCAG-3'
ACS2	F 5'-GGTTAGGTAAAAGGCACAAACAT-3'
	R 5'-GAATAGGTGACGAAAGTGGTGAC-3'
ACS4	F 5'-GATTTGCGGTCATTGTTGAAAG-3'
	R 5'-GCCTGGGCGAATCTAGTTTATTT-3'

ACS6	F 5'-CTCCTATGGTCCAAGCAAGG-3'
	R 5'-CGACATGTCCATAATTGAACG-3'
ETR3	F 5'-AGGGAACCACTGTCACGTTTG-3'
	R 5'-TCTGGGAGGCATAGGTAGCA-3'
ETR4	F 5'-GTAATCCCAAATCCAGAAGGTTT-3'
	R 5'-CAATTGATGGCCGCAGTTG-3'
ETR6	F 5’-ATTCCAAAGGCAGCCGTTAA-3’
	R 5’-GATGTGGATATGTGGGATTAGAA-3’
CCD1A	F 5’-ATGGGGAGAAAAGAAGATGATGGA-3’
	R 5’-ATTCAAGAACAAGCCAAACTGTGA-3’
CCD1B	F 5’-ATGGGGATGAATGAAGAAGATGGA-3’
	R 5’-ATTCAGGAGCAAGCCAAAATGTGA-3’
ADH2	F 5’-TGAGTACACCGTGGTTCATGTTG-3’
	R 5’-TCCAAGGCCTGTCGAAATTC-3’
LOXC	F 5’-GAGTTTGGAGTTCCAGGAGCAT-3’
	R 5’-CATCTTCGAGTGTGAGTGACTTGA-3’
HPL	F 5’-GTCCACCAGTACCAAGTCAATATGC-3’
	R 5’-GCTCCCCTTTCTTGATTTCGTAA-3’
GH3.1	F 5’-AGACAATGAGGGCAATGCAAAC-3’
	F 5’-CAAGAGATGGCAGTACAAAGATTGG-3’
GH3.2	F 5'-CAATGTTGTTTAGCGATGGAAGAG-3'
	R 5'-AATGGACCAATTGAGTTACAAGCA-3'
GH3.3	F 5'-TTCATCTCCTTTTGATCCCTCT-3'
	R 5'-CATTTTGTCGGATAACATCAACA-3'

4 RESULTS

4.1 SECTION 1

4.1.1 Consensus nomenclature for tomato ERF genes

The important role attributed to ethylene in triggering and coordinating the ripening of climacteric fruits and the central role assigned to ERFs in mediating the hormone action, prompted the search for the ERFs involved in ethylene responses during fleshy fruit ripening. Building on the achievement of the complete tomato genome sequence (SL2.40 genome sequence and ITAG2.30 whole protein sequences), we previously extended the total number of AP2/ERF genes from 112 (Sharma et al., 2010) to 146 (Pirrello et al., 2012). Among these, the nature of distinctive amino acid residues allowed to assign 77 genes to the ERF subfamily and 48 to DREBs (Dehydration-Responsive Element binding proteins) and the constructed phylogenetic tree clustered the 77 tomato ERF proteins into 9 subclasses (subclass A to J). To comply with the classification adopted for Arabidopsis, tomato ERF genes were given a letter (A to J) with reference to the subclass they belong to, and a number to distinguish between members of the same subclass, according to their position in the neighborhood phylogenetic tree (Pirrello et al., 2012). Since a link between the physiological function of ERFs and their structural features has been previously suggested (Nakano et al., 2006; Pirrello et al., 2012), the present study attempted to clarify the structure-based classification of all tomato ERFs in order to unify their nomenclature and make it compatible with that established for Arabidopsis. Table 3 provides the correspondence between the nomenclature proposed here and the Solyc chromosome address issued by ITAG 2.40 reference annotation (Tomato Genome Consortium, 2012) as well as the various names given in the literature to some tomato ERFs and the reported putative function of the few ERFs subjected to functional analysis are given (Table 3).

Table 3. Correspondence between the unified nomenclature of the *ERF* gene family and their Solyc ID. When relevant, others names proposed in the literature are also listed, as well as their reported putative function.

New names	Solyc ID	Other names	Reported function	References
Sl-ERF.A1	Solyc08g078180			

Sl-ERF.A2	Solyc03g093610			
Sl-ERF.A3	Solyc05g052050	pti4	Disease resistance	(1,2)
Sl-ERF.A4	Solyc08g078170			
Sl-ERF.A5	Solyc08g007230			
Sl-ERF.B1	Solyc05g052040			
Sl-ERF.B2	Solyc03g093560	ERF5	Drought and salt tolerance	(3)
Sl-ERF.B3	Solyc05g052030	LeERF4	Ethylene response and Fruit ripening	(4–6)
Sl-ERF.B4	Solyc03g093540			
Sl-ERF.B5	Solyc03g093550			
Sl-ERF.B6	Solyc01g090300			
Sl-ERF.B7	Solyc01g090310			
Sl-ERF.B8	Solyc01g090320			
Sl-ERF.B9	Solyc01g090340			
Sl-ERF.B10	Solyc01g090370			
Sl-ERF.B11	Solyc05g050790			
Sl-ERF.B12	Solyc09g066350			
Sl-ERF.B13	Solyc08g078190			
Sl-ERF.C1	Solyc05g051200	TERF1/JERF2	Salt tolerance	(7)
Sl-ERF.C2	Solyc04g014530			
Sl-ERF.C3	Solyc09g066360			
Sl-ERF.C4	Solyc09g089930	TSRF1	Pathogen resistance	(8)
Sl-ERF.C5	Solyc02g077360			
Sl-ERF.C6	Solyc02g077370	pti5	Disease resistance	(2)
Sl-ERF.C7	Solyc11g011740			
Sl-ERF.C8	Solyc11g011750			
Sl-ERF.C9	Solyc11g006050			
Sl-ERF.C10	Solyc03g005520			
Sl-ERF.D1	Solyc04g051360			
Sl-ERF.D2	Solyc12g056590			
Sl-ERF.D3	Solyc01g108240			
Sl-ERF.D4	Solyc10g050970			
Sl-ERF.D5	Solyc04g012050			
Sl-ERF.D6	Solyc04g071770			
Sl-ERF.D7	Solyc03g118190			
Sl-ERF.D8	Solyc12g042210			
Sl-ERF.D9	Solyc06g068830			
Sl-ERF.E1	Solyc09g075420	LeERF2	Ethylene response and seed germination	(9)
Sl-ERF.E2	Solyc06g063070	JERF1	Salt tolerance	(10)
Sl-ERF.E3	Solyc03g123500	JERF3	Salt tolerance	(11)
Sl-ERF.E4	Solyc01g065980	Sl-ERF6	Fruit ripening	(12)

Sl-ERF.E5	Solyc12g049560			
Sl-ERF.F1	Solyc10g006130	Sl-ERF36	Photosynthesis and growth regulation	(13)
Sl-ERF.F2	Solyc07g064890			
Sl-ERF.F3	Solyc07g049490			
Sl-ERF.F4	Solyc07g053740			
Sl-ERF.F5	Solyc10g009110	Sl-ERF3/ LeERF3b	Stress response	(4,14)
Sl-ERF.F6	Solyc12g005960			
Sl-ERF.F7	Solyc03g006320			
Sl-ERF.F8	Solyc01g067540			
Sl-ERF.F9	Solyc05g013540			
Sl-ERF.G1	Solyc01g095500			
Sl-ERF.G2	Solyc06g082590	pti6/SlCRF1	Hormone and stress response	(1,15)
Sl-ERF.G3	Solyc03g007460			
Sl-ERF.G4	Solyc06g051840			
Sl-ERF.H1	Solyc06g065820	LeERF1	Fruit ripening	(16)
Sl-ERF.H2	Solyc06g068360			
Sl-ERF.H3	Solyc03g116610			
Sl-ERF.H4	Solyc01g090560			
Sl-ERF.H5	Solyc05g050830			
Sl-ERF.H6	Solyc03g120840			
Sl-ERF.H7	Solyc06g066540			
Sl-ERF.H8	Solyc08g066660			
Sl-ERF.H9	Solyc07g042230			
Sl-ERF.H10	Solyc04g054910			
Sl-ERF.H11	Solyc12g056980			
Sl-ERF.H12	Solyc04g072900			
Sl-ERF.H13	Solyc12g013660			
Sl-ERF.H14	Solyc05g052410			
Sl-ERF.H15	Solyc06g050520			
Sl-ERF.H16	Solyc01g008890			
Sl-ERF.H17	Solyc01g014720			
Sl-ERF.H18	Solyc01g091760			
Sl-ERF.H19	Solyc02g067020			
Sl-ERF.J1	Solyc02g090770			
Sl-ERF.J2	Solyc02g090790			
Sl-ERF.J3	Solyc05g009450			

4.1.2 Identification of ERF genes exhibiting ripening-associated pattern of expression

Comprehensive transcriptomic profiling of tomato ERF genes in vegetative and reproductive tissues was carried out using the online TomExpress platform and associated data mining tools (http://gbf.toulouse.inra.fr/tomexpress). Heatmap representations were constructed in two different ways based on Spearman's correlation or Euclidian distance, in order to cluster genes according to their expression pattern or level of expression, respectively. Heatmap representation based on the expression pattern distributed the 77 tomato ERFs into 6 distinct clades (Figure 1). Clade I gathers 27 genes (Sl-ERF.A1,A3, B1-B3, B13, C1, D2, D6, D7, E1, E2, E4, E5, F1-F6, G3, H10, H12-H15, and H17) displaying an increase in their expression at the onset of ripening (Breaker stage, Br), peaking at 5 days post-breaker (Br5), and then declining at late ripening stages.

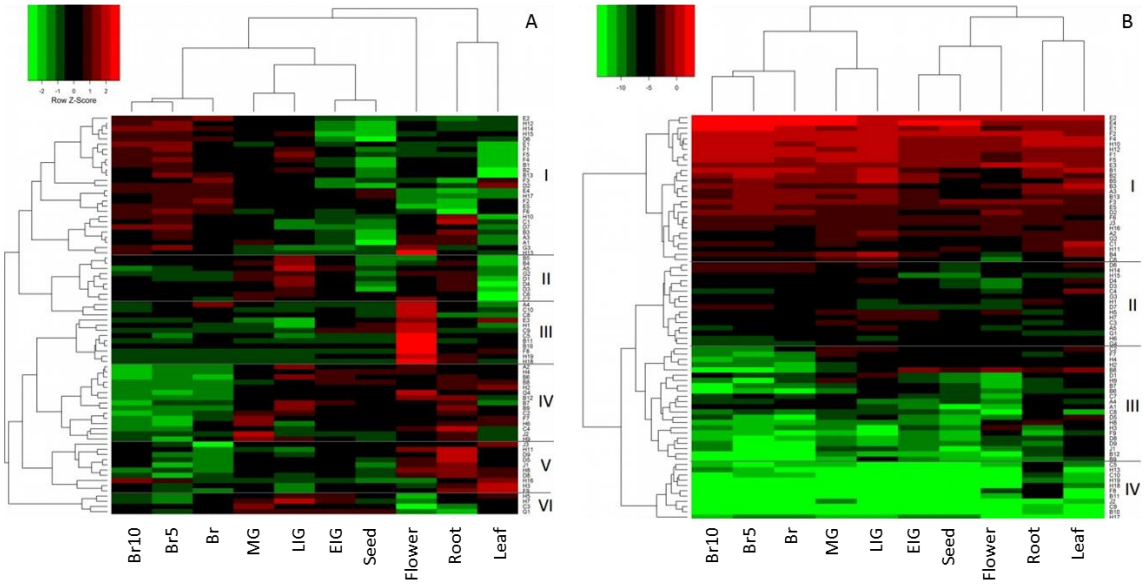

Figure 1. A, Heatmap of the expression pattern of tomato ERF family genes in different tissues and developmental stages. The distance used for the clustering is based on the Spearman correlation which allows to clustering gene expressions by patterns. For a given row of the heatmap, green and red colors correspond respectively to low and high values of expression of the considered gene which allows an easier comparison of similar patterns. B, Heatmap of the expression level of tomato ERF family genes in different tissues and developmental stages. The distance used for the clustering is based on the classical Euclidean which allows to cluster gene expressions by expression levels. Green and red colors correspond respectively to low and high values of all expressions. For a given gene and tissue or stage, the expression value corresponds to the mean of normalized expressions of all cultivars contained in the TomExpress platform (from all available RNA-Seq data sets). EIG, Early Immature Green; LIG, Late Immature Green; MG, Mature Green; Br, Breaker; Br5, 5 days post-breaker; Br10, 10 days post-breaker.

This pattern of expression suggests a potential role of these genes in regulating the ripening process. Clade II contains 9 genes (Sl-ERF.A5, B4, B5, C6, C7, D1, D3, D4, and G2) with preferential expression in young unripe fruits that declines at the onset of ripening. Genes from Clade III (Sl-ERF.A4, B10, B11, C5, C8-C10, E3, F8, H1, H18, and H19) show transcript accumulation mainly in roots suggesting their specific involvement in the developmental process of this organ. Clade IV is made of 15 genes (Sl-ERF.A2, B6-B9, B12, C2, C4, F7, G4, H2, H4, H6, H9, and J2) strongly down-regulated during ripening and exhibiting high expression in roots, leaves, flowers and immature fruits. Clade V genes (Sl-ERF.D5, D8, D9, F9, H3, H8, H11, H16, J1, and J3) display the highest expression in roots, leaves and flowers, whereas those of clade VI (Sl-ERF.C3, G1, H5, and H7) are highly expressed at pre-ripening stages including early immature green (EIG), late immature green (LIG) and mature green (MG). Most ERFs (55 out of 77) exhibit a ripening-associated pattern of expression with 27 genes being up-regulated and 28 genes down-regulated during fruit ripening. A second heatmap representation generated by applying the Euclidian distance method to emphasize the expression level, classified the 77 ERFs in 4 separate clades. Clade I gathers 28 genes corresponding to ERFs most highly expressed in both vegetative and reproductive tissues. By contrast, genes from clade IV display very weak expression level in all the tissues while ERFs from Clade II and III show intermediate expression levels.

By comparing the output of the two clustering methods taking into account both the level and pattern of expression, 19 genes (Sl-ERF.A3, B1, B2, B3, B13, C1, D2, E1, E2, E4, E5, F1, F2, F3, F4, F5,F6, H10, and H12) were selected as best candidates in actuating the ripening process based on their ripening-related pattern and high expression levels. Transcript accumulation patterns of the selected 19 ERF genes, assessed by qRT-PCR, fully match those obtained using the online TomExpress pipeline, hence, confirming the consistency and robustness of this platform (Figure 2). Of particular interest, members of subclass E (Sl-ERF.E1, E2, and E4) are the most highly expressed during fruit ripening, displaying a net up-regulation at the onset of ripening starting after the mature green stage (Figure 2A and B, upper panel). Eight ERF genes (Sl-ERF.B1, B2, F1, F2, F4, F5, H10, and H12) display up-regulation at the onset of ripening but have significantly lower level of expression than subclass E members (Figure 2A and B, middle panel). Eight other ERFs (Sl-ERF.A3, B3, B13, C1,

D2, E5, F3, and F6) show the lowest level of transcript abundance among the selected 19 ERF genes and display enhanced expression during ripening (Figure 2A and B; lower panel).

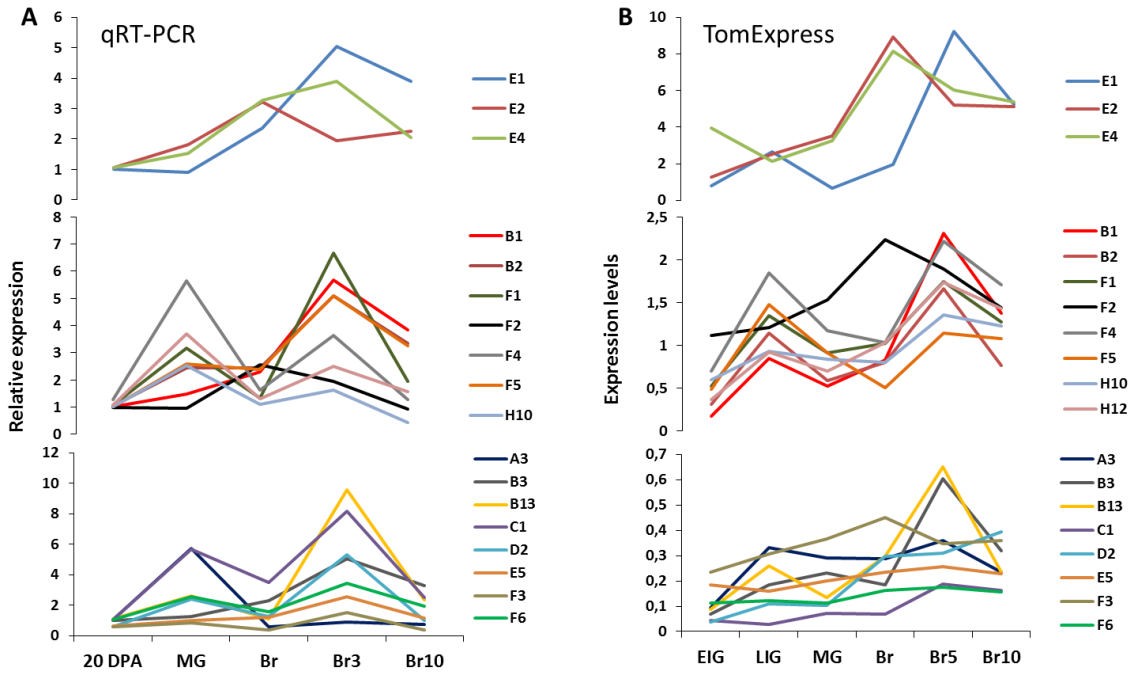

Figure 2. Quantitative RT-PCR results confirm the expression data of ERF genes obtained from the TomExpress platform. Panel A shows relative expression of ERFs during fruit developmental stages and ripening process in wild type assessed by qRT-PCR. The relative expression of each ERF gene at the 20 days post-anthesis (20 DPA) stage is standardized to 1 (referring to the Sl-Actin gene as an internal control). Panel B shows expression data obtained from the TomExpress platform developed by the GBF laboratory (from all available RNA-Seq data sets of Tomato transcriptome analyses). For each gene, the expression represents the mean of normalized counts of all tomato cultivars contained in TomExpress. Graphs in B are separated by scale. EIG, Early immature green; LIG, Late immature green; MG, Mature green; Br, Breaker; Br3, 3 days post-breaker; Br5, 5 days post-breaker; Br10, 10 days post-breaker.

4.1.3 Ripening-related ERF genes show altered expression in the tomato ripening mutants

To shed more light on the potential role of the selected ERFs in fruit ripening, we compared their expression in wild type and *rin*, *nor* and *Nr* ripening mutants at different ripening stages including 20 days post-anthesis (DPA), Mature Green (MG), Breaker (Br), 3 days post-breaker (Br3) and 10 days post-breaker (Br10). We took advantage of a newly generated tomato genetic resources where the *rin*, *nor* and *Nr* mutant loci have

been introgressed into the Micro-Tom genetic background. By minimizing the genotype effect, this plant material allows to assign more rigorously the changes in the expression of ERF genes to the ripening mutation rather than to a variation in the genetic background. It is important to mention that the ripening phenotypes of *Nr*, *rin* and *nor* in the Micro-Tom genetic background is strictly comparable to those described in other genotypes (Figure 3).

Figure 3. Ripening impaired mutants in Microtom genetic background. *Nr*, Never-ripening mutant; *rin*, ripening-inhibitor mutant; *nor*, non-ripening mutant; 20DPA, 20 days post-anthesis; MG, Mature green; Br, Breaker; Br3, 3 days post-breaker; Br10, 10 days post-breaker.

The qRT-PCR analyses indicated that among the 19 selected genes, the expression levels of 14 ERFs undergo alteration in the ripening mutants while, by contrast, the expression of 5 ERFs (Sl-ERF.A3, B1, B13, H10 and H12) did not show consistent change (Figure 4). Overall, eleven ERFs are consistently repressed (Sl-ERF.B3, C1, D2, E1, E2, E4, E5, F2, F3, F5 and F6) during the maturation phases in the ripening mutants (Figure 4B) whereas only three ERFs are consistently induced (Sl-ERF.B2, F1 and F4). Noteworthy, a higher number of ERFs are impacted in *rin* and *nor* mutants than in *Nr* throughout the ripening process, and all ERFs being down-regulated in *Nr* mutant are also down-regulated in *rin* and *nor* (Figure 4B). When considering specifically the onset of ripening (MG and Br stages), more ERFs are down-regulated in *rin* than in *nor* and

Nr (Table 4). Remarkably, four out of five members of ERF subclass E (Sl-ERF.E1, E2, E4 and E5) display reduced expression in the ripening mutants and the down-regulation of Sl-ERF.E1, E2 and E4 is ubiquitous to all ripening mutants suggesting their putative prominent role in the ripening process (Table 4). On the other hand, Sl-ERF.B2, Sl-ERF.F1 and Sl-ERF.F4, which exhibit down-regulation at the MG to Br transition in wild type fruit (Figure 2A and B, middle panel), are the only ERFs that undergo clear up-regulation in the ripening mutants suggesting that reduced expression levels of these ERFs at the onset of ripening might be required for normal ripening. Sl-ERF.B2 is the most consistently up-regulated ERF in all three *rin, nor* and *Nr* mutants throughout ripening indicating that high expression levels of this gene might contribute to refraining the ripening process. Likewise, the expression of Sl-ERF.F1 and Sl-ERF.F4, both encoding transcriptional repressors, show constant up-regulation in the ripening mutants suggesting they might be involved in inhibiting some essential ripening genes. Of particular interest, the expression of Sl-ERF.A3 displays strong down-regulation at the mature green stage in all ripening mutants and then retrieves normal expression level at later ripening stages, suggesting its putative role specifically in the transition from unripe to ripe fruit.

Table 4. ERF genes down-regulated at the onset of ripening in the tomato ripening mutants *rin, nor* and *Nr*. Numbers in parentheses correspond to the number of down-regulated ERFs in the corresponding ripening mutant(s).

Mutants	**ERFs down-regulated at MG and Br**
rin	**(8)** Sl-ERF.C1, Sl-ERF.E1, Sl-ERF.E2, Sl-ERF.E4, Sl-ERF.E5, Sl-ERF.F2, Sl-ERF.F5, Sl-ERF.F6
nor	**(6)** Sl-ERF.E1, Sl-ERF.E2, Sl-ERF.E4, Sl-ERF.E5, Sl-ERF.F2, Sl-ERF.F5
Nr	**(6)** Sl-ERF.C1, Sl-ERF.E1, Sl-ERF.E2, Sl-ERF.E4, Sl-ERF.F2, Sl-ERF.F6
rin-nor-Nr	**(4)** Sl-ERF.E1, Sl-ERF.E2, Sl-ERF.E4, Sl-ERF.F2
rin-nor	**(6)** Sl-ERF.E1, Sl-ERF.E2, Sl-ERF.E4, Sl-ERF.E5, Sl-ERF.F2, Sl-ERF.F5
rin-Nr	**(6)** Sl-ERF.C1, Sl-ERF.E1, Sl-ERF.E2, Sl-ERF.E4, Sl-ERF.F2, Sl-ERF.F6
nor-Nr	**(4)** Sl-ERF.E1, Sl-ERF.E2, Sl-ERF.E4, Sl-ERF.F2

4.1.4 Sl-ERF.E1, Sl-ERF.E2, Sl-ERF.E4, and Sl-ERF.F2 are the main ripening-associated ERFs

When specifically considering MG and Br stages that are essential for the initiation of ripening, the under-expression of Sl-ERF.E1, Sl-ERF.E2, Sl-ERF.E4 and Sl-ERF.F2 emerges as a common feature of *rin*, *nor* and *Nr* mutants (Table 4), indicating that these ERFs might be instrumental to fruit ripening. Noteworthy, the expression pattern of Sl-ERF.E2 and Sl-ERF.E4 matches that of RIN with the three genes displaying parallel increase between MG and Br stages, while the upraise of Sl-ERF.E1 expression occurs later at post-breaker stages (Figure 5). The search for conserved Cis-regulatory elements indicated that the promoter regions of these four ERF genes (Figure 6A) contain typical ethylene-response-elements (ERE) and putative RIN binding-sites (known as CArG-box). Accordingly, Sl-ERF.E1, Sl-ERF.E2 and Sl-ERF.E4 display ethylene-induced expression in mature green fruit (Figure 7). Indeed, treatment with the ethylene precursor ACC results in up to 6-fold increase in Sl-ERF.E1 transcript accumulation and 2-fold increase in the case of Sl-ERF.E2 and E4 but has no effect on Sl-ERF.F2 expression. Correlation studies, using the TomExpress platform and associated co-expression tools, indicated that expression of Sl-ERF.E2, Sl-ERF.E4 and Sl-ERF.F2 is highly correlated (Figure 8). Furthermore, Sl-ERF.E2 and Sl-ERF.E4 expression show a high coefficient of correlation with major ripening-associated and key ripening regulator genes including NOR, NR, CTR1, ACS4, AP2a, E4, E8, ERF.E2, PG2a, PSY1, PDS, AAT1, AAT2, α-amylase2 and α-amylase1 (Figure 8).

To investigate whether Sl-ERF.E1, E2, E4, F2 and RIN are involved in the same regulatory network, we first tested the ability of RIN protein to regulate the promoter activity of the four ERF genes. To this purpose, tobacco BY-2 protoplasts were co-transformed with the effector construct carrying the RIN coding sequence driven by the 35S constitutive promoter and with reporter constructs consisting of the GFP coding sequence driven by Sl-ERF.E1, E2, E4 or F2 promoters (Figure 6).

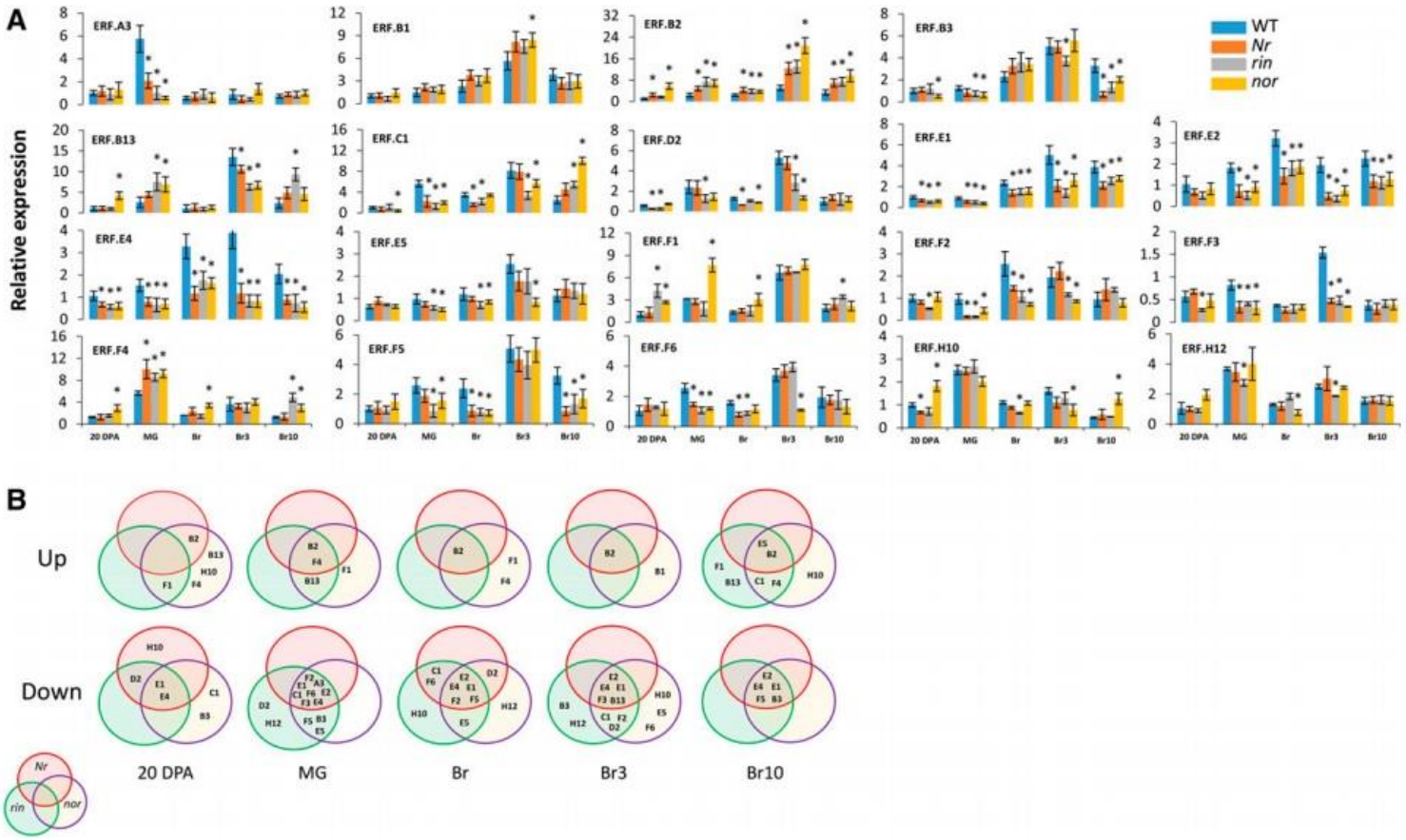

Figure 4. Modulated expression of ERF genes in *Nr*, *rin* and *nor* ripening mutants. A, the levels of transcripts were assessed by quantitative real-time PCR (qRT-PCR) and values represent the means of three biological replicates. Vertical bars represent ±SD of the means. *, $P < 0.05$ (Student's t-test). B, regulation of ERF genes at different fruit developmental and ripening stages in ripening mutants shown by Venn diagram. Up, up-regulation in ripening mutants compared with WT. Down, down-regulation in mutants compared with WT. In each color circle, genes, regulated in the corresponding, mutant are indicated. *Nr*, Never-ripening mutant in red; *rin*, ripening-inhibitor mutant in green; *nor*, non-ripening mutant in purple. 20DPA, 20 days post-anthesis; MG, Mature green; Br, Breaker; Br3, 3 days post-breaker; Br10, 10 days post-breaker.

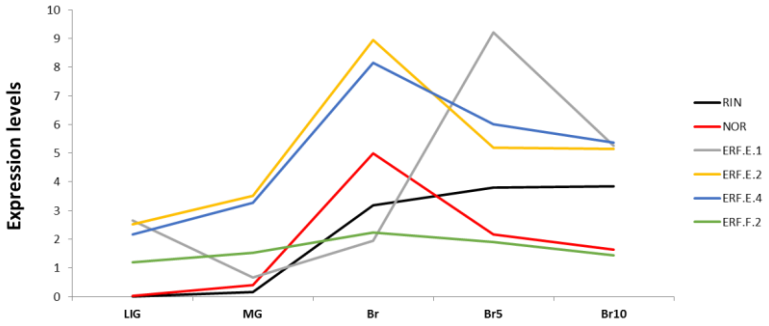

Figure 5. Expression data obtained from the TomExpress platform of *RIN*, *NOR*, *ERF.E1*, *E2*, *E4* and *F2*. For each gene, the expression represents the mean of normalized counts of all tomato cultivars contained in TomExpress. LIG, late immature green; MG, mature green; Br, breaker; Br5, 5 days post-breaker; Br10, 10 days post-breaker.

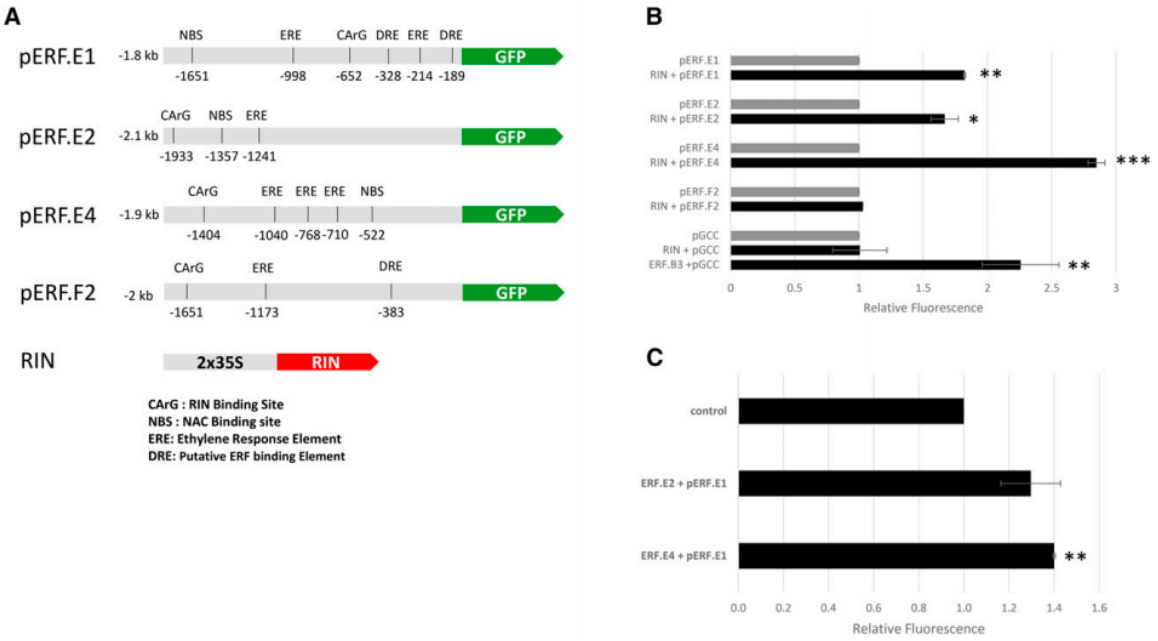

Figure 6. Transcriptional regulation of ERF.E1, ERF.E2, and ERF.E4. A, Presence of putative RIN-binding sites [CArG, C(C/T)(A/T)$_6$(A/G)G; NAC-binding site (NBS), CATGTG; and ERE, A(A/T)TTCAAA] and a putative ERF-binding element (DRE) in the promoters of ERF genes. The cis-acting elements identified are represented by black bars and localized from ATG. B, Transactivation of ERF promoters by RIN. Protoplasts were cotransfected with the GFP reporter fused to the promoters of ERFs (ERF.E1, E2, E4, and F2) and an effector plasmid expressing RIN under the control of the 35S promoter. In the transactivation assay of the pGCC synthetic promoter (4x GCC box) by RIN and ERF.B3, protoplasts were cotransfected with the GFP reporter fused to the synthetic promoter and an effector plasmid expressing either RIN or ERF.B3 under the control of the 35S promoter. Gray bars correspond to the control for each GFP reporter. Values represent means of three biological replicates. Error bars represent SD. C, Transactivation of ERF.E1 promoters by ERF.E2 and ERF.E4. Protoplasts were cotransfected with the GFP reporter fused to the promoter of ERF.E1 and an effector plasmid expressing either ERF.E2 or ERF.E4 under the control of the 35S promoter. Values represent means of three biological replicates. Error bars represent SD. *, $P < 0.01$; **, $P < 0.001$; and ***, $P < 0.0001$.

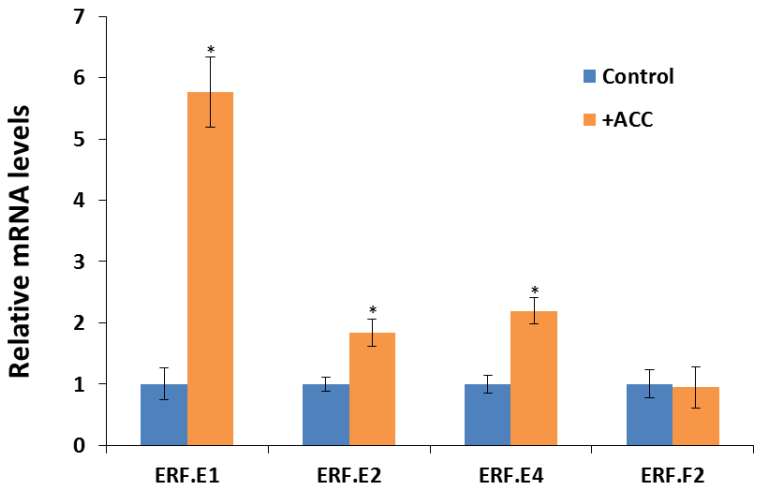

Figure 7. Ethylene regulation of ripening-associated ERFs. WT fruits at MG stage were treated with ACC solution by direct injection through the calyx end (Su et al., 2015) and then RNA was extracted 96 h after treatment. Control samples were injected only with buffer solution. The levels of transcripts were assessed by quantitative real-time PCR (qRT-PCR) and values represent the means of three biological replicates. Vertical bars represent ±SD of the means. *, $P < 0.05$ (Student's t-test).

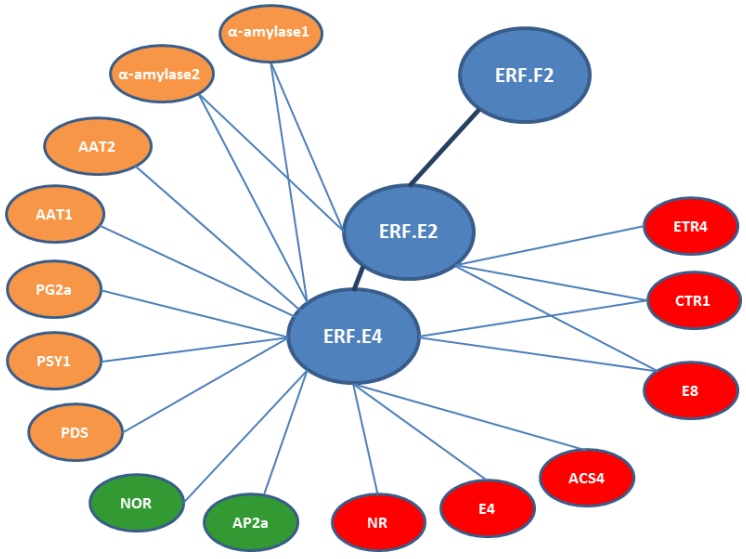

Figure 8. Correlation network of ERFs and ripening-associated genes. The network was generated from the analysis of co-expressions of ERFs and ripening-related genes in the TomExpress platform (with a correlation threshold > 0.85). ERF genes are shown as blue ovals, ethylene-related genes are shown as red ovals, ripening regulators are shown as green ovals, and genes encoding enzymes involved in fruit development and ripening are shown as orange ovals. ACS4, aminocyclopropane-1-carboxylic acid synthases; E4, E8, ethylene response genes; CTR1, ethylene signaling component; NR, ETR4, ethylene receptors; AP2a, NOR, ripening regulators; PSY1, phytoene synthase; PDS, phytoene desaturase; PG2a, polygalacturonase; AAT, alcohol acyltransferase.

Transactivation assays indicated that RIN is capable to act as positive regulator of the promoter activity of Sl-ERF.E1, E2 and E4, but has no impact on that of Sl-ERF.F2 promoter (Figure 6B). RIN has the strongest impact on Sl-ERF.E4 (2.8-fold increase) compared to Sl-ERF.E1 (1.8-fold increase) and Sl-ERF.E2 (1.6-fold increase). The activation of Sl-ERF.E1, E2 and E4 by RIN is consistent with the presence of a RIN binding site in their promoter regions (Figure 6A). To assess whether RIN activates the transcription of the target ERF.E genes via inducing the ethylene pathway, we tested the effect of RIN on the ethylene-inducible GCC box-containing promoter. As shown in Figure 6B, the activity of this highly ethylene-inducible promoter is not induced by RIN, thus ruling out the possibility that RIN activates ERF.E transcription through inducing ethylene production. In the same experiment, the synthetic ethylene-responsive promoter containing a GCC box was strongly induced by ERF.B3, shown previously to be an efficient activator of the GCC box (Pirrello et al., 2012). Further supporting the idea that these ERF.E genes undergo direct regulation by RIN, the use of chromatin immunoprecipitation (ChIP) sequencing and ChIP-chip approaches revealed the direct binding of RIN to ERF.E1 and ERF.E4 promoters (Fujisawa et al., 2013; Zhong et al., 2013). On the other hand, the delayed expression of SlERF.E1 compared with that of RIN (Figure 5) and its strong ethylene-induced expression suggest the putative regulation of this ERF gene by SlERF.E2 or SlERF.E4, whose expression precedes that of SlERF.E1. Therefore, we tested the ability of SlERF.E2 and SlERF.E4 proteins to regulate the transcriptional activity of SlERF.E1 (Figure 6C). The data indicate that SlERF.E4, but not SlERF.E2, is able to significantly enhance the transcriptional activity of the SlERF.E1 promoter.

4.1.5 Cloning and plant transformation for a set of ERF genes

Based on the results described above that led to potential ripening-related ERF genes, a set of genes (*ERF.B3*, *ERF.E1*, *ERF.E2*, *ERF.E4*, *ERF.F2*) were cloned by Golden Braid strategy for further Agrobacterium-mediated plant transformation of ripening-impaired tomato mutants such as *rin* and *Nr*. The expression pattern of these selected *ERFs* in wild type tomato fruit revealed consistent up-regulation during ripening especially after breaker stage. Most notably, in the mentioned ripening-impaired mutants they were all sharply repressed. In this context, the transformation of *rin* and *Nr*

plants by overexpressing ERF genes down-regulated in these mutants relies on the hypothesis of ripening-related metabolic activities could be recovered by the overexpression of potential regulators thus shedding light on the role of ERFs in tomato ripening.

For this purpose, genetic constructions containing the selected ERF genes were generated (Table 5). The special design features of GB vectors allowed the direct modulation of the transcriptional units leading to exchangeable building blocks that could be assembled into binary vectors further used for plant transformation. Two notable constructions were designed combining *ERF.E1* + *ERF.E2* or *ERF.E1* + *ERF.E2* + *ERF.E4* overexpressing simultaneously under the 35S promoter. It aimed to reinforce the overexpression effects of these genes acting in concert considering that some phenotypes underlie on the activation of more than one ERF.

Table 5. Genetic constructions containing transcriptional units of ERF genes generated by Golden Braid strategy used for WT, *rin* and *Nr* plants transformation.

Constructions (sense)	**Golden Braid vectors**	**WT, *rin*, *Nr* transformation**
ERF.B1	pUPD	
ERF.B3	pUPD	
ERF.E1	pUPD	
ERF.E2	pUPD	
ERF.E4	pUPD	
ERF.F2	pUPD	
2x35S_ERF.B3_Tnos	pDGB1alfa2	
2x35S_ERF.E2_Tnos	pDGB1alfa2	
2x35S_ERF.E4_Tnos	pDGB1alfa2	
2x35S_ERF.E1_Tnos	pDGB1alfa1	
NPTII_2x35S_ERF.B3_Tnos	pDGB1omega1	X
NPTII_2x35S_ERF.E2_Tnos	pDGB1omega1	X
NPTII_2x35S_ERF.E4_Tnos	pDGB1omega1	X
NPTII_2x35S_ERF.E1_Tnos	pDGB1omega1	
2x35S_ERF.E1+ERF.E2_Tnos	pDGB1omega2	
NPTII_2x35S_ERF.E1+ERF.E2+ERF.E4_Tnos	pDGB1alfa2	X

Transgenic plants were then generated using the wild type and *Nr* and *rin* mutants under the MicroTom background by Agrobacterium-mediated transformation. Up to date, 12

transgenic lines were developed: overexpressed lines for *ERF.B3, ERF.E2, ERF.E4* or *ERF.E1 + ERF.E2 + ERF.E4*, each of them in wild type, *rin* and *Nr* tomato plants (Table 5). Unfortunately, for all these transgenic lines, no significant visual effects were achieved in plant development and fruits from *rin* and *Nr* transformed mutants showed the same ripening-impaired phenotype. However, these results are in preliminary steps and many questions remain to be understood. Further studies on this direction will be addressed.

4.2 SECTION 2

4.2.1 Auxin delays ethylene production and peel colour shift

In order to assess the role of the auxin/ethylene interplay in controlling climacteric fruit ripening, tomato fruit samples were picked at mature green stage and randomly divided into four groups according to hormonal treatments: IAA (direct infiltration of IAA solution in the fruit tissue), ETHYLENE (exposure to the gaseous hormone), ETHYLENE + IAA (exposure to ethylene followed by IAA infiltrations), and CTRL (injection with buffer solution only).

All groups were evaluated on each day of the experiment in terms of ethylene levels and peel colour. These features characterize the ripening stages since the climacteric ethylene production and the accumulation of red pigment due to lycopene are strongly related to tomato ripening (Figure 1). In the control fruits, the ethylene production started to increase concurrently with the peel colour shift on D4, thus defining the breaker stage. However, it was found that both auxin and ethylene treatments altered the ripening behaviour. As predicted by the consolidated literature concerning the role of ethylene in tomato fruit ripening, the exposure to exogenous ethylene induced the ethylene production, the levels of which remained higher than the control throughout ripening. In addition, the transition from green to orange/red peel colour was accelerated in ethylene-treated fruits compared with the control fruits. By contrast, exogenous auxin delayed the burst of climacteric ethylene and the peel colour shift revealed by the colour evaluation which fruits remained predominantly green until D6. The reduced lycopene accumulation in IAA-treated fruits can also be observed in Figure 5A. Interestingly, considering the profile of ethylene production and the peel colour, combined treatment with both hormones led to an intermediate phenotype between fruits treated with only ethylene or auxin, clearly indicating that auxin hampers the effect of ethylene on promoting ripening.

Indeed, the expression patterns of ethylene-related genes elicited that auxin has an opposing role in regulating key effectors of ripening (Figure 2). ACS6 and ACS1a, genes associated to system I of ethylene production, were induced by IAA in the first days after treatment, whereas ACS2 and ACS4, known to be related to system II and up-regulated by ethylene, were down-regulated by auxin until breaker stage at D4. Furthermore, in fruits treated with ethylene and IAA, the responses were similar to

IAA-treated fruits, which revealed that the presence of auxin repressed ethylene-inducible genes. It highlights epistatic effects of auxin over ethylene.

Particularly notable is that the transcripts levels of an ACO related to tomato ripening (Van de Poel et al., 2012) were induced by ethylene treatment in D1 whereas IAA treatment seems to have had no effect on its expression at the transcriptional level. On the contrary: the expression of ACO4 – also associated to tomato ripening (Kumar et al., 2014) – was up-regulated by IAA, which suggests the role played by auxin in its regulation.

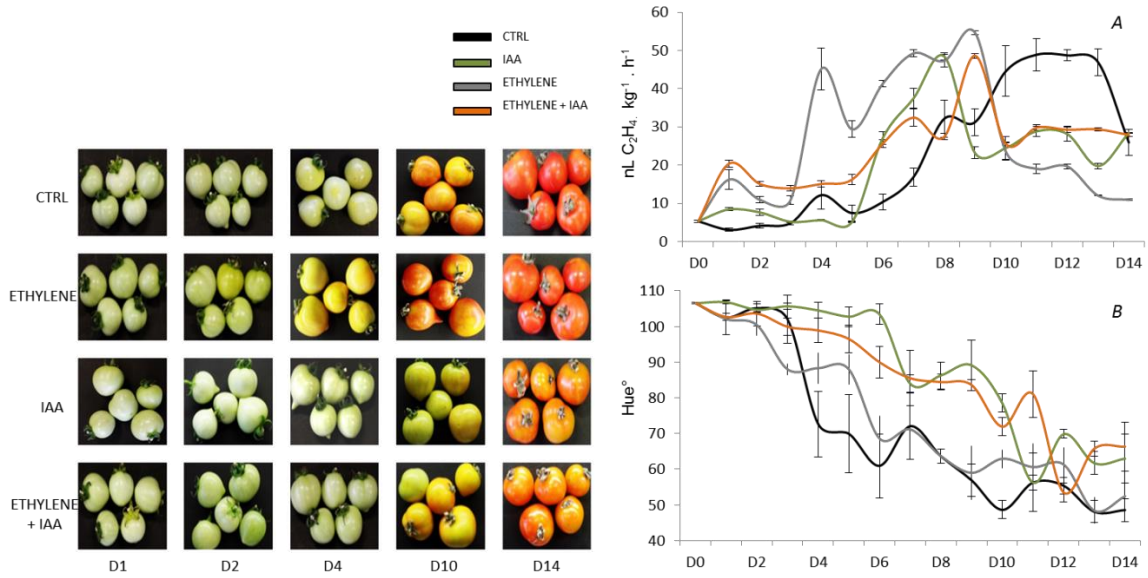

Figure 9. Pictures of tomato fruit from each experimental group: CTRL, IAA, ETHYLENE, ETHYLENE + IAA. A, ethylene production; B, peel colour expressed by hue angle. Each value is presented as mean (n=5). Vertical bars represent ±SD of the means. D followed by a number represents the day of experiment after hormonal treatments.

4.2.2 Auxin affects ethylene perception and signalling

The results revealed that auxin affected the ethylene perception, which is consistent with the ripening delay. The transcription of the ethylene receptors predominantly expressed in tomato fruit ripening, ETR3, ETR4 and ETR6 (Kevany et al., 2007), was down-regulated by IAA treatments (Figure 2). Moreover, those genes are known to be ethylene-regulated, thus confirming the induction observed in ethylene-treated fruits. In

fruits treated with ethylene followed by IAA infiltration, however, the effect of ethylene was repressed, indicating a potentially direct regulation by auxin of the ethylene perception and downstream signal transduction.

Ethylene responsive factors (ERFs) were also affected by auxin. Liu et al. (2016) reported that ERF.E1, ERF.E2 and ERF.E4 are important transcription factors of the ERF family in tomatoes displaying a consistent ripening-associated expression pattern. In the present study, results concerning the expression of these three genes revealed up-regulation by ethylene in the first days of ripening until breaker stages, whereas IAA treatments were found to block the ethylene effect, repressing the transcription. The expression of APETALA2a gene (AP2a), an important component of the regulatory network of tomato fruit ripening that acts downstream of RIN and member of the AP2/ERF superfamily (Chung et al., 2010), was also induced by ethylene and repressed by auxin, and maintained levels of the AP2a transcript close to those of the control. Once these genes can directly regulate ripening events associated to several metabolisms such as colour shift, ethylene biosynthesis or volatile production, these findings can be correlated with the overall ripening delay observed for auxin treatments.

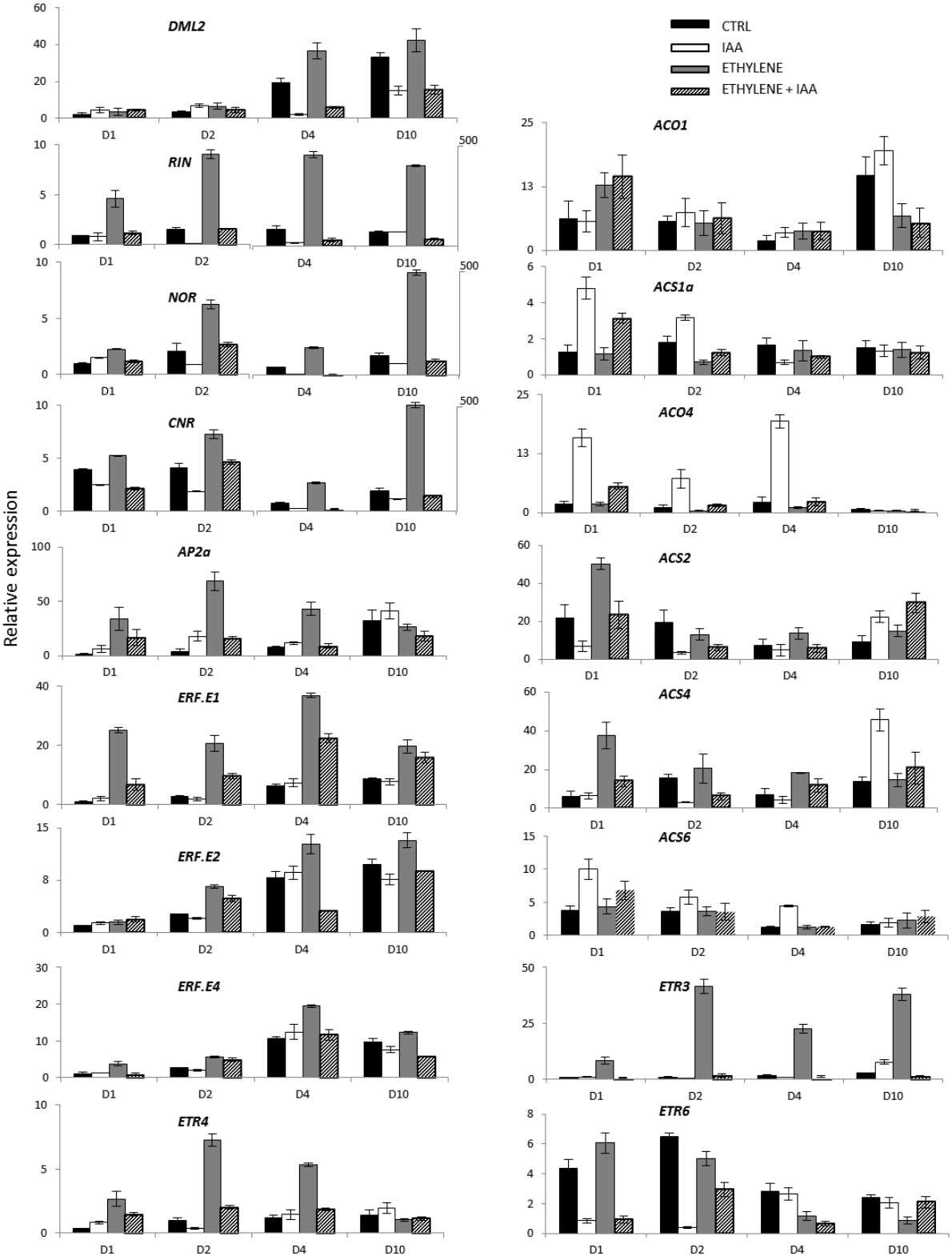

Figure 10. Relative expression of ripening-related genes in tomato fruit from each experimental group: CTRL, IAA, ETHYLENE, ETHYLENE + IAA. The levels of transcripts were assessed by quantitative real-time PCR (qRT-PCR) and values represent the means of three biological replicates. Vertical bars represent ±SD of the means. D followed by a number represents the day of experiment after hormonal treatments.

4.2.3 Antagonistic effects of ethylene and auxin on key ripening regulators

Altogether, the phenotypic data and the global expression profiling support the idea that auxin opposes the ethylene-induced ripening in the tomato fruit. In order to gain more insight into the mechanisms by which auxin treatment affects the ripening process, the expression of important ripening-related genes was verified (Figure 2). In the control fruits, transcript accumulation of central regulators of tomato fruit ripening – RIN, CNR and NOR – were strongly up-regulated during ripening, especially at breaker stages (Figure 2). By contrast, auxin treatments led to down-regulation of these genes, most notably in fruits treated with both hormones. This reinforces the idea of the predominant effect of auxin over ethylene on regulating genes known to be ethylene-responsive and furthermore highlights that the expression of master regulators of ripening is also under auxin control.

Similar results were observed for DML2 (DEMETER-like DNA demethylase): up-regulation at breaker/red stages in controls and ethylene-treated fruits and down-regulation in IAA- and ethylene + IAA-treated fruits. It has been proposed that DML2 is necessary for the active demethylation of RIN, NOR and CNR promoter regions (Liu et al., 2015) and our results provide some evidence for auxin acting in different steps of the ripening regulation network, even upstream of the so-called master regulators, thus supporting the relevant role of auxin in controlling ripening.

4.2.4 Ethylene-auxin crosstalk on IAA conjugation

Our results concerning IAA conjugation showed that free IAA levels in control fruits were reduced in the first days of ripening, whereas the ester- and amide-linked conjugated forms increased in the same time period, therefore reflecting that active auxin removal is required for normal ripening (Figure 3). Nonetheless, ethylene-treated fruits revealed an accelerated conjugation: compared with the control, fewer contents of free IAA were detected in the first days, while conjugated forms, especially amide-linked, had already been detected.

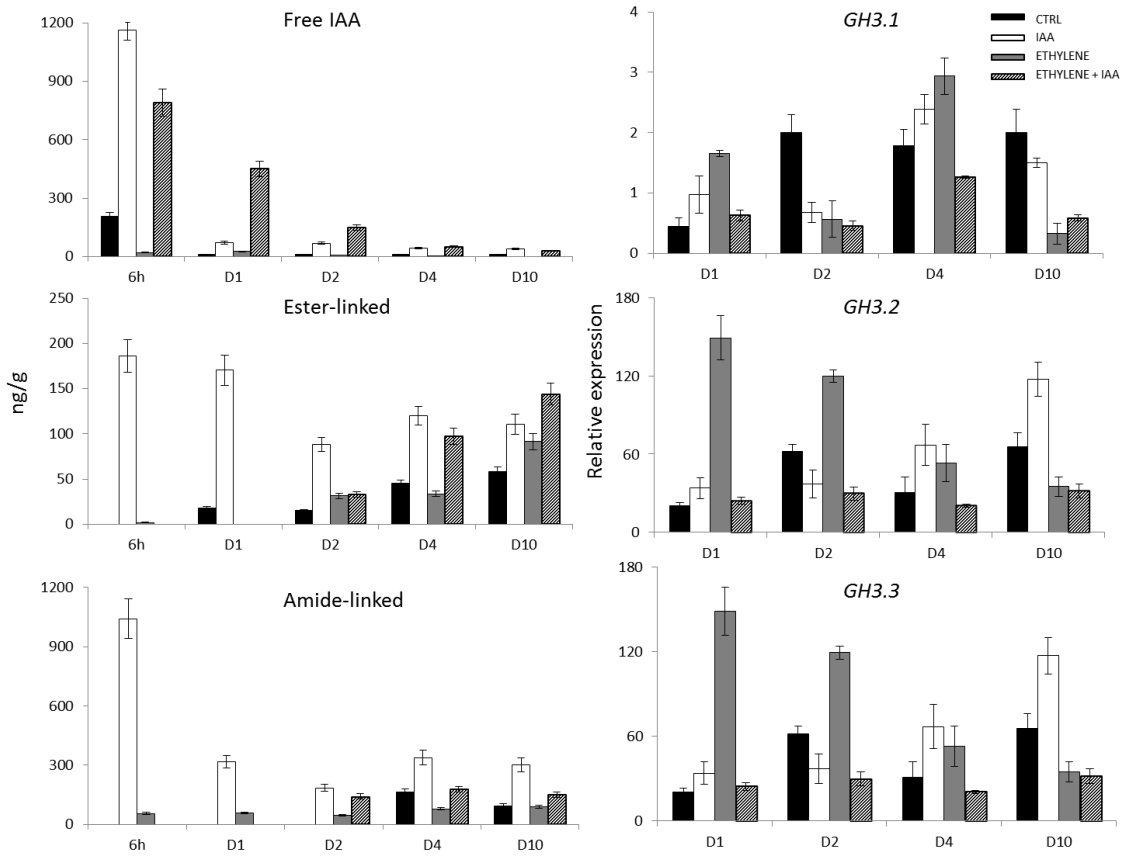

Figure 11 Free IAA levels, ester- and amide-linked IAA conjugated forms and relative expression of GH3 genes (GH3.1, GH3.2, GH3.3) in tomato fruit from each experimental group: CTRL, IAA, ETHYLENE, ETHYLENE + IAA. Free IAA determination was assessed by the method proposed for Ludwig-Müller et al. (2008) with modifications. Conjugated forms were assessed by solid phase extraction according to a method adapted from Chen et al. (1988). The levels of transcripts were assessed by quantitative real-time PCR (qRT-PCR). All values represent the means of three biological replicates. Vertical bars represent ±SD of the means. D followed by a number represents the day of experiment after hormonal treatments.

In order to verify the auxin and ethylene responsiveness of genes encoding Gretchen Hagen proteins (GH3) – IAA-amide synthetases known to be associated with IAA conjugation (Korasick et al., 2013) – transcripts levels were assessed for three GH3 genes: GH3.1, GH3.2 and GH3.3 (Figure 3). These genes have shown a ripening-related expression pattern and up-regulation by ethylene in the first days after treatment. Notably, auxin treatments seem to have no inducible effects in the first ripening stages, but only after D4. Altogether, these results suggest that ethylene might induce auxin conjugation, possibly through up-regulation of ethylene-inducible GH3 genes, shedding light on a potential crosstalk point between auxin and ethylene.

4.2.5 Tomato fruit volatile profile is affected by auxin

Previous studies have demonstrated the effects of auxin on tomato peel colour (Su et al., 2015) and, in this study it was further evaluated the impact of auxin on the tomato fruit volatile profile throughout ripening by performing SPME analyses. The tomato's volatile profile here is represented by 22 detected compounds (Table 6): 6 out of the 22 compounds derived from the isoprenoid pathway (β-ionone; β-cyclocitral; citral; α-copaene; 2,2,6-trimethylcyclohexanone; geranyl acetone), 2 derived from carbohydrates (2-ethylfurane; 2-pentylfurane), 6 derived from amino acids (butylated hydroxyl toluene; 2-ethylhexanol; styrene; methyl salicylate; methyl heptanoate; 2-isobutylthiazole) and 8 derived from the lipoxygenase pathway (cis-3-hexenal; cis-3-hexenol; hexanal; nonanal; trans-2-octenal; trans-2-heptenal; trans-2-hexenal; trans, trans-2,4-hexadienal).

Principal component analysis (PCA) was applied to highlight the differences caused by the hormonal treatments at the breaker stage (Figure 4A) and at the red stage (Figure 4B). Considering the breaker stage, the first two principal components (PC1 and PC2) accounted for about 72% of the total variability among the samples when volatile compounds were used as variables. The samples for the four experimental groups (CTRL, IAA, ETHYLENE, ETHYLENE + IAA) were placed separately in different regions of the chart (Figure 4A), providing evidence that the volatile profile of the fruits was affected by the hormonal treatments. PC1, representing 48.12% of the total variability, clearly separated the control samples on the positive quadrant at the right side of the chart, which suggests a predominance of the compounds clustered at the same region of the chart, elicited by the Biplot PCA (Cluster 1; Figure 4A). Samples from the group ETHYLENE were mainly separated by the PC2 and placed on the positive side of the chart next to the compounds nonanal, β-ionone and 2-isobutylthiazole, indicating that these substances tended to increase in ethylene-treated fruits. By contrast, the samples from the IAA and ETHYLENE + IAA groups were placed in the negative region of the chart, quite separate from the other groups but next to each other, revealing that: (i) fruits from these groups retained a similar volatile profile, (ii) the overall compounds tended to decrease in auxin-treated samples and (iii) auxin treatment was epistatic over ethylene treatment.

Table 6. Volatile compounds detected by SPME in MicroTom tomato fruit throughout ripening.

RI	Compounds	Precursor	Odor description*
1157	cis-3-hexenal	Fatty acids	Green, Herbaceous
1388	nonanal	Fatty acids	Citrus, Floral
1425	trans-2-octenal	Fatty acids	Green, Herbaceous
1085	hexanal	Fatty acids	Green, Herbaceous
1321	trans-2-heptenal	Fatty acids	Green, Herbaceous
1222	trans-2-hexenal	Fatty acids	Green, Fruity
1407	trans-2,4-hexadienal	Fatty acids	Green, Citrus
1381	cis-3-hexenol	Fatty acids	Green, Herbaceous
1393	2-isobutylthiazole	Amino acids	Green, Herbaceous, Tomato
1284	methyl heptanoate	Amino acids	Fruity
1761	methyl salicylate	Amino acids	Menthol
1248	styrene	Amino acids	Fruity, Sweet
1487	2-ethylhexanol	Amino acids	Floral
1905	BHT (butylated hydroxyl toluene)	Amino acids	Camphor
1211	2-pentylfurane	Carbohydrates	Fruity
1701	2-ethylfurane	Carbohydrates	Sweety
1924	β-ionone	Isoprenoids	Fruity
1601	β-ciclocitral	Isoprenoids	Herbaceous
1726	citral	Isoprenoids	Citrus
1850	geranyl acetone	Isoprenoids	Floral, Fruity
1305	2,2,6-trimethylcyclohexanone	Isoprenoids	Woody
1475	α-copaene	Isoprenoids	Woody

*Source: http://www.thegoodscentscompany.com/.

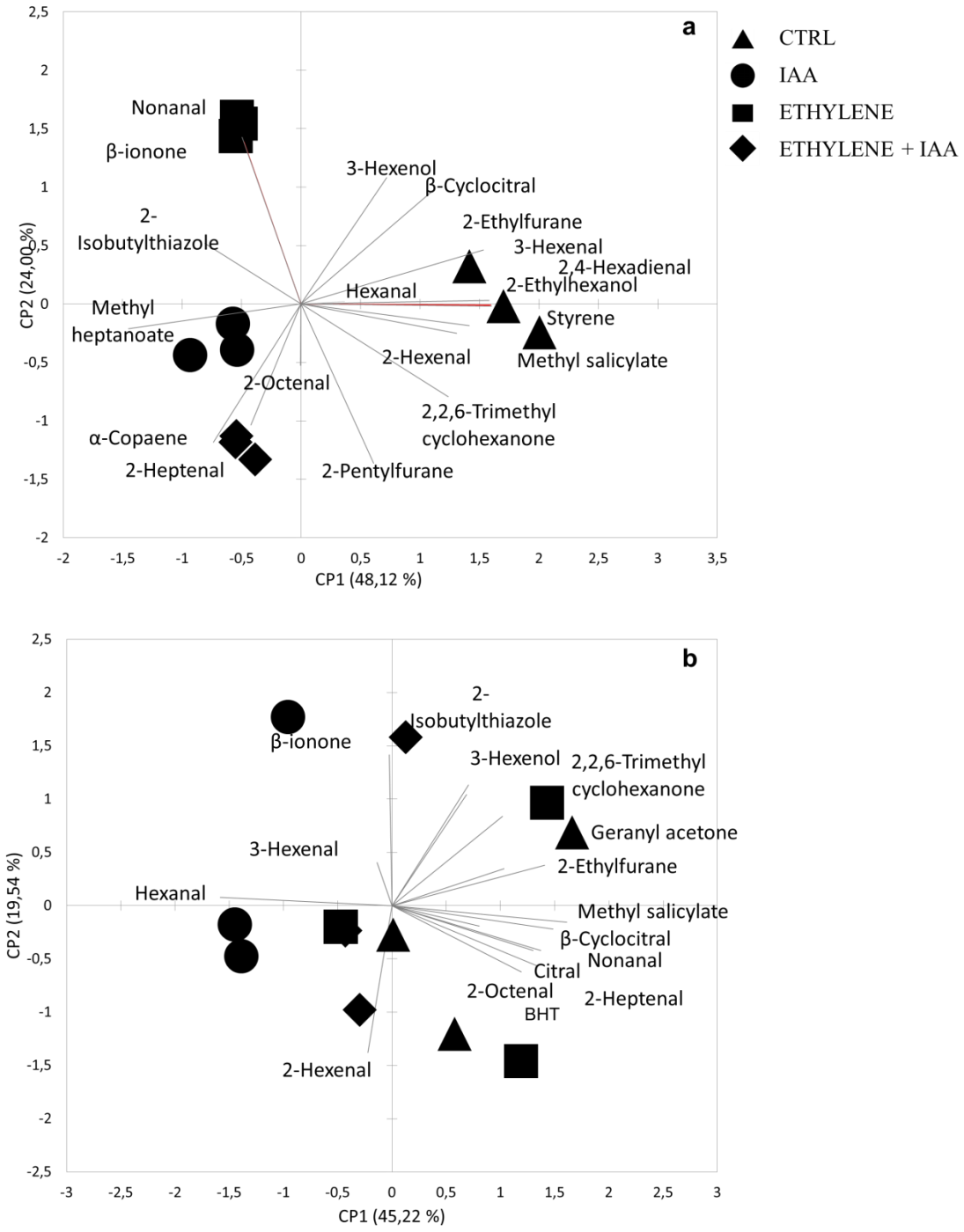

Figure 12. PCA of volatile compounds detected by SPME in MicroTom tomato fruit from each experimental group: CTRL, IAA, ETHYLENE, ETHYLENE + IAA. (a) Samples representing breaker stage of ripening, (b) samples representing red stage of ripening. The data input represents the means of three biological replicates.

Indeed, PCA summarized the differences caused by the hormonal treatments in the volatile profile through the identified compounds then characterizing the samples at the breaker stages (Figure 4A). It is worth mentioning that IAA- and ethylene + IAA-treated fruits are likely to retain a similar profile in comparison with CTRL and ETHYLENE groups due to its distribution in the PCA chart. Conversely, Biplot PCA for the red stage fruits showed different results: samples were not clearly separated or clustered in the PCA chart, indicating that there were no profound differences on the volatile profile of the fruits at that ripening stage (Figure 4B). Moreover, it suggests that fruits at the red stage tended to recover the volatile profile regardless of the ripening delay caused by the auxin treatments.

4.2.6 Carotenoids' contents and volatile-related genes

In order to shed more light on the effects of the auxin and ethylene treatments on the volatile compounds, the abundance of substances derived from the isoprenoid pathway (β-ionone; β-cyclocitral; citral; α-copaene; 2,2,6-trimethylcyclohexanone; geranyl acetone) were evaluated as well as the contents of lycopene and β-carotene. The relative expression of the genes related to carotenoids cleavage dioxygenases such as *CCD1A* and *CCD1B* (Simkin et al., 2004) were also evaluated (Figure 5). Lycopene contents in D10 and D14 were lower in IAA- and ethylene + IAA-treated fruits and higher in ethylene-treated fruits, which is in accordance with the peel colour measurements and the pictures shown in Figure 1. In addition, no significant differences in the β-carotene levels were found between the groups (Figure 5A). Su et al. (2015) achieved similar results concerning the carotenoids' levels once auxin exposure had affected only the lycopene contents in tomato fruit.

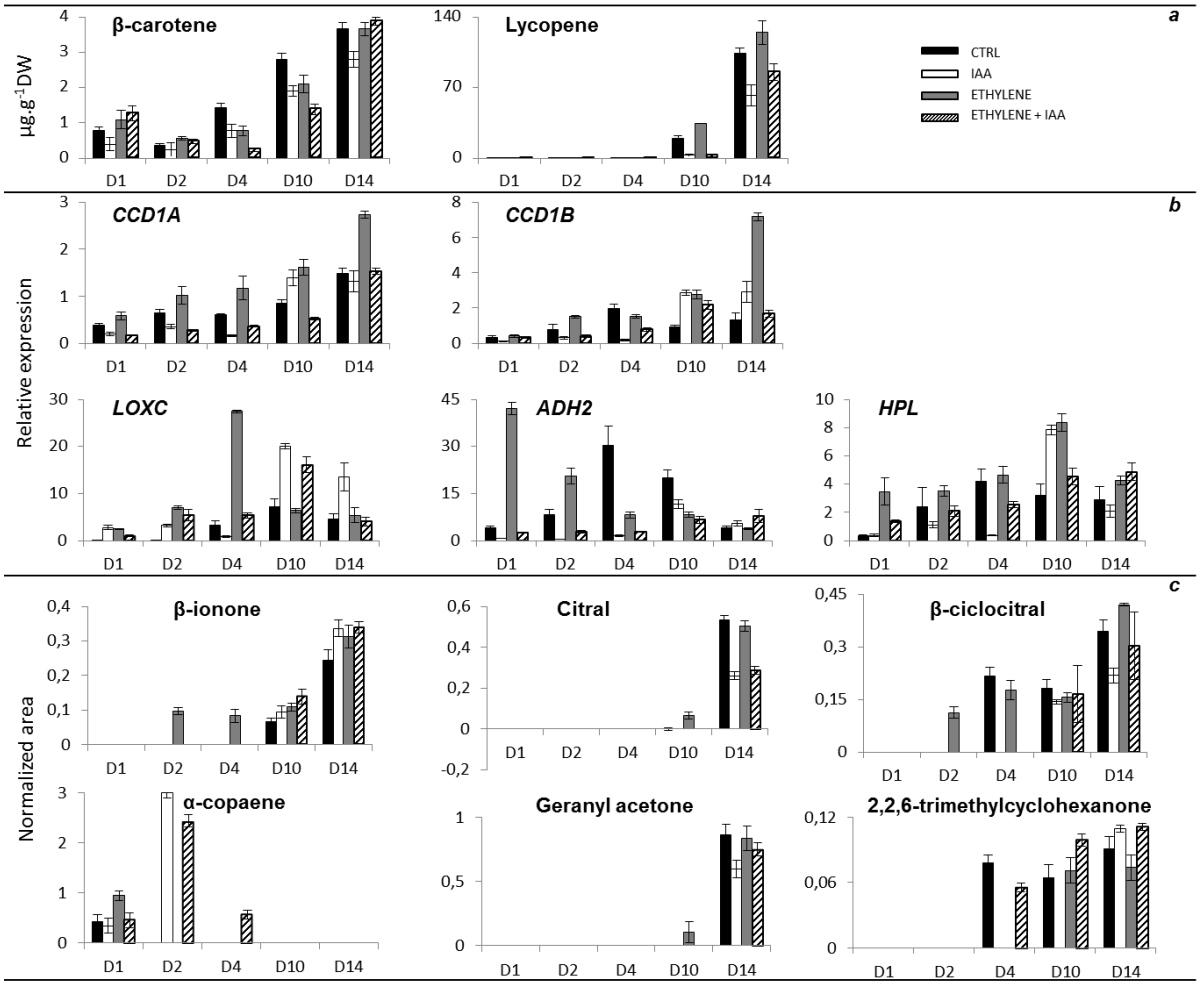

Figure 13. (a) Assessment of lycopene and β-carotene contents by the method proposed Sérino et al. (2009). (b) Relative expression of volatile-related genes in tomato fruit from each experimental group: CTRL, IAA, ETHYLENE, ETHYLENE + IAA. (c) Representation of volatile compounds abundance according to the normalized area of the chromatogram as detected by SPME. The levels of transcripts were assessed by quantitative real-time PCR (qRT-PCR). All values represent the means of three biological replicates. Vertical bars represent ±SD of the means. D followed by a number represents the day of experiment after hormonal treatments.

CCD1A and *CCD1B* transcripts levels were also down-regulated by the IAA and up-regulated by the ethylene, whereas treatment with both hormones led to a similar expression pattern of IAA-treated fruits (Figure 5B). Once the enzymes' encoding by these genes use carotenoids as substrates for the volatile substances' biosynthesis, the reduction or the delay in the production of the volatile compounds (Figure 5C) in IAA-treated fruits, especially those derived from the isoprenoid pathway, these results are in

agreement with the lower carotenoids contents and the down-regulation of the mentioned volatile-related genes.

Following the same pattern which auxin represses and ethylene induces, the expression of other volatile-related genes such as ADH2, LOXC and HPL – both related to the lipoxygenase pathway (Paliyath et al., 2008) – were also found to be up-regulated by the ethylene, especially at the breaker stages. By contrast, IAA delayed the increase of the transcripts levels until D10 (Figure 5B). Altogether, these results support the retarding effects of the IAA treatments on the tomato fruit's volatile profile, and consolidate the role of auxin in delaying ripening.

5 DISCUSSION

5.1 SECTION 1

Although ethylene is known since a long time to be a key factor in initiating and orchestrating climacteric fruit ripening (Giovannoni, 2004), the molecular mechanisms by which this hormone recruits the ripening-associated genes remain poorly understood. The ERF transcription factors are downstream components of ethylene signaling, known to regulate the expression of ethylene-responsive genes (Solano et al., 1998; Pirrello et al., 2012). It is widely accepted that ethylene is instrumental to climacteric ripening and ERFs have been assigned a central role in mediating ethylene responses. Nevertheless, so far, little is known about the role of the ERF family members in fruit ripening and, strikingly, reports describing ripening mutants affected in ERF genes are lacking, likely due to functional redundancy among members of this large gene family. Up to 77 ERF genes are found in the tomato genome (Pirrello et al., 2012), but the functional significance of the overwhelming majority of these still awaits elucidation. The comprehensive expression profiling of tomato ERF genes performed in the present study, combined to the use of the ripening-impaired mutants Nr, rin and nor (Lanahan et al., 1994; Vrebalov et al., 2002; Giovannoni, 2004) allowed the identification of a small subset of ERF genes whose expression is highly linked to the ripening process. Overall, 19 ERFs exhibited ripening-associated patterns and elevated levels of expression in fruit. Among these, four ERFs (Sl-ERF.E1, Sl-ERF.E2, Sl-ERF.E4 and Sl-ERF.F2), which display dramatic down-regulation in *rin, nor* and *Nr* ripening mutants, emerge as strong candidates to play a key role in climacteric fruit ripening.

The cumulative RNA-seq data processed by TomExpress pipeline indicated that a high number of tomato ERF genes exhibit fruit development- and ripening-associated pattern of expression (Fig.1). Indeed, among the 77 tomato ERFs, up to 55 members show high correlation with fruit ripening which may explain why the majority of the ERF genes identified so far were reported to exhibit a ripening-related pattern of expression (Tournier et al., 2003; Chen et al., 2008; Sharma et al., 2010; Lee et al., 2012; Pirrello et al., 2012; Liu et al., 2014). Among the 77 tomato ERFs, 27 show enhanced expression at the onset of ripening, while 28 others display a decreased expression during ripening, suggesting that different ERFs may have contrasting roles in fruit ripening. Interestingly, we show here that some genes belonging to the same clade, exhibit similar

expression profiles, suggesting a link between structural subclasses and physiological function. Of particular note, most ERFs from subclasses E (4 out of 5) and F (6 out of 9) show a ripening-related pattern and a high expression levels, suggesting their prominent role in fruit ripening. The potential role of members of subclass E and F is consistent with the ripening-associated pattern previously described for Sl-ERF.E1 (named LeERF2 in Tournier et al., 2003) and Sl-ERF.F5 (named LeERF3b in Chen et al., 2008). In addition, Sl-ERF.E1 was described as ethylene-inducible and as a positive regulator of a feedback regulation loop via the control of ethylene biosynthesis genes ACS and ACO (Zhang et al., 2009). Another subclass E member, Sl-ERF.E4 (named Sl-ERF6 in Lee et al., 2012), has been reported to play an important role in fruit ripening by integrating ethylene and carotenoid pathways. This is in agreement with previous report on RAP2.2, a subclass E Arabidopsis ERF, shown to regulate the expression of carotenoid biosynthesis genes via binding to the ATCTA cis-element in the promoter regions of PSY and PDS (Welsch et al., 2007). Moreover, correlation analysis revealed that the expression of Sl-ERF.F1 is positively correlated with α-carotene accumulation suggesting the involvement of subclass F members in controlling fruit ripening through the regulation of carotenoid accumulation (Lee et al., 2012). More recently, a dominant repression strategy showed that Sl-ERF.B3 controls fruit ripening through regulating climacteric ethylene production and carotenoid accumulation (Liu et al., 2014). On the other hand, Sl-ERF.H1 (named LeERF1 in Li et al., 2007) was reported to affect some ripening aspects like fruit softening in tomato even though it failed to show a typical ripening-related expression pattern (Li et al., 2007). Likewise, Sl-ERF.C2 display low expression in fruit, but shows negative correlation with trans-lycopene accumulation suggesting its putative role in fruit ripening (Lee et al., 2012).

Interestingly, most ERF genes selected in the present study based on their consistent ripening-associated pattern of expression, also display altered expression in Nr, rin and nor tomato ripening mutants, further suggesting their putative involvement in ethylene-mediated ripening regulatory networks. Among these, members of subclass E seem to be the most active, sustaining the hypothesis that this subclass might play a central role in controlling fruit ripening. The data corroborate previous studies showing that Sl-ERF.E2 is down-regulated in Nr mutant (Alba et al., 2005) and that Sl-ERF.E1 and Sl-ERF.E2 (previously named Sl-ERF71 and Sl-ERF72 in Kumar et al., 2012) are dramatically down-regulated at different ripening stages in the rin mutant. Likewise, the

transcript level of Sl-ERF.E4 was reported to undergo significant decrease in all ripening-impaired mutants including Nr, rin and nor (Lee et al., 2012). In contrast to subclass E members, Sl-ERF.B2, F1 and F4 display consistent up-regulation in the ripening mutants, suggesting a possible requirement for the down-regulation of these ERFs in normal ripening. The coordinated up-regulation of these ERFs in the rin mutant is consistent with the reported assumption that RIN plays a role in irreversibly promoting ripening via the negative regulation of some transcription factors (Fujisawa et al., 2013). Of particular interest, Sl-ERF.B3, previously reported to have contrasting effects on tomato fruit ripening (Liu et al., 2013; Liu et al., 2014), exhibits a distinct expression pattern in the ripening mutants with a significant down-regulation at 10 days post-breaker in all ripening mutants but up-regulation at the Breaker stage suggesting that its role in controlling ripening is possibly stage-dependent. On the other hand, Sl-ERF.D1 which displays a typical down-regulation during fruit ripening (Fig.1), was reported to be strongly down-regulated during ripening of OrrDs/ORR heterozygous mutant (Nashilevitz et al., 2010), indicating that ERFs from different subclasses might contribute to the ripening process. Taking together, these data suggest that the coordinated expression of some ERFs is central to fruit ripening. Strikingly, ERFs from subclass F, encoding transcriptional repressors, also emerge as major regulators of fruit ripening in the tomato. Sl-ERF.F2, F3 and F5, which show ripening-associated expression, were also significantly down-regulated in all tomato ripening-mutants (Fig. 5; Table 2). This supports the hypothesis that these repressor ERFs may inhibit the expression of some negative regulators whose repression is instrumental to the layout of the ripening program. Expression correlation analysis supports this last hypothesis, with Sl-ERF.E2 and Sl-ERF.E4 emerging as positive regulators of ripening whereas Sl-ERF.F2, whose expression is highly correlated to these 2 genes, might act through the down-regulation of a negative regulator of Sl-ERF.E2 and Sl-ERF.E4.

Of particular note, ERF genes that display clear down-regulation in the ripening mutant Nr are also down-regulated in rin and nor (Table 2), supporting that NOR and RIN act upstream of NR. The down-regulation of Sl-ERF.E1, E2 and E4 in the ripening mutants, together with the presence of conserved RIN-binding sites in their promoter regions (Fig.8A), indicate that subclass E ERFs can be among the direct target genes regulated by RIN protein. This is in agreement with the Chip-seq studies showing that Sl-ERF.E1 and Sl-ERF.E4 are potential target of RIN (Zhong et al., 2013). In addition to Sl-

ERF.E1 and E4, 21 other ERFs were reported to be potential targets of RIN (Fujisawa et al., 2013; Zhong et al., 2013). The transactivation assays performed in the present study confirm that RIN is capable of inducing the transcriptional activity of Sl-ERF.E1, E2 and E4 promoters (Fig. 8). Since RIN and NOR have been reported to play a crucial role in the attainment of competence to ripen (Osorio et al., 2011; Fujisawa et al., 2013; Zhong et al., 2013), it is conceivable that these master regulators affect fruit ripening through direct regulation of a subset of ERF genes. Further supporting the possibility of a direct regulation of ERFs by RIN and NOR, the promoter regions of the ripening-associated ERF genes harbor well conserved RIN-binding sites and putative NAC protein binding motif (Fig. 8A). Of particular interest, Sl-ERF.F5 was reported to be a potential target of RIN although it lacks a typical RIN-binding site (CArG box) in its promoter (Fujisawa et al., 2013). On the other hand, Sl-ERF.F2 harbors a typical RIN binding site in its promoter but failed to show a RIN-mediated transcriptional (Fig.8B). These data illustrate the high complexity of the network of regulation connecting RIN and ERFs. Moreover, the shift in the expression kinetics between Sl-ERF.E4 and Sl-ERF.E1 along with the ability of Sl-ERF.E4 to activate the transcriptional activity of the Sl-ERF.E1 promoter in a transactivation assay, support the hypothesis that the expression of some ERFs is interconnected. Taking together, the data suggest a complex RIN-dependent mechanism of regulation of ERFs where RIN initiates a cascade of events by turning on Sl-ERF.E4 expression, which in turn activates the transcription of Sl-ERF.E1.

Although an increasing number of studies addressing the functional significance of ERF genes are now becoming available, yet, little is known about their position in the regulatory network triggering and orchestrating the ripening process. Overall, the present study identifies a subset of ERF genes as being potentially important in controlling fruit ripening via both ethylene-dependent and RIN/NOR-mediated mechanisms. The data designate the selected ERFs as priority target for further functional characterization aiming to position these transcription factors in the gene regulatory networks underlying fruit ripening. In particular, it is important to further clarify whether specific roles are devoted to different ERF subclasses during fleshy fruit ripening and how these ERFs interact with key regulators like RIN and NOR. The implementation of new approaches, such as in vivo chromatin immune-precipitation (ChIP) coupled to high-throughput sequencing, is anticipated to yield essential

information on the direct target genes of the candidate ERFs, and hence to provide clues on the specific pathway(s) in which these transcriptional regulators are involved.

5.2 SECTION 2

Fruit ripening is driven by a dynamic interplay between hormones and several key developmental factors. Despite the fact that the concurrence of multiple hormones controlling ripening is a common theme in plant development, the mechanisms underlying these interactions are not always clear. The results achieved here demonstrated that auxin played an opposing role to ethylene and affected several aspects of fruit ripening: peel colour shift and lycopene accumulation, climacteric ethylene levels, volatile production and the transcription control of known master regulators of tomato ripening. In addition, the delaying effects of auxin seemed to overcome the role of ethylene in inducing ripening, which was revealed when fruits were submitted to treatments with both hormones and displayed similar phenotypes to auxin-treated samples. Taking into account that active IAA levels decreased at the beginning of ripening-associated changes, the modulation of IAA levels appears to play an important part in ripening control. Indeed, results regarding the measurements of IAA-conjugated forms suggested that IAA conjugation is possibly induced by ethylene through the activation of GH3-related genes. Thus, it provides further evidence for the notion that the decrease of IAA levels is necessary to trigger the onset of ripening then leading to the ethylene enhancing, which in turn modulates active IAA levels by conjugation.

Colour shift in the tomato peel was clearly delayed by auxin. Colour changes in fruits are part of the typical process of ripening, yet still little is known about its complete regulation. It is already clear that ethylene controls some of the mechanisms related to these pathways. Ethylene treatments, or even treatments with the ethylene perception inhibitor 1-MCP (1-methylcicloprοpene), are known to markedly affect fruit colour (Liu et al. 2015, Klee and Giovannoni 2011, Bramley 2002). However, there are also ethylene-independent factors in the control of colour metabolisms as tomato mutants like Nr (Wilkinson et al., 1995), in which ethylene perception is compromised while fruits still accumulate lycopene and β-carotene. In this manner, auxin can regulate at least a part of the mechanisms that regulate colour changes associated with fruit development and ripening. Su et al. (2015) demonstrated that ethylene and auxin act to induce and repress, respectively, the expression of genes related to colour metabolisms such as psy1 and β-Lcy1 (lycopene β-cyclase), therefore oppositely influencing the lycopene accumulation and the peel colour of tomato fruit. Additionally, in silico

analyses of cis-regulatory elements for the presence of auxin-responsive elements in the upstream regulatory region of ripening-related genes (data not shown) revealed that β-Lcy1 might be regulated directly by ARFs.

On the role of ARFs that coordinate biochemical metabolisms, it was found that the downregulation of the tomato ARF4 leads to a fruit phenotype with enhanced firmness, increased chlorophyll content and displayed formation of large chloroplasts (Jones et al. 2002). Sagar et al. (2013) reported that ARF4 is involved in the regulation of starch and sugar metabolism in tomato fruit. Additionally, these authors have demonstrated that the "blotchy" phenotype in the antisense lines of ARF4 fruit is related to up-regulation of a GLK1 (GOLDEN-LIKE 1) transcription factor, suggesting that ARF4 is a transcriptional repressor of GLK1.

Ethylene synthesis genes are also differently regulated by ethylene and auxin, and this seems to vary depending on the timing of the expression. Findings from auxin treatments in tomatoes revealed induction of ACS6 expression, a gene encoding ACS synthase expressed during the pre-climacteric phase and down-regulated by ethylene at that stage (Barry et al., 2000). In the present study, ACS6 and ACS1a were both up-regulated by auxin in the first days of tomato ripening. Conversely, ASC2 and ACS4 were repressed by auxin. System I relies on ACS1A and ACS6, both being down-regulated by ethylene, while the up-regulation of ACS2 and ACS4 through positive feedback by ethylene is responsible for the activation of system II (Barry et al., 2000). The idea that auxin acts to prevent the burst of ethylene production then delaying the onset of ripening is perfectly supported by the positive regulation of system I-related genes by auxin; by contrast, system II genes are repressed. Also worthy of note is that ACO4 expression was considerably induced by auxin. It has been reported that ACO4 expression slightly increased during ripening (Van de Poel et al., 2012), which corresponds to the pattern achieved in this study amongst the control samples. Notably, no significant effects in ethylene-treated fruits were observed and, furthermore, ACO4 was found to contain auxin responsive elements in its promoter region, which suggests that this gene is potentially regulated at the transcriptional level by auxin.

The effects of auxin on ethylene biosynthesis have been observed in several fruits (Tatsuki et al., 2013; Choudhury et al., 2008; Kondo et al., 2009; Trainotti et al., 2007). The increase in auxin levels in the pre-climacteric stage of peach fruit appear to be

crucial for triggering the molecular mechanisms necessary for system II autocatalytic ethylene production (Tatsuki et al., 2013). In addition, auxin-related genes have been found to be up-regulated during peach ripening (Trainotti et al., 2007). The induction of ripening caused by auxin in peaches seems to be an exception in this fruit since the most common feature found in auxin is that it delays ripening changes. Undoubtedly, however, even in peaches, auxin levels need to decrease to allow ripening to proceed.

In addition to the synthesis, the ethylene perception seemed to be repressed by auxin once ETR3, ETR4 and ETR6 transcriptions were down-regulated after IAA treatments. Also worthy of note is that, in fruits treated with ethylene + IAA, the expected induction of expression by ethylene was not observed, thereby suggesting that IAA had a predominant effect over ethylene. This inhibitory effect of auxin on gene receptors expression is very complex in the context of the proposed model for receptors acting as negative regulators of ethylene signal transduction, especially considering that IAA-treated fruits provided an impaired-ripening phenotype in the afore-mentioned study. However, when the mRNA levels of the receptors are low, the associated proteins do not necessarily follow this trend (Kevany et al., 2007). Moreover, receptor proteins' degradation upon ethylene binding appears to be essential for positively activating the mechanisms that provide feedback on the ethylene synthesis and signalling (Kevany et al., 2007). Within this context, an important question arises: could auxin block the receptors degradation leading to stabilized proteins which then negatively feedback the ethylene signalling? This seems possible. Nonetheless, further studies in this area are necessary to verify the exact role of auxin on ethylene perception.

Downstream elements of ethylene signalling closely associated to tomato ripening – ERF.E1, ERF.E2 and ERF.E4 (Liu et al., 2015) – were negatively affected by auxin. Despite the influence of ethylene in regulating these genes, the involvement of other hormones such as auxin is also likely to be important in tuning ERFs' expression during fruit ripening. This is supported by the recent study which shows that SlARF2 is an important component of the regulatory mechanism controlling tomato fruit ripening (Hao et al., 2015). Interestingly, a high number of ERFs, including ERF.E1 and ERF.E4, are significantly down-regulated in the SlARF2 ripening-impaired mutant (Hao et al., 2015), evidencing a possible crosstalk involving ERFs and ARFs. Ethylene and auxin not only affect each other's regulatory pathways during ripening, but together can also control the expression of several genes and, furthermore, directly regulate

metabolisms such as colour changes or volatile production. Ripening-related genes known to be ethylene-responsive, whose transcripts levels were also affected by IAA (ETR3, ACS4, ACO4, CCD1B, LOXC, NOR), contain auxin responsive elements in their promoter region (data not shown), which suggests a potential regulation by ethylene (as inducer) and auxin (as repressor), and thereby represents a secondary crosstalk.

Other key effectors of tomato fruit ripening regulation were monitored at the transcriptional level and were noticeably affected by the hormonal treatments in the present study. The trend observed in the expression patterns amongst control samples for RIN, NOR, CNR, DML2 and AP2a was up-regulation throughout ripening. Ethylene exposure led to a marked increase on the transcripts accumulation of RIN, NOR and CNR, especially at the breaker stage, in accordance with previous findings (Liu et al., 2015; Fujisawa, et al. 2013; Manning et al. 2006; Giovannonni, 2004). Ethylene-inducible expression was also observed in DML2 and AP2a. The induction by ethylene consistently confirms the known role of these regulators on the overall control of ripening via an ethylene-dependent pathway, although it is widely thought that they also play a role in an ethylene-independent pathway upstream of ethylene signalling. That is, auxin emerges as a potentially strong regulator of the ethylene-independent mechanisms involved in the expression of DML2, RIN, NOR, CNR and AP2a once IAA treatments have blocked the ethylene induction, as was shown when transcripts levels in IAA- and ethylene + IAA-treated fruits were kept similar to the controls.

It has been reported that RIN interacts directly with the promoter of several ripening-associated genes, such as those related to ethylene biosynthesis and perception, cell-wall metabolism and volatiles and carotenoids pathways (Ito et al., 2008; Fujisawa et al., 2011; Martel et al., 2011; Qin et al., 2012). Furthermore, CNR is required in RIN-binding activity, and a systems biology approach pointed out that they both act in the same regulatory pathways as NOR (Osorio et al., 2011). CNR also directly binds in vitro to the promoter of AP2a, a transcription factor belonging to the ERF family and suggested to negatively regulate ethylene biosynthesis (Karlova et al. 2011). However, the repression of AP2a expression in RNAi lineages led to a number of down-regulated genes, such as those related to carotenoid biosynthesis, volatile production and cell wall disassembly, indicating that AP2a has a positive regulatory effect on fruit ripening (Karlova et al. 2011). Besides that, considering the down-regulation of DML2 by IAA,

it seems that auxin might contribute to the maintenance of high methylation levels in the DNA and thus inhibit or delay ripening by affecting the transcriptional activation in a process mainly mediated by DML2. Therefore, it is plausible to infer that the delay in the onset of ripening caused by auxin (revealed by the retarding of the peel colour shift), the delay in the increase of ethylene production and the changes in the volatile profile might be attributed, at least in part, to the down-regulation of these afore-mentioned key effectors.

The most remarkable changes in the volatile profile of tomato fruit were observed at the breaker stages. Indeed, at that stage, the ripening of IAA-treated fruits was clearly delayed compared to controls: the peel colour was greener, the contents of carotenoids were lower, the volatile profile was represented by different compounds and the expression of key ripening effectors and several ripening-related genes was down-regulated. During tomato ripening, colour changes from green to red and the conversion of chloroplasts into chromoplasts were temporarily associated with the major changes of aromatic volatile profile. This suggests that a key role is played by substrate availability in the regulation of the biosynthesis of volatile-related compounds (Klee, 2010; Vogel et al., 2010). Indeed, volatile products derived from open-chain carotenoids such as phytoene and lycopene correlate strongly with the levels of the carotenoid precursors (Lewinsohn et al., 2005). In this study, results suggested that IAA delayed the lycopene accumulation while CCD1A and CCD1B expression were lower at the breaker stages. This may explain the delayed production of volatile substances derived from carotenoids such as β-ionone, β-cyclocitral and citral. Notably, those compounds are important aroma contributors (Wang et al., 2016).

RIN's loss of function affected the production of aromatic compounds, and it is known that RIN binds to the promoter of LOXC gene (which is closely associated to tomato ripening). These findings suggest that RIN modulates aroma formation through the direct regulation of gene expression in the LOX pathway (Qin et al., 2012). Furthermore, ripening regulators such as RIN and CNR were shown to function upstream of AP2a and to positively regulate its expression. In the AP2ai transgenic fruits, several ripening-associated genes encoding proteins such as those in the carotene biosynthesis pathway, including LOXC, were down-regulated, indicating that AP2a has positive ripening regulatory functions in volatile formation (Chung et al., 2010; Karlova et al., 2011). The transcripts levels of LOXC, ADH2 and HPL were reduced at the

breaker stage. At this stage, the expression of what have been considered the "master" regulators of ripening (including RIN, CNR and AP2a) was compromised by IAA treatments. This indicates that differences found in the volatile profile of IAA-treated tomato fruit were caused at least partly by altering the transcription of those key ripening effectors.

By contrast, ethylene-treated fruits revealed a different volatile profile. It is known in related literature that ethylene induces changes in biochemical pathways related to volatile production, which leads to changes in the general profiles of not only tomato fruit, but also of other climacteric and non-climacteric fruits (Wang et al., 2016; Rambla et al., 2014). Interestingly, after ten days of the experiment (D10), the ethylene production was found to have recovered in IAA-treated fruits. In addition, these fruits showed a clear tendency to recover the ripening program and restore the biochemical parameters, a result which was elicited by the volatile profile at the red stage (D14), when no significant differences in the treated fruits were achieved. Meanwhile, the expression of transcription factors which have been considered ripening regulators acting upstream of ethylene also tending to increase. Thus, other ethylene-independent pathways can also be part of the regulation of volatile by modulating RIN-associated regulatory pathways.

Thus, bringing together all the data concerning volatile-related genes, compounds and some precursors (such as carotenoids like lycopene), as well as the monitored ripening-associated genes, it is possible to assume that auxin treatment at the mature green stage of tomato fruit was capable of inducing significant changes in volatile formation at the breaker stage, possibly through modulating ethylene-dependent and -independent mechanisms. Those differences, however, were resolved in fruit that reached the red stage. This also points to the effect auxin treatment has in delaying ripening whilst not harming the volatile formation of important compounds of tomato aroma.

Evidencing another important crosstalk point between auxin and ethylene, three genes encoding GH3 were induced by ethylene in tomato fruit concurrently with the detection of the conjugated forms detected earlier when compared to the control. Conversely, IAA led to expression tendencies similar to those of the controls. The auxin conjugation has been associated to initiating ripening in tomato and grape fruits (Böettcher et al., 2010, 2013; Kumar et al., 2012). In the tomato, GH3 genes showed a ripening-associated

transcriptional pattern, and this expression can be associated with an increase in ethylene production (Kumar et al., 2012). The expression of a pepper (Capsicum chinense) GH3 gene in tomatoes revealed that it is ethylene inducible as well as linked to decreased levels of auxin (Liu et al., 2005). In addition, applying exogenous ethylene to overexpressed GH3 in tomato fruit accelerated ripening. Our results showed that ethylene might induce auxin conjugation, possibly through the up-regulation of ethylene-inducible GH3 genes. Due to the role of auxin as a negative regulator of ripening, it is reasonable to suggest that ethylene has a pivotal role in removing active IAA from the fruit through activating conjugation mechanisms – which can be essential to triggering ripening.

The application of exogenous IAA affected the transcription of DML2, which acts upstream of RIN, NOR and CNR, and AP2a, which acts downstream. Likewise, IAA affected the transcripts levels of genes related to ethylene synthesis, perception and signalling. This provides evidence that auxin may act at several stages of the network regulation of tomato fruit ripening. Nevertheless, the exact mechanisms underlying ripening are still unclear and there is much scope for future study. The present study has shown that auxin can act in an ethylene-dependent way by disturbing, for instance, the ethylene perception. It thus blocks the transduction flow and creates a negative-feedback loop which down-regulates global ripening regulators and represses the autocatalytic synthesis, signalling and related responses. It has shown that auxin can also directly affect the expression of upstream master regulators in an ethylene-independent way and consequently affect ripening parameters such as colour and volatile profile. Nonetheless, it is feasible to suggest that a combination of both situations could be used in a synergic mechanism in which target genes can be regulated by both hormones through distinct pathways. Besides that, ethylene also seems to have an important role as an inducer of IAA conjugation through the activation of GH3 genes, which provides new evidence regarding auxin/ethylene interplay. Indeed, free auxin removal appears to be crucial to allowing ripening to proceed. Enhancing scientific knowledge underlying the precise spatial and temporal expression of the factors coordinating fruit development and ripening is fundamental to the design of strategies that extend fruit shelf life and prevent spoilage losses without compromising quality traits.

6 CONCLUSIONS

The present study identifies a subset of ERF genes as being potentially important in controlling fruit ripening via both ethylene-dependent and RIN/NOR-mediated mechanisms. The data designate the selected ERFs as priority target for further functional characterization aiming to position these transcription factors in the gene regulatory networks underlying fruit ripening. In addition to that, the results corroborate to the role of auxin in delaying the onset of ripening further showing epistatic effects over the influence of ethylene. Several components of the ethylene signalling, including other ripening-related genes, were affected by auxin suggesting potential crosstalk points between the two hormones. Moreover, ethylene appears as potential part of the auxin regulation by inducing IAA conjugation.

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